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Depolarization-induced Rapid Generation of 2-Arachidonoylglycerol, an Endogenous Cannabinoid Receptor Ligand, in Rat Brain Synaptosomes

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2-Arachidonoylglycerol (2-AG) is an endogenous ligand for the cannabinoid receptors with a variety of potent biological activities. In this study, we first examined the effects of potassium-induced depolarization on the level of 2-AG in rat brain synaptosomes. We found that a significant amount of 2-AG was generated in the synaptosomes following depolarization. Notably, depolarization did not affect the levels of other molecular species of monoacylglycerols. Furthermore, the level of anandamide was very low and did not change markedly following depolarization. It thus appeared that the depolarization-induced accelerated generation is a unique feature of 2-AG. We obtained evidence that phospholipase C is involved in the generation of 2-AG in depolarized synaptosomes: U73122, a phospholipase C inhibitor, markedly reduced the depolarization-induced generation of 2-AG, and the level of diacylglycerol was rapidly elevated following depolarization. A significant amount of 2-AG was released from synaptosomes upon depolarization. Interestingly, treatment of the synaptosomes with SR141716A, a CB1 receptor antagonist, augmented the release of glutamate from depolarized synaptosomes. These results strongly suggest that the endogenous ligand for the cannabinoid receptors, *i.e.* 2-AG, generated through increased phospholipid metabolism upon depolarization, plays an important role in attenuating glutamate release from the synaptic terminals by acting on the CB1 receptor.

Key words: anandamide, 2-arachidonoylglycerol, cannabinoid, monoacylglycerol, synaptosomes.

Abbreviations: 2-AG, 2-arachidonoylglycerol; DFP, diisopropylfluorophosphate; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; LTD, long-term depression; Δ^9-THC, Δ^9-tetrahydrocannabinol.

Δ^9-Tetrahydrocannabinol (Δ^9-THC), a major psychoactive ingredient of marijuana, interacts with specific binding sites, *i.e.* the cannabinoid receptors (CB1 and CB2), thereby eliciting various pharmacological responses such as reduced spontaneous motor activity, analgesia, heightened sensory awareness, euphoria, hypothermia and impairment of short-term memory in experimental animals and humans (*1*).

Two types of arachidonic acid-containing molecules have thus far been identified as endogenous ligands for the cannabinoid receptors. The first endogenous cannabinoid receptor ligand to be found was anandamide (*N*-arachidonoylethanolamine) which was isolated from pig brain by Devane *et al*. in 1992 (*2*). To date, a number of investigators have demonstrated that anandamide induces various cannabimimetic activities *in vitro* and *in vivo* (*3*), although it acted as a partial agonist in most cases. On the other hand, we (*4*) and Mechoulam *et al*. (*5*) identified 2-arachidonoylglycerol (2-AG) as another endogenous ligand for the cannabinoid receptor in 1995. 2-AG exhibited various cannabimimetic activities similar to those of anandamide (*6*, *7*). Notably, in contrast to anandamide, 2-AG acted as a full agonist in various assay systems (*8–14*). Based on the results of structure–activity relationship experiments, we proposed that 2-AG rather than anandamide is the true natural ligand for the cannabinoid receptors (CB1 and CB2) (*9–11*).

There is growing evidence that 2-AG plays important physiological and pathophysiological roles in various mammalian tissues and cells, especially those in the nervous system (*6*, *7*). For instance, 2-AG provoked a Ca^{2+} transient in NG108-15 neuroblastoma × glioma hybrid cells (*8–10*), the suppression of a depolarization-induced elevation of intracellular free Ca^{2+} concentrations in differentiated NG108-15 cells (*15*), and the inhibition of voltage-gated Ca^{2+} channels in the transverse tubule membrane from rabbit skeletal muscle (*16*) and rat sympathetic neurons (*17*). 2-AG also induced the suppression of long-term potentiation (*18*) and the attenuation of the synaptic transmission (*19*) in rat hippocampal slices and the activation of inwardly

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rectifying K^+ channels in rat sympathetic neurons (*17*). As for the generation of 2-AG, several investigators provided evidence that it takes place in various stimulated tissues and cells such as ionomycin-stimulated cultured neural cells (*20*) and electrically stimulated rat hippocampal slices (*18*). Despite these preceding studies, however, the available information concerning 2-AG is not yet satisfactory for a thorough elucidation of the mechanism underlying the biosynthesis as well as the physiological significance of 2-AG in the nervous system. Many important issues remain to be determined. For example, it is still not clear whether 2-AG actually acts as an intercellular messenger molecule in the synapse in living animals. Inasmuch as the CB1 receptor is abundantly expressed in the brain (*21*, *22*) and 2-AG is the true natural ligand for the CB1 receptor (*9*, *10*), further detailed studies on the physiological functions of 2-AG in the nervous tissues are indispensable.

In the present study, we investigated in detail the generation and release of 2-AG in depolarized rat brain synaptosomes. We found that rapid and selective generation of 2-AG takes place in depolarized synaptosomes. We also found that a significant amount of 2-AG was released from synaptosomes upon depolarization. Possible physiological meanings of the depolarization-induced rapid generation of 2-AG in the synapses are discussed.

MATERIALS AND METHODS

Chemicals—Arachidonic acid, heptadecanoic acid and essential fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). A23187 was acquired from Merck (Darmstadt, Germany). L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (PDC) was purchased from Tocris Bioscience (Bristol, UK). SR144528 was a generous gift from Sanofi-Synthelabo Recherche (Montpellier, France). Glutamate dehydrogenase was obtained from Calzyme, Inc. (San Luis Obispo, CA, USA). 1-Anthroyl cyanide, butylated hydroxytoluene (BHT), NADP, ω-conotoxin GVIA, ω-conotoxin MVIIC and nifedipine were purchased from Wako Pure Chem. Ind. (Osaka, Japan). Quinuclidine was from Molecular Probes, Inc. (Eugene, OR, USA). GF/B filters were acquired from Whatman, Inc. (Florham Park, NJ, USA). Ficoll PM 400 was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). U73122 and SR141716A were obtained from Biomol (Plymouth Meeting, PA, USA). Anandamide was synthesized from ethanolamine and arachidonic acid by the method of Devane *et al*. (*2*). 2-AG and 2-heptadecanoylglycerol were synthesized from 1,3-benzylideneglycerol and arachidonic acid and heptadecanoic acid, respectively, as previously described (*10*).

