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2-ARACHIDONOYLGLYCEROL: A POSSIBLE ENDOGENOUS CANNABINOID RECEPTOR LIGAND IN BRAIN

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Summary: The effects of anandamide, 2-arachidonoylglycerol and related compounds on the specific binding of a radiolabeled cannabinoid receptor ligand, [^{3}H]CP55940, to synaptosomal membranes were examined. Anandamide, an endogenous cannabinoid receptor ligand, reduced the specific binding of [^{3}H]CP55940 to synaptosomal membranes in a dose-dependent manner: the Ki value was 89 nM. 2-Arachidonoylglycerol was also shown to bind appreciably to the cannabinoid receptor in competitive inhibition experiments. The apparent binding affinity was markedly increased when the binding assay was carried out in the presence of the esterase inhibitor DFP or at 0^{o}C. Free arachidonic acid and N-palmitoylethanolamine were almost inactive in terms of binding to the cannabinoid receptor in synaptosomal membranes. 2-Arachidonoylglycerol may be an endogenous cannabinoid receptor ligand in the brain.

Anandamide (N-arachidonoylethanolamine) is an endogenous cannabinoid receptor ligand first described by Devane et al. (1) in 1992. Anandamide has been shown to possess potent cannabimimetic activity in various biological systems either in vitro or in vivo (1-5). The structure-activity relationship of anandamide has been explored extensively by Mechoulam and co-workers (6) and by others (2).

We have recently studied the actions of N-acylethanolamine phosphate on human platelets and found that this compound is a potent agonist toward lysophosphatidic acid receptor (7). In this series of experiments, we noticed that N-acylethanolamine phosphate and alkyllysophosphatidic acid have rather similar biological activity even though their chemical structures are

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Abbreviations: DFP, diisopropyl fluorophosphate; BSA, bovine serum albumin; Fatty acids are designated in terms of number of carbon atoms:number of double bonds, e.g., 20:4 for arachidonic acid.

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considerably different from each other: the latter compound contains a glycerol backbone, while the former one contains the N-acylethanolamine structure and lacks a glycerol moiety. This observation prompted us to examine whether analogues of anandamide containing a glycerol backbone possess cannabimimetic activities. In a previous communication (8), we reported that 2-arachidonoylglycerol exhibits binding affinity toward cannabinoid receptor in rat brain synaptosomes and we suggested that this compound may function as an endogenous cannabinoid receptor ligand at some sites in the brain. However, its binding affinity estimated under our former experimental conditions (without inhibitors of esterases) was much weaker than that observed for anandamide estimated under the same experimental conditions, probably because of the susceptibility of 2-arachidonoylglycerol to lipase(s), which may degrade 2-arachidonoylglycerol to release arachidonic acid during the binding assay. In the present study, we compared in detail the binding affinities of anandamide, 2-arachidonoylglycerol and several other analogues to synaptosomal membranes in the presence or absence of diisopropyl fluorophosphate (DFP), which is a potent inhibitor of various types of serine esterases, including monoacylglycerol lipase (9-11), or at 0^{o}C. We established that 2-arachidonoylglycerol possesses relatively high binding affinity toward cannabinoid receptor in synaptosomal membranes and that its amount in brain is about a thousand times higher than that of anandamide. These results strongly suggest that 2-arachidonoylglycerol is another candidate for an endogenous cannabinoid receptor ligand.

MATERIALS AND METHODS

Materials: A non-classical cannabinoid receptor radiolabeled ligand [^{3}H]CP55940 (113 Ci/mmol) was purchased from Dupont-NEN (Boston, MA). Arachidonic acid (20:4), heptadecanoic acid (17:0), oleic acid (18:1), pentadecanoic acid (15:0) methyl ester and essentially fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Palmitoyl chloride was prepared by treating palmitic acid with oxalyl chloride. Anandamide was purchased from Research Biochemicals International (Natick, MA). N-Palmitoylethanolamine was prepared by reacting palmitoyl chloride with ethanolamine and purified by TLC developed with petroleum ether:diethyl ether:acetone:acetic acid (30:40:20:1, v/v). DFP, polyethylenimine P-70 and other chemicals (analytical grade) were from Wako Pure Chem. Ind. (Osaka, Japan). Lipase (Rhizopus delemar) was from Seikagaku Kogyo (Tokyo, Japan). GF/B filters were purchased from Whatman (Maidstone, England). 2-Arachidonoylglycerol, 2-oleoylglycerol and 2-heptadecanoylglycerol were

