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Review

2-Arachidonoylglycerol and the cannabinoid receptors

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Abstract

2-Arachidonoylglycerol (2-AG) is a unique molecular species of monoacylglycerol isolated from rat brain and canine gut as an endogenous cannabinoid receptor ligand (Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., Waku, K., 1995. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem. Biophys. Res. Commun. 215, 89–97; Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., Pertwee, R.G., Giffin, G., Bayewitch, M., Brag, J., Vogel, Z., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50, 83–90). 2-AG binds to the cannabinoid receptors (CB1 and CB2) and exhibits a variety of cannabimimetic activities in vitro and in vivo. Recently, we found that 2-AG induces Ca^{2+} transients in NG108-15 cells, which express the CB1 receptor, and in HL-60 cells, which express the CB2 receptor, through a cannabinoid receptor- and Gi/Go-dependent mechanism. Based on the results of structure-activity relationship experiments, we concluded that 2-AG but not anandamide is the natural ligand for both the CB1 and the CB2 receptors and both receptors are primarily 2-AG receptors. Evidences are gradually accumulating that 2-AG is a physiologically essential molecule, although further detailed studies appear to be necessary to determine relative importance of 2-AG and anandamide in various animal tissues. In this review, we described mainly our previous and current experimental results, as well as those of others, concerning the tissue levels, bioactions and metabolism of 2-AG.

Keywords: 2-Arachidonoylglycerol; Anandamide; Cannabinoid; Monoacylglycerol; Δ^9-Tetrahydrocannabinol; Lysophosphatidic acid

1. Cannabinoid receptors

Δ^9-Tetrahydrocannabinol (Δ^9-THC), a major psychoactive ingredient of marijuana, possesses a variety of pharmacological activities. When administered orally or intravenously, Δ^9-THC induces diverse biological responses such as reduced spontaneous motor activity, immobility, analgesia, heightened sensory awareness, euphoria, hypothermia and impairment of short-term memory (Dewey, 1986). Δ^9-THC is also known to exert profound effects on several biological systems other than the central nervous system: for example, Δ^9-THC exhibits a potent immunosuppressive

Abbreviations: 2-AG, 2-arachidonoylglycerol; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; LPA, lysophosphatidic acid; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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activity (Hollister, 1992). Until recently, however, the mechanism of the action of Δ^9-THC in the central nervous system, as well as peripheral tissues, remained uncertain.

In the late 1980s, Howlett and co-workers provided evidence that a specific binding site(s) for cannabinoids is present in the brain, using a radiolabeled synthetic cannabinoid [^{3}H]CP55940 (Devane et al., 1988). Their finding strongly suggested that various cannabimimetic molecules, as well as Δ^9-THC itself, bind to a specific receptor site(s) thereby eliciting a variety of responses. This had also been suggested by Mechoulam and co-workers based on the observation that $(-)$-Δ^9-THC exhibits potent pharmacological activities, whereas its stereoisomer $(+)$-Δ^9-THC is far less potent (reviewed in Mechoulam et al., 1992).

A decade ago, Matsuda et al. (1990) cloned a cDNA encoding a cannabinoid receptor (CB1) from a rat brain cDNA library. The CB1 receptor is a seven-transmembrane, G protein-coupled receptor, and contains 472 (human) or 473 (rat) amino acids. Soon after, Munro et al. (1993) cloned another cDNA encoding a cannabinoid receptor (CB2) from a HL-60 cell cDNA library. The CB2 receptor is also a seven-transmembrane, G protein-coupled receptor, and consists of 360 amino acid residues. The CB1 receptor and the CB2 receptor share 44% overall identity (68% identity for the transmembrane domains). Importantly, the tissue distributions of these cannabinoid receptors are quite different from each other; the CB1 receptor is expressed primarily in the nervous system and the CB2 receptor is expressed mainly in the immune system (Matsuda and Bonner, 1995; Matsuda et al., 1990; Munro et al., 1993; Herkenham, 1995). Thus, there is likely to be a clear distinction between the physiological roles of these two subtypes of cannabinoid receptors. The CB1 receptor is abundant in substantia nigra, globus pallidus, molecular layer of cerebellum, hippocampus and cerebral cortex, and is assumed to be involved in the regulation of cognition, memory and motor activity (Matsuda and Bonner, 1995; Matsuda et al., 1990; Herkenham, 1995). On the other hand, the CB2 receptor is present abundantly in several types of leukocytes such as macrophages/monocytes, B lymphocytes and natural killer cells, and is assumed to participate in the regulation of immune responses and/or inflammatory reactions (Munro et al., 1993; Galiegue et al., 1995; Matsuda and Bonner, 1995), though the details remain to be elucidated. In any case, because of the high abundance of these receptors in mammalian tissues, it is reasonable to assume that both subtypes of cannabinoid receptors are physiologically essential, and that their endogenous ligand(s) plays a key role in diverse mammalian tissues and cells.