Preparation of Rat Brain Synaptosomes—Rat brain synaptosomes were prepared by the modified method of Nicholls (*23*). Briefly, male Wistar rats (350–400 g body weight) were killed by decapitation. The brain was removed and homogenized in 0.32 M sucrose-5 mM TES buffer (pH 7.2) containing 0.5 mM EDTA (solution A). The homogenate was centrifuged at 900 *g* for 5 min. The supernatant was aspirated and further centrifuged at 17 000 *g* for 10 min. After the removal of the supernatant, the pellet was dissolved in solution A and carefully layered on a discontinuous Ficoll gradient [upper, 6% Ficoll in solution A (w/v); medium, 9% Ficoll in solution A (w/v); lower, 12% Ficoll in solution A (w/v)]. The tubes were then centrifuged at 75 000 *g* for 30 min using a Hitachi swinging rotor (RPS27-2). The interfacial layer between 9% and 12% Ficoll was carefully collected, diluted with solution A, and further centrifuged at 75 000 *g* for 10 min. The pellet was dissolved in 0.25 M sucrose-5 mM TES buffer (pH 7.2) (solution B). The protein content was determined by the method of Lowry *et al*. (*24*). The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Teikyo University and the Japanese Pharmacological Society.

Incubation of Rat Brain Synaptosomes—Rat brain synaptosomes (5 mg protein) were incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM (control) or 80 mM KCl (high K^+) or 3mM KCl plus 2 μM A23187, 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM diisopropylfluorophosphate (DFP) at 30°C for 30 s. The incubation was terminated by adding 18.8 ml of chloroform : methanol (1 : 2, v/v). Total lipids were extracted by the method of Bligh and Dyer (*25*). In the experiments where the effects of Ca^{2+} channel blockers and a phospholipase C inhibitor were examined, synaptosomes were preincubated with these inhibitors at 4°C for 5 min and then incubated at 30°C for 30 sec as described above.

Estimation of the Amounts of Individual Molecular Species of Monoacylglycerols and N-acylethanolamines—For the analysis of monoacylglycerols, 2-heptadecanoylglycerol (0.1 nmol) was added to the total lipids as an internal standard. Total lipids were then fractionated by TLC with development using petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v) in a sealed tank containing N_2 gas. The area corresponding to the standard monoacylglycerol was scraped off the TLC plate and extracted from the silica gel by the method of Bligh and Dyer (*25*). The extraction was conducted in the presence of BHT (0.001%, w/v) in an N_2 gas-sealed tube. The purified monoacylglycerols were converted to their 1-anthroyl derivatives and then analysed with an HPLC system equipped with a reverse-phase column (CAPCELL PAK C18 SG120, 4.6 mm × 250 mm, Shiseido Co., Tokyo, Japan) and a fluorescence detector (excitation at 370 nm; emission at 470 nm). The mobile phase was acetonitrile : 2-propanol : water (90 : 4 : 6, v/v), and the flow rate was 1.4 ml/min as previously described (*26*). For the analysis of *N*-acylethanolamines, *N*-heptadecanoylethanolamine (0.1 nmol) was added as an internal standard. Total lipids were fractionated by TLC using development with chloroform : methanol : NH_4OH (80 : 20 : 2, v/v) in a sealed tank containing N_2 gas. The area corresponding to the standard *N*-acylethanolamine was scraped off the TLC plate and extracted from the silica gel by the method of Bligh and Dyer (*25*). The extraction was conducted in the presence of BHT (0.001%, w/v) in an N_2 gas-sealed tube.

The *N*-acylethanolamine was further purified by TLC with petroleum ether : diethyl ether : acetone : acetic acid (30 : 40 : 20 : 1, v/v) and by subsequent TLC with an organic layer of ethyl acetate : petroleum ether : diethyl ether : acetic acid : water (100 : 50 : 20 : 100, v/v). *N*-Acylethanolamines were then converted to their 1-anthroyl derivatives and analysed with an HPLC system equipped with a reverse-phase column (CAPCELL PAK C18 SG120, 4.6 mm × 250 mm × 2, Shiseido Co., Tokyo, Japan) and a fluorescence detector (excitation at 370 nm; emission at 470 nm). The mobile phase was acetonitrile : 2-propanol : water (80 : 3 : 17, v/v), and the flow rate was 1.4 ml/min as previously described (*26*). The amounts of monoacylglycerols and *N*-acylethanolamines were expressed as pmol per mg protein.

Analysis of the Tert-butyldimethylsilyl (tBDMS) Derivatives of Monoacylglycerols by Gas Chromatography-mass Spectrometry (GC-MS)—Rat brain synaptosomes (125 mg protein) were incubated in 125 ml of 20 mM TES buffer (pH 7.2) containing 80 mM KCl, 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for 30 s. The incubation was terminated by adding 470 ml of chloroform : methanol (1 : 2, v/v), and the total lipids were extracted by the method of Bligh and Dyer (*25*). Total lipids were then fractionated by TLC using petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v) as the solvent system in a sealed tank containing N_2 gas. The area corresponding to the standard monoacylglycerol was scraped off the TLC plate, and extracted from the silica gel using the method of Bligh and Dyer (*25*). The extraction was conducted in the presence of BHT (0.001%, w/v) in a N_2 gas-sealed tube. The monoacylglycerols were further purified by TLC using development with petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v). Monoacylglycerols were then treated with 72 mg of *tert*-butyldimethylchlorosilane dissolved in 100 μl of dimethylformamide containing 20 mg of imidazole at 65°C for 60 min and converted to the *t*BDMS derivatives. A portion of the *t*BDMS derivatives of monoacylglycerols was analysed by GC-MS using a JEOL JMS-SX 102A mass spectrometer (accelerating voltage, 10 kV; ionizing current, 300 μM; ionizing voltage, 70 eV) coupled with a gas chromatograph equipped with a silica fused column (J & W Scientific, DB-1, 30 m × 0.25 mm ID, 0.25 μm thickness). The column temperature was increased from 200°C to 280°C at a rate of 10°C/min. The temperatures of the injection port, interface and ion source were 250°C.

Estimation of the Amounts of Diacylglycerols—Rat brain synaptosomes (5 mg protein) were incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM or 80 mM KCl, 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for 30 s. The incubation was terminated by adding 18.8 ml of chloroform : methanol (1 : 2, v/v). Total lipids were extracted by the method of Bligh and Dyer (*25*). Total lipids were then fractionated by TLC with development using petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v) in a sealed tank containing N_2 gas. The area corresponding to the standard diacylglycerol was scraped off the TLC plate and extracted from the silica gel by the method of Bligh and Dyer (*25*). The extraction was conducted in the presence of BHT (0.001%, w/v) in an N_2 gas-sealed tube. The fatty acyl moieties of the purified diacylglycerols were then converted to fatty acid methyl esters and analysed by GLC using a heptadecanoic acid methyl ester as an internal standard.