prepared as follows. Fatty acids (100 mg) dissolved in 1 ml of chloroform (containing 2-methyl-2-butene as a stabilizer) were first converted to fatty acid anhydride by treatment with 100 mg of dicyclohexylcarbodiimide (12) and then mixed with 20 mg of glycerol, 20 mg of dimethylaminopyridine and 0.1 ml of pyridine in a tube sealed under N_2 gas. The mixture was stirred overnight. The resultant triacylglycerol was purified by TLC developed with petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). Triacylglycerol (50 mg) was dispersed in 3 ml of 50 mM sodium acetate-acetic acid buffer (pH 5.6) containing 0.1 M NaCl and 10 mM $CaCl_2$ by brief sonication and then hydrolyzed by exposure to lipase (30 mg) for 60 min under vigorous stirring. Monoacylglycerol was purified by TLC developed with petroleum ether:diethyl ether:acetic acid (20:80:1, v/v). The content of monoacylglycerol was estimated by quantitating its fatty acyl moiety by GLC after conversion to fatty acid methyl esters by treatment with 0.5 M methanolic sodium methoxide. 15:0 Fatty acid methyl ester was used as an internal standard.

Preparation of synaptosomes: Rat brain synaptosomal membranes were prepared by the modified method of Whittaker et al. (13). Briefly, rats (Wistar, male, 350-400 g) were killed by decapitation and the brain was removed. The cerebrum was isolated and homogenized in 0.32 M sucrose/10 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was taken and further centrifuged at 17000 x g for 55 min. This supernatant was aspirated and the pellet was dissolved in 0.32 M sucrose and carefully layered on a discontinuous sucrose gradient (upper, 0.8 M sucrose; lower, 1.2 M sucrose). Then, the tubes were centrifuged at 63000 x g for 120 min using a Hitachi swinging rotor (SRP28SA). The interfacial layer between 0.8 M sucrose and 1.2 M sucrose was carefully taken, and diluted with distilled water and further centrifuged at 105000 x g for 60 min. The pellet was dissolved in 16.6 mM sucrose/25 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and stored at $-80^{\circ}C$. This fraction was used as synaptosomal membranes. The protein content was estimated by the method of Lowry et al. (14).

Binding assay: The binding assay was carried out by the modified method of Devane et al. (15). Briefly, synaptosomal membranes (50 μg protein) were incubated with 50 fmol of [^{3}H]CP55940 (12500 dpm) in 500 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 3 mM $MgCl_2$ in the presence or absence of varying concentrations of anandamide, 2-arachidonoylglycerol and other compounds (dissolved in 2.5 % BSA, the final concentration of BSA in the incubation mixture was 0.25 %) at $30^{\circ}C$ for 60 min. In some experiments, synaptosomal membranes (100 μg protein) were incubated in the same buffer at $0^{\circ}C$ for 240 min. In the case of the buffer containing 1 mM DFP, synaptosomal membranes (75 μg protein) were incubated with [^{3}H]CP55940 and various compounds at $30^{\circ}C$ for 60 min. Non-specific binding of the radiolabeled ligand was determined from the binding in the presence of 20 μM anandamide. After the incubation, 2.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.05 % BSA was added to the tube and the mixture was immediately filtered on a GF/B filter which had been pre-treated with 0.1 % polyethylenimine. The filter was further washed with 2.5 ml of the same buffer 4 times and dried prior to measurement of the radioactivity.

Analysis of monoacylglycerol in brain: Rats were killed by decapitation and the brains were removed. The brains were homogenized in chloroform:methanol (1:2, v/v), and the total lipids were extracted by the method of Bligh and Dyer (16). 2-Heptadecanoylglycerol (25 nmol) was added as an internal standard. Lipids were applied to a silicic acid column and neutral lipids were eluted with chloroform. Neutral lipids were then separated by TLC developed with petroleum ether:diethyl ether:acetic acid (20:80:1, v/v). The monoacylglycerol fraction was extracted from the silica gel by the method of Bligh and Dyer (16) and further purified by TLC developed with the same solvent system. The fatty acyl moiety of monoacylglycerol was converted to fatty acid methyl ester with sodium methoxide and analyzed by GLC.

RESULTS AND DISCUSSION

First, we examined the kinetics of the binding of [^{3}H]CP55940 to synaptosomal membranes. The binding of [^{3}H]CP55940 to synaptosomal membranes was saturable as reported earlier (15). The Kd value estimated at 30°C was 0.83 ± 0.06 nM and the Bmax was 2.25 ± 0.64 pmol/mg protein (the means ± SD of three separate experiments, with 10-12 determinations each (n=3)). The Kd value and the Bmax estimated in the presence of 1 mM DFP were 4.29 ± 1.20 nM and 2.05 ± 0.22 pmol/mg protein, respectively (the means ± SD of three separate experiments, with 10-12 determinations each (n=6)), and those estimated at 0°C were 2.53 nM and 0.72 pmol/mg protein, respectively (the means of two separate experiments, with 10-12 determinations each (n=6)).

Next we examined the effects of anandamide and related lipid molecules on the specific binding of [^{3}H]CP55940. Fig. 1 shows the competitive inhibition of the binding of [^{3}H]CP55940 to synaptosomal membranes by various compounds. The specific binding of [^{3}H]CP55940 to synaptosomal membranes was markedly decreased with increased concentrations of anandamide (Fig. 1 (a)). The Ki value was calculated to be 89 nM. A similar value (99 nM) was observed when the binding experiments were carried out in the presence of 1 mM DFP (Fig. 1 (b)). It should be noted that 2-arachidonoylglycerol also inhibits the specific binding of [^{3}H]CP55940 in a dose-dependent manner. The Ki value for 2-arachidonoylglycerol estimated in the presence of 1 mM DFP was 2.4 μM (Fig. 1 (d)), indicating that 2-arachidonoylglycerol has a binding affinity which is about 24 times lower than that of anandamide estimated under the same experimental conditions. We also observed that the Ki value for 2-arachidonoylglycerol estimated at 0°C was about 4.6 times higher than that for anandamide estimated under the same assay condition (Fig. 2). However, the Ki value for 2-arachidonoylglycerol was elevated to 48 μM (540 times higher than that for anandamide), if DFP was omitted from the assay system and the incubation was carried out at 30°C (Fig. 1 (c)). We also found that arachidonoylethyleneglycol has a similar binding activity to that of 2-arachidonoylglycerol, while 2-oleoylglycerol fails to inhibit the specific