Release of 2-AG from Rat Brain Synaptosomes—Rat brain synaptosomes (10 mg protein) were incubated in 10 ml of 20 mM TES buffer (pH 7.2) containing 3 mM or 80 mM KCl, 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose, 0.25% BSA and 1 mM DFP at 30°C for 30 sec. The incubation mixture was then filtered through a GF/B filter. Total lipids were extracted from both the filtrate and the synaptosomes trapped on a GF/B filter by the method of Bligh and Dyer (*25*). The amounts of 2-AG in both fractions were estimated using reverse-phase HPLC.

Binding of 2-AG to Rat Brain Synaptosomes—2-AG (1 nmol) was incubated in 10 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl, 127 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM EGTA, 10 mM glucose, 15 mM sucrose, 0.25% BSA and 1 mM DFP in the presence or absence of rat brain synaptosomes (10 mg protein) at 30°C for 30 s. EGTA was added to block the generation of 2-AG in the synaptosomes during the incubation. The incubation mixture was then filtered through a GF/B filter. Total lipids were extracted from both the filtrate and the GF/B filter with or without synaptosomes by the method of Bligh and Dyer (*25*). The amounts of 2-AG in both fractions were estimated using reverse-phase HPLC.

Effects of Cannabinoid Receptor Antagonists on Glutamate Release from Depolarized Rat Brain Synaptosomes—Rat brain synaptosomes (1 mg protein) were suspended in 300 μl of 20 mM TES buffer (pH 7.2) containing 3 mM KCl, 127 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose and 15 mM sucrose and kept at 4°C for 30 min. Following the addition of SR141716A or SR144528 (each final concentration, 1 μM) or dimethyl sulfoxide (DMSO) (vehicle) (final, 0.2%, v/v), DFP (final, 1 mM), PDC (a glutamate uptake inhibitor) (final, 10 μM) and KCl (final, 80 mM) to synaptosomes, the mixture was incubated at 30°C for 1 min. It was then cooled to 4°C and centrifuged at 17 000 *g* for 1 min. The supernatant was collected and further centrifuged at 17 000 *g* for 1 min to remove the contaminated membrane fraction. The amount of glutamate in the supernatant was estimated as follows. The supernatant was incubated with 1 mM NADP and 20 U of glutamate dehydrogenase at 37°C for 25 min. Following the incubation, the fluorescence intensity of a reaction product NADPH was determined using a Hitachi fluorescence spectrophotometer (F-2500) (excitation 360 nm; emission 455 nm). For the calibration, various amounts of glutamate (0–6 nmol) were added to the tubes and incubated with 1 mM NADP and 20 U of glutamate dehydrogenase at 37°C for 25 min. The amount of glutamate released upon depolarization was estimated by subtracting the amount of glutamate

present in the supernatant at time 0 from that detected following depolarization.

Statistical Analysis—Statistical analysis was performed using Dunnett's test (Fig. 1), Tukey's test (Figs. 4–6, 9) and Student's *t*-test (Fig. 7). A *P*-value of <0.05 was considered to be significant.

RESULTS

Effects of Depolarization and the Stimulation with A23187 on the Levels of 2-AG, 1(3)-AG, and Anandamide in Rat Brain Synaptosomes—First, we examined the effects of depolarization and the stimulation with A23187, a Ca^{2+} ionophore, on the amounts of 2-AG, 1(3)-AG and anandamide in rat brain synaptosomes. As shown in Fig. 1A, the level of 2-AG in the synaptosomes was markedly elevated following the depolarization evoked by treatment with high K^+ (80 mM): 1.6-fold over the control level [low K^+ (3 mM)]. Treatment of the synaptosomes with 2 μM A23187 also augmented the level of 2-AG (1.8-fold). On the other hand, the level of 1(3)-AG, an isomer of 2-AG, was very low and did not change markedly after high K^+-induced depolarization or the stimulation with A23187 (Fig. 1B). The level of anandamide was also very low and did not change markedly after depolarization or the stimulation with A23187 (Fig. 1C).

Effects of Depolarization on the Levels of Individual Molecular Species of Monoacylglycerols in Rat Brain Synaptosomes—Figure 2 shows the effects of depolarization on the levels of individual molecular species of monoacylglycerols in rat brain synaptosomes. Depolarization provoked a rapid increase in the level of 2-AG (Fig. 2A). On the other hand, it did not affect the levels of 2-palmitoylglycerol plus 2-oleoylglycerol plus 2-*cis*-vaccenoylglycerol (Fig. 2C), 1(3)-palmitoylglycerol plus 1(3)-oleoylglycerol plus 1(3)-*cis*-vaccenoylglycerol (Fig. 2D) and 1(3)-stearoylglycerol (Fig. 2H). The levels of other molecular species such as 1(3)-AG (Fig. 2B), 2-docosahexaenoylglycerol (Fig. 2E), 1(3)-docosahexaenoylglycerol (Fig. 2F) and 2-stearoylglycerol (Fig. 2G) were very low and did not change markedly following depolarization. It is apparent, therefore, that the depolarization-induced augmented generation is a 2-AG-specific event.

GC-MS Analysis of the Monoacylglycerols Obtained from Depolarized Rat Brain Synaptosomes—In order to obtain more direct evidence of the occurrence of various molecular species of monoacylglycerols including 2-AG, the monoacylglycerol fraction obtained from depolarized rat brain synaptosomes was further investigated by GC-MS. Figure 3 shows the mass chromatograms ($[M-$*tert*-butyl$]^+$) of the *t*BDMS derivatives of the major molecular species of the monoacylglycerols obtained from depolarized synaptosomes: *m*/*z* 501 for palmitoylglycerols (16:0 acyl) ($[M-$*tert*-butyl$]^+$) (the retention time was 10. 5 min for 2-palmitoylglycerol and 10.9 min for 1(3)-palmitoylglycerol), *m*/*z* 527 for oleoylglycerols and *cis*-vaccenoylglycerols (18:1 acyl) ($[M-$*tert*-butyl$]^+$) (the retention times for 2-oleoylglycerol, 2-*cis*-vaccenoylglycerol, 1(3)-oleoylglycerol and 1(3)-*cis*-vaccenoylglycerol were 12.6, 12.7, 13.1, and 13.3 min, respectively), *m*/*z* 529 for stearoylglycerols (18:0 acyl) ($[M-$*tert*-butyl$]^+$) (the retention time was 13.0 min for 2-stearoylglycerol and 13.5 min for 1(3)-stearoylglycerol) and *m*/*z* 549 for AGs (20:4 acyl) ($[M-$*tert*-butyl$]^+$) (the retention time was 14.8 min for 2-AG and that 15.5 min for 1(3)-AG).

Effects of Ca^{2+} Channel Blockers on Depolarization-induced Generation of 2-AG in Rat Brain Synaptosomes—We then examined the effects of Ca^{2+} channel blockers (N-type, P/Q-type and L-type) on the generation of 2-AG in rat brain synaptosomes. As demonstrated in Fig. 4, treatment of the synaptosomes with Ca^{2+} channel blockers markedly reduced the depolarization-induced generation of 2-AG. The addition of Ca^{2+} channel blockers did not affect the basal level of 2-AG formation, suggesting that the depolarization-induced augmented generation of 2-AG is due, at least in part, to the entry of Ca^{2+} through voltage-gated Ca^{2+} channels.

Effects of U73122 on Depolarization-induced Generation of 2-AG in Rat Brain Synaptosomes—The effects of U73122, a phospholipase C inhibitor, on the generation of 2-AG were examined next. As demonstrated in Fig. 5, treatment of the synaptosomes with

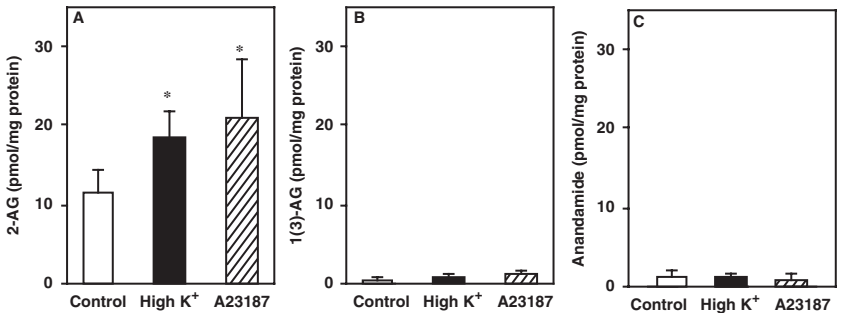

Fig. 1. **Effects of depolarization and the stimulation with A23187 on the levels of 2-AG, 1(3)-AG and anandamide in rat brain synaptosomes.** Rat brain synaptosomes (5 mg protein) were incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl (control) or 80 mM KCl (high K^+) or 3 mM KCl plus 2 μM A23187, 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for 30 s. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and the total lipids were extracted. Monoacylglycerols and *N*-acylethanolamines were analysed as described in 'MATERIALS AND METHODS'. (A) 2-AG; (B) 1(3)-AG; (C) anandamide. Control, open bar; high K^+, closed bar; A23187, hatched bar. The data are the means ± SD of four determinations. $^*P<0.05$ (compared with control).

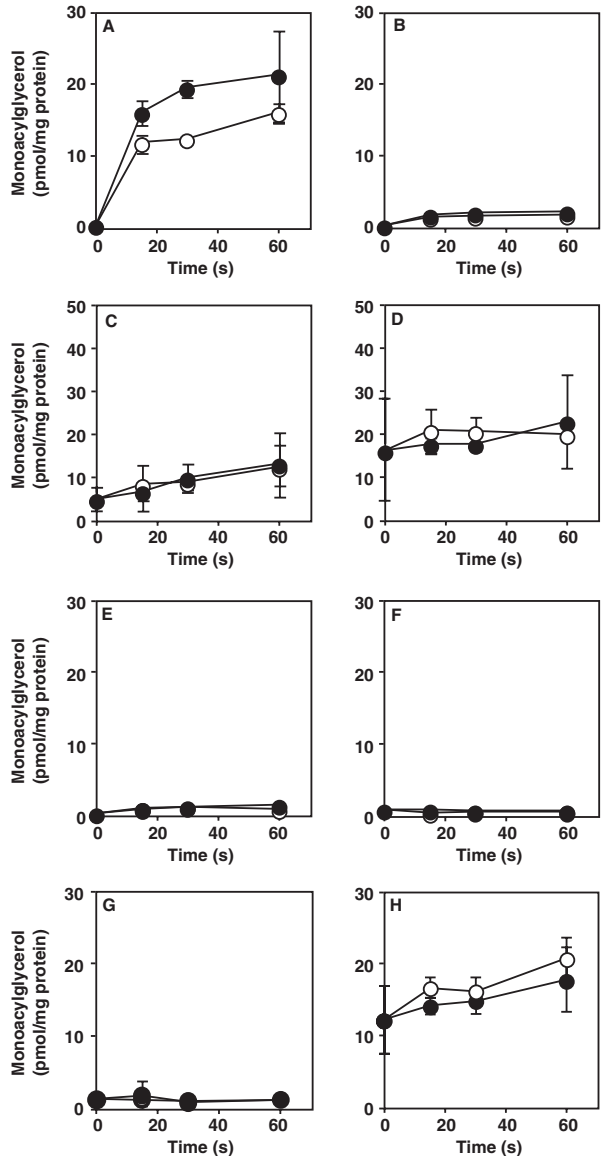

Fig. 2. **Effects of depolarization on the levels of individual molecular species of monoacylglycerols in rat brain synaptosomes.** Rat brain synaptosomes (5 mg protein) were incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl (control) or 80 mM KCl (high K^+), 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for the indicated periods of time. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and the total lipids were extracted. Monoacylglycerols were analysed as described in 'MATERIALS AND METHODS'. (A) 2-AG; (B) 1(3)-AG; (C) 2-palmitoylglycerol plus 2-oleoylglycerol plus 2-*cis*-vaccenoylglycerol; (D) 1(3)-palmitoylglycerol plus 1(3)-oleoylglycerol plus 1(3)-*cis*-vaccenoylglycerol; (E) 2-docosahexaenoylglycerol; (F) 1(3)-docosahexaenoylglycerol; (G) 2-stearoylglycerol; (H) 1(3)-stearoylglycerol. Control, open circle; high K^+, closed circle. The data are the means ± SD of four determinations.

U73122 diminished the formation of 2-AG following depolarization. It is thus conceivable that phospholipase C is closely involved in the depolarization-induced generation of 2-AG in rat brain synaptosomes.