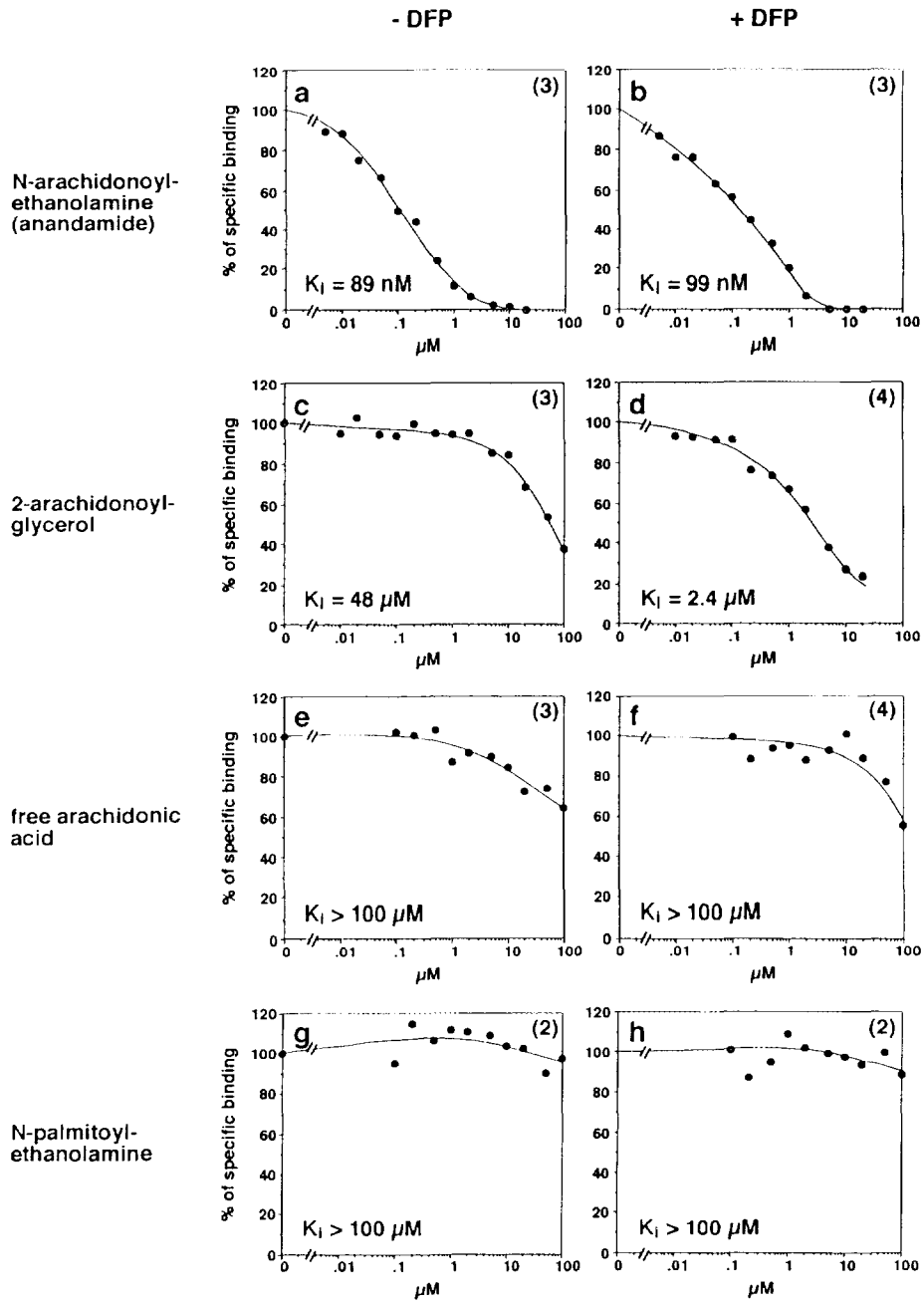

Fig. 1. Effects of anandamide, 2-arachidonoylglycerol and related molecules on the specific binding of [^{3}H]CP55940 to synaptosomal membranes in the presence or absence of 1 mM DFP at 30°C. The data are the means of two to four separate experiments (in parenthesis) each done in triplicate or quadruplicate.

binding of [^{3}H]CP55940 to synaptosomal membranes (data not shown). Free arachidonic acid itself did not exhibit high affinity for cannabinoid receptor, either in the presence or absence of DFP (Fig. 1 (e) and (f)). Further, N-

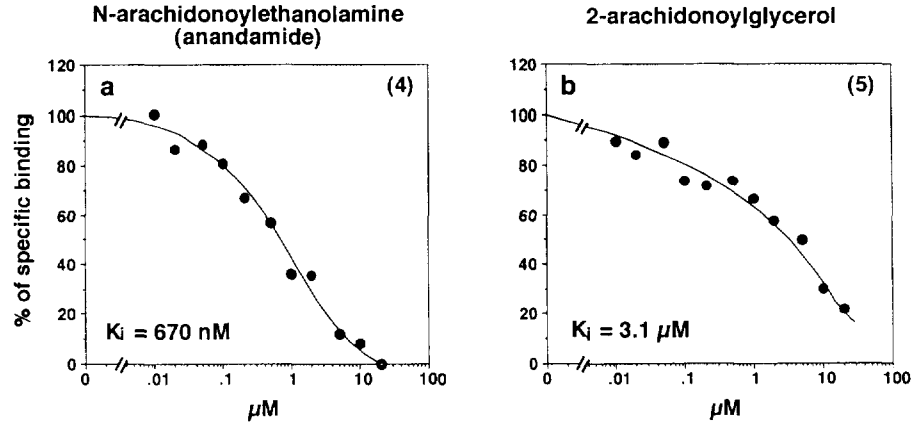

Fig. 2. Effects of anandamide and 2-arachidonoylglycerol on the specific binding of [^{3}H]CP55940 to synaptosomal membranes at 0°C. The data are the means of four to five separate experiments (in parenthesis) each done in quadruplicate.

palmitoylethanolamine (Fig. 1 (g) and (h)) was virtually inactive, at least in terms of binding to the cannabinoid receptor in synaptosomal membranes.

Then, we examined the level and the fatty acid profile of monoacylglycerol in brain. As shown in Table 1, rat brain contains monoacylglycerol at the level of 6.86 nmol/g wet tissue. Notably, the proportion of arachidonic acid-containing species was very high; arachidonic acid-containing species account for as much as 47 % of total monoacylglycerol. The concentration of arachidonoylglycerol in this tissue is, thus, approximately 4 μM, supposing

TABLE 1

Fatty acid composition of monoacylglycerol in rat brain

Fatty acyl moiety	%	nmol/g wet weight
16:0	11.2	0.77 ± 0.46
16:1	0.4	0.03 ± 0.01
18:0	5.5	0.38 ± 0.09
18:1	12.4	0.85 ± 0.44
18:2	0.9	0.06 ± 0.03
20:3	0.7	0.05 ± 0.01
20:4	47.4	3.25 ± 0.52
22:4	2.2	0.15 ± 0.04
22:5	1.8	0.12 ± 0.10
22:6	17.5	1.20 ± 0.56
total	100	6.86 ± 2.37

The data are the means ± SD of five separate experiments.

that 80 % of the tissue consists of water and that arachidonoylglycerol is homogeneously distributed in this tissue.