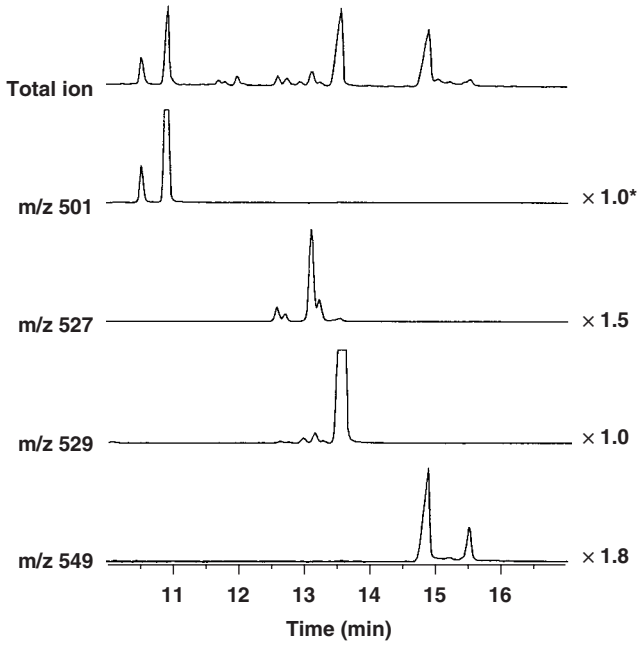

Fig. 3. **GC-MS analysis of the monoacylglycerols obtained from depolarized rat brain synaptosomes.** Rat brain synaptosomes (125 mg protein) were incubated in 125 ml of 20 mM TES buffer (pH 7.2) containing 80 mM KCl, 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for 30 s. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and total lipids were extracted. Monoacylglycerols were analysed by GC-MS as described in 'MATERIALS AND METHODS'. The data are the mass chromatograms ($[M - tert\text{-butyl}]^+$) of the *t*BDMS derivatives of the major molecular species of the monoacylglycerols obtained from depolarized rat brain synaptosomes. *Magnification for the intensity of ion.

Effects of Depolarization on the Levels of Individual Fatty Acyl Moieties of Diacylglycerols in Rat Brain Synaptosomes—We then examined the effects of depolarization on the levels of diacylglycerols, especially those containing arachidonic acid, in rat brain synaptosomes, because arachidonic acid-containing species of diacylglycerols are regarded as one of the major precursors of 2-AG. We found that the levels of diacylglycerols in rat brain synaptosomes were rapidly elevated following depolarization: 1115 pmol/mg protein for before incubation, 1260 pmol/mg protein for control synaptosomes (3 mM K^+, 30 s) and 1740 pmol/mg protein for depolarized synaptosomes (80 mM K^+, 30 s). Changes in the levels of individual fatty acyl residues of the diacylglycerols are depicted in Fig. 6. The level of stearic acid esterified in diacylglycerols was elevated in the depolarized synaptosomes (Fig. 6B). A similar but less prominent increase was observed for palmitic acid (Fig. 6A), and a more pronounced increase was observed for arachidonic acid (Fig. 6E). On the other hand, the level of oleic acid plus *cis*-vaccenic acid remained unchanged in depolarized synaptosomes (Fig. 6C). The levels of linoleic acid and docosahexaenoic acid were very low and also did not change markedly following depolarization (Fig. 6, D and F). These results

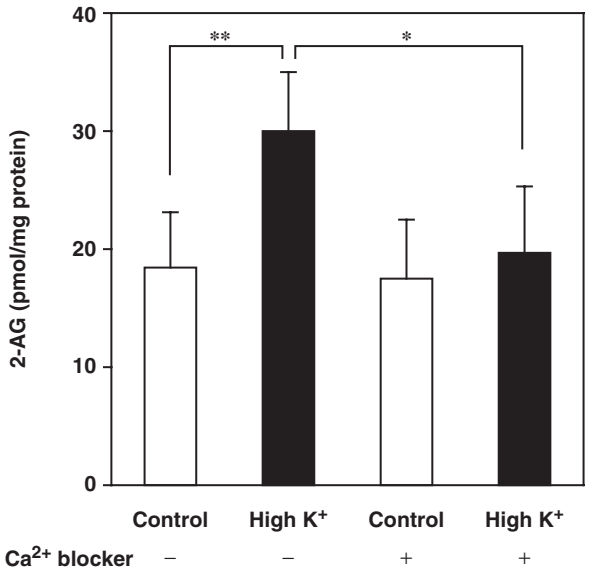

Fig. 4. **Effects of Ca^{2+} channel blockers on depolarization-induced generation of 2-AG in rat brain synaptosomes.** Rat brain synaptosomes (5 mg protein) were preincubated in 0.3 ml of 0.25 M sucrose-5 mM TES buffer (pH 7.2) in the presence or absence of Ca^{2+} channel blockers (a mixture of ω-conotoxin GVIA, ω-conotoxin MVIIC, and nifedipine, 1 μM each) at 4°C for 5 min. Synaptosomes were then incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl (control) or 80 mM KCl (high K^+), 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP in the presence or absence of Ca^{2+} channel blockers (a mixture of ω-conotoxin GVIA, ω-conotoxin MVIIC and nifedipine, 1 μM each) at 30°C for 30 s. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and total lipids were extracted. Monoacylglycerols were analysed as described in 'MATERIALS AND METHODS'. Control, open bar; high K^+, closed bar. The data are the means ± SD of five to six determinations. $^*P < 0.05$, $^{**}P < 0.01$.

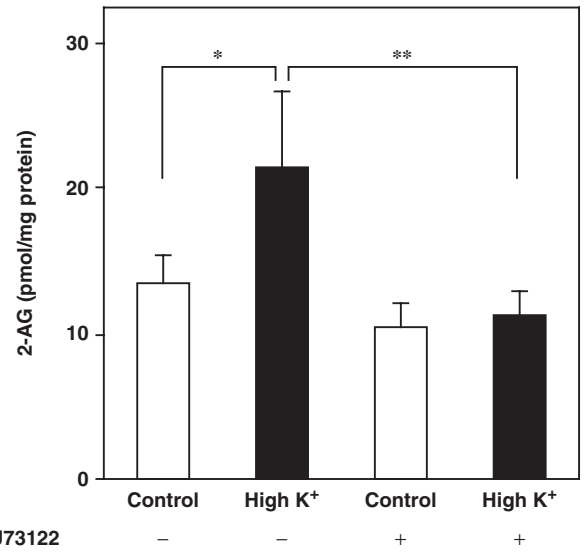

Fig. 5. **Effects of U73122, a phospholipase C inhibitor, on depolarization-induced generation of 2-AG in rat brain synaptosomes.** Rat brain synaptosomes (5 mg protein) were preincubated in 0.3 ml of 0.25 M sucrose-5 mM TES buffer (pH 7.2) in the presence or absence of 5 μM U73122 at 4°C for 5 min. Synaptosomes were then incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl (control) or 80 mM KCl (high K^+), 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP in the presence or absence of 5 μM U73122 at 30°C for 30 s. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and total lipids were extracted. Monoacylglycerols were analysed as described in 'MATERIALS AND METHODS'. Control, open bar; high K^+, closed bar. The data are the means ± SD of four determinations. $^*P < 0.05$, $^{**}P < 0.01$.

suggest that 1-stearoyl-2-arachidonolyl-*sn*-glycerol as well as 1-palmitoyl-2-arachidonoyl-*sn*-glycerol were rapidly generated in depolarized rat brain synaptosomes.

Release of 2-AG from Rat Brain Synaptosomes—We next examined whether 2-AG is released from synaptosomes. As shown in Fig. 7A, a small amount (3.2 pmol/mg protein) of 2-AG was released from control synaptosomes which were incubated with low K^+ (3 mM). Notably, the amount of 2-AG released from synaptosomes was augmented by 1.7-fold over the control level following treatment of the synaptosomes with high K^+ (80 mM) (5.4 pmol/mg protein). It is thus evident that a significant part of the newly generated 2-AG can be rapidly released from synaptosomes. Meanwhile, the amount of 2-AG retained in the synaptosomes was also increased following depolarization (1.7-fold over the control) (Fig. 7B); it appears, therefore, that the proportion of the amount of 2-AG released from synaptosomes to that retained in the synaptosomes was not markedly affected by depolarization.

Binding of 2-AG to Rat Brain Synaptosomes—It is well-known that a number of lipid molecules non-specifically bind to membranes. If this is also the case for 2-AG, the amount of 2-AG passed through the filter did not necessarily reflect the actual amount of 2-AG released from synaptosomes. We then examined whether 2-AG binds to rat brain synaptosomes. As shown in Fig. 8A, most of the exogenously added 2-AG passed through the filter in the absence of synaptosomes. On the other hand, a large amount of 2-AG was trapped on the filter in the presence of synaptosomes (Fig. 8B). Considering this, it seems plausible that the actual amount of 2-AG once released from synaptosomes was much higher than the apparent amount of 2-AG released from synaptosomes shown in Fig. 7A.

Effects of Cannabinoid Receptor Antagonists on Depolarization-induced Rapid Release of Glutamate from Rat Brain Synaptosomes—Finally, we examined the effects of cannabinoid receptor antagonists on the depolarization-induced glutamate release from rat brain synaptosomes. The addition of high K^+ (80 mM) to the synaptosomes triggered depolarization and elicited a rapid release of glutamate (Fig. 9). Notably, treatment of the synaptosomes with SR141716A, a CB1 receptor antagonist, significantly enhanced the release of glutamate from depolarized synaptosomes (1.3-fold). In contrast, treatment of the synaptosomes with SR144528, a CB2 receptor antagonist, did not exert apparent effects. These results strongly suggest that the endogenous ligand for the cannabinoid receptors, *i.e.* 2-AG, interacts with the CB1 receptor to attenuate the release of glutamate upon depolarization.

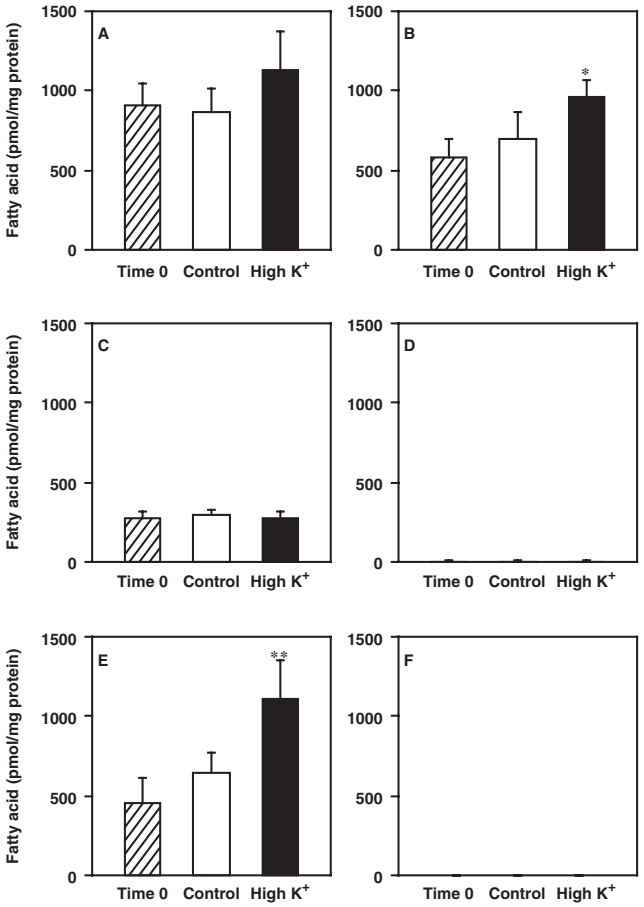

Fig. 6. **Effects of depolarization on the levels of individual fatty acyl moieties of diacylglycerols in rat brain synaptosomes.** Rat brain synaptosomes (5 mg protein) were incubated in 5 ml of 20 mM TES buffer (pH 7.2) containing 3 mM (control) or 80 mM KCl (high K^+), 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose and 1 mM DFP at 30°C for 30 s. The incubation was terminated by adding chloroform : methanol (1 : 2, v/v) and total lipids were extracted. The fatty acyl moieties of the purified diacylglycerols were analysed by GLC as described in 'MATERIALS AND METHODS'. (A) palmitic acid; (B) stearic acid; (C) oleic acid plus *cis*-vaccenic acid; (D) linoleic acid; (E) arachidonic acid; (F) docosahexaenoic acid. Time 0, hatched bar; control open bar; high K^+, closed bar. The data are the means ± SD of five determinations. $^*P < 0.05$, $^{**}P < 0.01$ (compared with control).