The binding affinity of 2-arachidonoylglycerol estimated in the absence of DFP at 30°C was considerably lower than that of anandamide. This can be attributed, at least in part, to possible hydrolysis of 2-arachidonoylglycerol by monoacylglycerol lipase, which is known to be present in brain tissues (17), during the incubation. In fact, in the presence of DFP, a potent inhibitor of monoacylglycerol lipase (9-11), the apparent binding affinity of 2-arachidonoylglycerol to cannabinoid receptor was increased (Fig. 1). Furthermore, we confirmed that the apparent binding activity of 2-arachidonoylglycerol was also markedly increased when the binding experiments were carried out at 0°C (Fig. 2). It is clear, therefore, that the actual activity of 2-arachidonoylglycerol is much higher than that observed in experiments where the activity of monoacylglycerol lipase is not blocked.

2-Arachidonoylglycerol can be formed through several metabolic pathways in brain (Fig. 3). First, inositol phospholipids are hydrolyzed by phospholipase C to release diacylglycerol enriched in arachidonic acid such as 1-stearoyl-2-arachidonoylglycerol. Then, this unique molecular species of diacylglycerol is hydrolyzed through the action of diacylglycerol lipase to release 2-arachidonoylglycerol. Another synthetic pathway involves the hydrolysis of phosphatidylinositol by phosphatidylinositol-specific phospholipase A_1, which

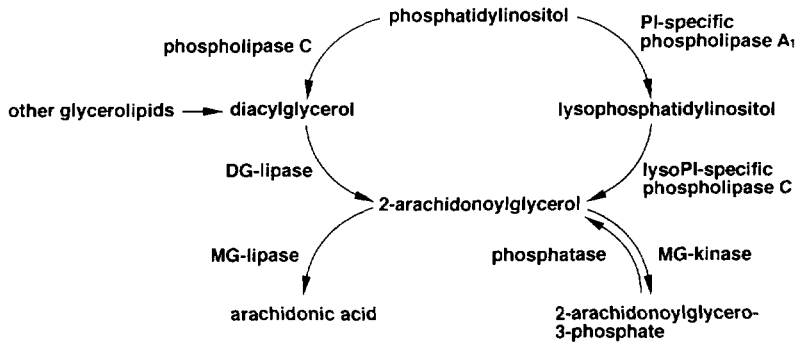

Fig. 3. Possible synthetic and metabolic pathways of 2-arachidonoylglycerol in brain.

is known to be present in brain (18,19). Alternatively, phosphatidylinositol may be hydrolyzed by a CoA-dependent degradation pathway to yield arachidonic acid-containing lysophosphatidylinositol in addition to stearic acid-containing species (20). Then, the resultant arachidonic acid-containing lysophosphatidylinositol is hydrolyzed by lysophosphatidylinositol-specific phospholipase C, which is also known to be present in brain (19), especially in synaptosomes (21). In both cases, the end product is 2-arachidonoylglycerol. The presence of such synthetic pathways is consistent with the observation that monoacylglycerol present in this tissue is particularly enriched in arachidonic acid-containing species (Table 1). Thus, it appears that 2-arachidonoylglycerol is not merely an end product of inositol phospholipid metabolism, but has some physiological role.

The observation that 2-arachidonoylglycerol has cannabimimetic binding activity is interesting in view of the possible linkage of enhanced inositol phospholipid metabolism within neuronal cells and the regulation of synaptic function through cannabinoid receptor expressed on the cell surface. It has already been demonstrated that arachidonoylglycerol is released from cells into the extracellular fluid upon stimulation (22). Recently, we found that the amount of anandamide in brain is of the order of pmol/g wet weight tissue (Sugiura, T. and Kondo, S., unpublished data), which is about a thousand times smaller than that of 2-arachidonoylglycerol found in the present study (Table 1), although little is so far known concerning the localization of these lipid molecules in tissues. In any case, the relative importance of anandamide and 2-arachidonoylglycerol in this tissue remains to be determined.

Not much attention has so far been paid to 2-arachidonoylglycerol, an interesting molecule from various biochemical and pharmacological viewpoints. Studies on possible physiological roles of this unique lipid mediator are in progress in our laboratory.

During the preparation of this manuscript, Mechoulam et al. (23) reported that 2-arachidonoylglycerol binds to cannabinoid receptors on rat spleen cells and receptor gene-transfected COS-7 cells.

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