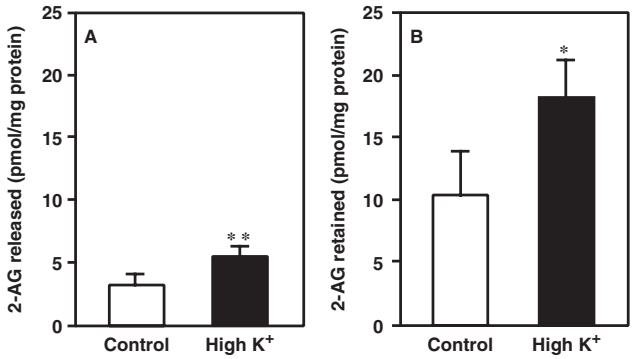

Fig. 7. **Release of 2-AG from rat brain synaptosomes.** Rat brain synaptosomes (10 mg protein) were incubated in 10 ml of 20 mM TES buffer (pH 7.2) containing 3 mM (control) or 80 mM KCl (high K^+), 127 mM or 50 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 15 mM sucrose, 0.25% BSA and 1 mM DFP at 30°C for 30 s. The incubation mixture was then filtered through a GF/B filter. Total lipids were extracted from both the filtrate and the synaptosomes trapped on a GF/B filter. The amounts of 2-AG in both fractions were analysed as described in 'MATERIALS AND METHODS'. (A) released; (B) synaptosomes-associated. Control, open bar; high K^+, closed bar. The data are the means ± SD of four determinations. $^*P < 0.05$, $^{**}P < 0.01$ (compared with control).

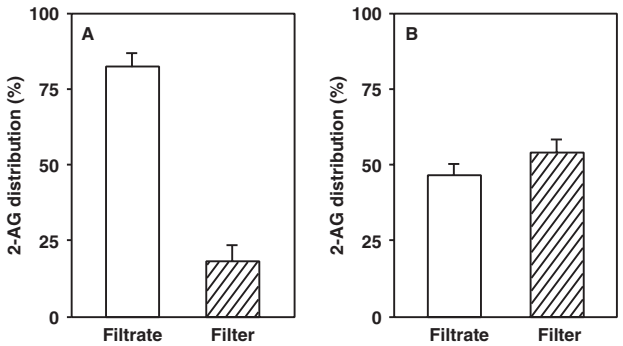

Fig. 8. **Binding of 2-AG to rat brain synaptosomes.** 2-AG (1 nmol) was incubated in 10 ml of 20 mM TES buffer (pH 7.2) containing 3 mM KCl, 127 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM EGTA, 10 mM glucose, 15 mM sucrose, 0.25% BSA and 1 mM DFP in the presence or absence of rat brain synaptosomes (10 mg protein) at 30°C for 30 s. The incubation mixture was then filtered through a GF/B filter. Total lipids were extracted from both the filtrate and the GF/B filter with or without synaptosomes. The amounts of 2-AG in both fractions were analysed as described in 'MATERIALS AND METHODS'. (A) without synaptosomes; (B) with synaptosomes. Filtrate, open bar; GF/B filter, hatched bar. The data are the means ± SD of four determinations.

DISCUSSION

The CB1 receptor is abundantly expressed in the brain (*21*, *22*). Among the various brain regions, the CB1 receptor is particularly abundant in the substantia nigra, globus pallidus, molecular layer of the cerebellum, hippocampus and cerebral cortex (*22*), suggesting that the CB1 receptor is involved in the regulation of motor activity, memory and cognition. Notably, the CB1 receptor is mainly located presynaptically (*22*). This strongly suggests that it plays an essential role in the attenuation of synaptic transmission, like other presynaptically expressed receptors (*27*, *28*). In support of this, various cannabimimetic molecules have been shown to inhibit voltage-gated Ca^{2+} channels, activate an inwardly rectifying K^+ current, and suppress the release of neurotransmitters from presynaptic terminals (*27*, *28*). Thus, detailed studies on the physiological functions of the CB1 receptor are essential for a thorough elucidation of the precise regulatory mechanism underlying synaptic transmission in certain regions of the brain.

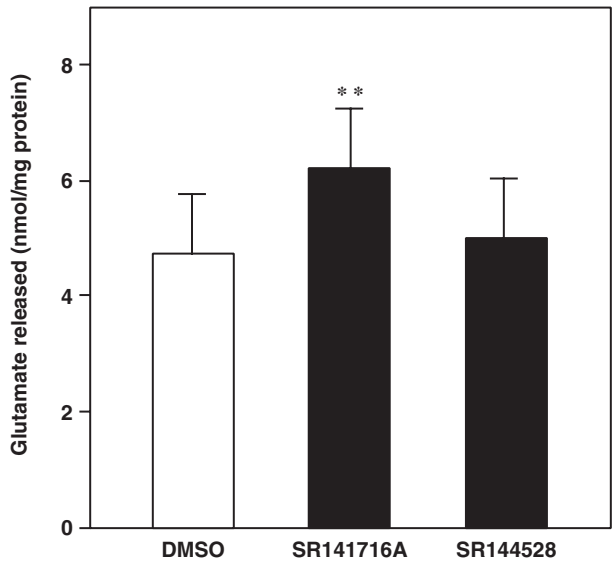

Fig. 9. **Effects of cannabinoid receptor antagonists on the depolarization-induced rapid release of glutamate from rat brain synaptosomes.** Rat brain synaptosomes (1 mg protein) were suspended in 300 µl of 20 mM TES buffer (pH 7.2) containing 3 mM KCl, 127 mM NaCl, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose and 15 mM sucrose and kept at 4°C for 30 min. Following the addition of SR141716A or SR144528 (each final concentration, 1 µM) or DMSO (vehicle) (0.2%, v/v), DFP (1 mM), PDC (10 µM) and KCl (80 mM) to synaptosomes, the mixture was incubated at 30°C for 1 min. It was then cooled to 4°C and centrifuged at 17,000*g* for 1 min. The supernatant was collected and further centrifuged at 17,000*g* for 1 min to remove the contaminated membrane fraction. The amount of glutamate in the supernatant was estimated as described in 'MATERIALS AND METHODS'. The amount of glutamate released upon depolarization was estimated by subtracting the amount of glutamate present in the supernatant at time 0 (6.7 nmol/mg protein) from that detected following depolarization. DMSO, open bar; SR141716A or SR144528, closed bar. The data are the means ± SD of 10 determinations. $^{**}P < 0.01$ (compared with DMSO (vehicle) and SR144528).

Previously, we investigated in detail the structure–activity relationship of the cannabinoid receptor agonists using NG108-15 cells which express the CB1 receptor (*8–10*). We found that 2-AG is the most efficacious agonist among various structural analogs. We concluded that 2-AG rather than anandamide is the true natural ligand for the CB1 receptor (*8–10*). We then examined the generation of 2-AG in the brain using a brain homogenate and an animal model of seizure. We found that 2-AG was rapidly generated in rat brain homogenate when incubated in the presence of Ca^{2+} (*29*) and in rat brain following the administration of picrotoxinin which induces convulsions (*30*). We also found that the rapid generation of 2-AG took place in rat brain after decapitation (*31*). Based on these experimental results, together with those by other investigators, we proposed that the physiological role of 2-AG in the synapse is as follows: 2-AG, generated through increased metabolism of inositol phospholipids upon synaptic transmission, plays an essential role in attenuating subsequent neurotransmitter release from the presynaptic terminals by acting on the CB1 receptor (*32*). In this scheme, 2-AG was first rapidly formed from inositol phospholipids in neurons during accelerated synaptic transmission. The 2-AG generated was subsequently released into the synaptic cleft, because 2-AG is a membrane-permeable molecule as mentioned before. The released 2-AG then binds to the CB1 receptor expressed in presynaptic terminals and stimulates Gi/o to inhibit voltage-gated Ca^{2+} channels and reduce the intracellular free Ca^{2+} concentration, thereby diminishing neurotransmitter release (*32*). This hypothesis was attractive from a variety of viewpoints and appeared to be very likely, although the details are yet to be verified.

In this study, we first examined whether depolarization triggers the generation of 2-AG. We found that depolarization of synaptosomes induced a rapid generation of 2-AG (Figs 1 and 2). This observation is particularly of interest, because such a rapid generation is essential for 2-AG to act as a fast signalling molecule in the brain. Notably, arachidonic acid-containing diacylglycerols were also generated concomitant with 2-AG upon depolarization (Fig. 6). On the other hand, the level of oleic acid or *cis*-vaccenic acid-containing species did not change markedly. Moreover, the levels of linoleic acid-containing species and docosahexaenoic acid-containing species were very low. We also found that the ratio of the amount of arachidonic acid to that of docosahexaenoic acid is 1.3, 0.5 and 13.0 for phosphatidylcholine fraction, phosphatidylethanolamine fraction and phosphatidylinositol fraction, respectively (Oka, S. and Sugiura, T., unpublished data). These results strongly suggest that the diacylglycerol derived from increased inositol phospholipid turnover acts as the predominant precursor for 2-AG in depolarized synaptosomes.

The finding that a significant part of the newly generated 2-AG was released from synaptosomes (Fig. 7) is also noteworthy, because such a molecular characteristic is essential for an intercellular mediator in the synapse. Several investigators have also demonstrated that 2-AG was rapidly released from stimulated tissues and cells *in vitro* (*20*, *33*). Further studies are needed to clarify the mechanism underlying rapid release of 2-AG from synaptosomes and neuronal cells. Importantly, treatment of the synaptosomes with SR141716A, a CB1 receptor antagonist, and not with SR144528, a CB2 receptor antagonist, enhanced the depolarization-induced release of glutamate (Fig. 9), which points to a possible essential role of the endogenous ligand for the CB1 receptor, that is 2-AG, in the regulation of glutamatergic synaptic transmission. These results support the hypothesis mentioned above that 2-AG, derived from stimulated neurons through the augmented breakdown of inositol phospholipids, has an important role in attenuating neurotransmission via a negative feedback mechanism.

This appears not to be the case with anandamide. Firstly, anandamide acted as a partial agonist toward the CB1 receptor whereas 2-AG acted as a full agonist (*6*, *7*). Secondly, the amounts of anandamide generated in stimulated tissues and cells were usually very small (*7*). Thirdly, in contrast to 2-AG, no selective and efficient synthetic pathways have thus far been reported for anandamide (*6*, *7*). All these characteristics do not favour

the notion that anandamide acts as a CB1 receptor agonist of great physiological significance in the brain.

Concerning 2-AG and synaptic transmission, Stella *et al*. (*18*) reported that 2-AG (20 μM) suppressed long-term depression (LTD) induced by high-frequency stimulation of Schaffer collaterals in the CA1 stratum radiatum in a CB1 receptor-dependent manner. Ameri and Simmet (*19*) also demonstrated that 2-AG (10–50 μM) attenuated the amplitude of the orthodromic population spike and the slope of the field excitatory postsynaptic potential. As for the generation of 2-AG, Stella and Piomelli demonstrated that it took place in electrically stimulated rat hippocampal slices (*18*), ionomycin-stimulated neurons (*18*) and *N*-methyl-D-aspartic acid-stimulated rat cortical neurons (*34*), and Bisogno *et al*. (*20*) demonstrated it in ionomycin-stimulated N18TG2 cells. These results do not conflict with the experimental results concerning the generation and the action of 2-AG shown in the present study and those in our previous studies (*29–31*). In any case, as mentioned before, 2-AG appears to possess an important physiological role in attenuating excitatory neurotransmission thereby preventing excessive excitability which might be harmful to neurons.

Recently, there has arisen another possible function of the endogenous ligand for the CB1 receptor in the brain: that is, a role as a mediator of depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE), both of which involve the suppression of synaptic transmission upon the depolarization of post-synaptic neurons. In 2001, three groups independently reported that the endogenous ligand for the CB1 receptor acts as a retrograde messenger in DSI and DSE (*35–37*). A number of studies have thus far been carried out on DSI and DSE (*38–42*); yet the molecule actually involved in DSI and DSE remained ambiguous until recently. Despite the need for further studies to fully solve this problem, several lines of evidence obtained from recent studies employing several enzyme inhibitors and gene-knockout mice strongly suggest that 2-AG is an effector molecule of DSI and DSE (*43–46*). The results of the present investigation appear to be in favour of the notion that the relevant molecule in DSI and DSE is 2-AG rather than anandamide, because depolarization triggered the generation of 2-AG but not anandamide (Fig. 1) and a CB1 receptor antagonist augmented the depolarization-induced release of glutamate from synaptosomes (Fig. 9).

Very recently, another possibility has arisen that the endogenous ligand for the CB1 receptor takes part in LTD. Several investigators demonstrated that inhibitors of 2-AG synthesis, such as RHC-80267, a diacylglycerol lipase inhibitor, and U73122, a phospholipase C inhibitor, prevented the induction of LTD in rat hippocampal slices (*47*) and rat or mouse cerebellar slices (*48*). These results strongly suggest that 2-AG is closely involved in the induction of LTD, while the details are yet to be determined.

More than 10 years have passed since the identification of 2-AG as an endogenous ligand for the cannabinoid receptors. At first, 2-AG did not receive much attention, probably because anandamide was in the spotlight at that time. Now, the situation has changed and increasing attention is being directed to 2-AG. Multiple lines of evidence strongly suggest that 2-AG plays important regulatory roles in the nervous system, yet the available information is not sufficient for a full understanding of the physiological and pathophysiological significance of 2-AG. The CB1 receptor is one of the most abundant G protein-coupled receptors in the brain (*22*). Further detailed studies on 2-AG and the CB1 receptor in the brain are thus essential for a thorough elucidation of the precise regulatory mechanisms underlying advanced brain functions such as cognition, memory and control of movement.

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