2. Identification of 2-AG as a cannabinoid receptor ligand

The discovery of the specific receptors for cannabinoids stimulated the search for endogenous ligand(s). Devane et al. (1992) isolated *N*-arachidonoylethanolamine (anandamide) (Fig. 1) from porcine brain as the first endogenous cannabinoid receptor ligand. They demonstrated that anandamide exhibits several cannabimimetic activities in vitro and in vivo. Anandamide appears to be an important lipid mediator in the nervous system, as well as in other systems (Mechoulam and Fride, 1995; Mechoulam et al., 1998a; Hillard and Campbell, 1997; Di Marzo, 1998b; Di Marzo et al., 1998a; Piomelli et al., 1998). However, we (Sugiura et al., 1996a,c) and others (Schmid et al., 1995; Felder et al., 1996; Kempe et al., 1996) have found that the levels of anandamide are very low in several mammalian tissues. In addition, the biosynthetic pathways for anandamide, either the *N*-acylphosphatidylethanolamine pathway (Di Marzo et al., 1994; Hansen et al., 1995; Sugiura et al., 1996a,c; Cadas et al., 1997) or the condensation pathway (Deutsch and Chin, 1993; Devane and Axelrod, 1994; Kruszka and Gross, 1994; Ueda et al., 1995; Sugiura et al., 1996c), do not appear able to provide large amounts of anandamide, at least under normal conditions in vivo, because the availabilities of the substrates are usually low. Furthermore, several investigators found that anandamide is produced mainly in the post-mortem period in the brain (Schmid et al., 1995; Felder et al., 1996; Kempe et al., 1996). Thus, the physiological significance or meaning of

anandamide, especially in the brain, has been questioned, despite its high binding affinity toward the cannabinoid receptor(s).

We started studies on 2-AG (Fig. 1) in 1993. Previously, we noticed that *N*-acylethanolamine phosphate (Fig. 1) and lysophosphatidic acid (LPA) (Fig. 1) interact with a common receptor site (LPA receptor) on human platelets and exhibit similar biological activities even though their chemical structures are considerably different from each other: the latter compound contains a glycerol backbone, while the former compound contains the *N*-acylethanolamine structure and lacks a glycerol moiety (Sugiura et al., 1994b). This prompted us to postulate that an analogue of anandamide containing a glycerol backbone, i.e. 2-AG, possesses cannabimimetic activities. This hypothesis was attractive in terms of possible linkage between the function of the cannabinoid receptors and the enhanced inositol phospholipid turnover, because 2-AG is a well-known degradation product of inositol phospholipid metabolism (Prescott and Majerus, 1983). We synthesized 2-AG and examined its binding activity toward the cannabinoid receptor in rat brain synaptosomes. We found that 2-AG possesses binding activity toward the cannabinoid receptor ($K_i = 15$ μM), although its activity was considerably lower than that of anandamide. We also found that AG is actually present in rat brain at concentrations of the order of nmol/g tissue. Based on these experimental results, we pointed out that arachidonic acid-containing monoacylglycerol may function as an endogenous cannabinoid receptor ligand at some sites in the brain (Sugiura et al., 1994a).

The reason for the relatively high apparent K_i value of 2-AG in that study may be due, at least in part, to possible hydrolysis of 2-AG during the incubation; we therefore examined the effect of the addition of diisopropyl fluorophosphate (DFP), an esterase inhibitor. The K_i value of 2-AG estimated in the presence of DFP was 2.4 μM and that of anandamide was 99 nM, indicating that the binding activity of 2-AG was 24 times less potent than that of anandamide. We also confirmed that rat brain contains 3.25 nmol/g tissue of AG, a level about 800 times higher than that of anandamide in the same tissue. These results support our idea that 2-AG is an important candidate for the endogenous ligand of the cannabinoid receptor. We reported these results at a satellite meeting of the 15th biennial meeting of the International Society for Neurochemistry (Lipid Messengers in the Nervous System Symposium) and published them in 1995 (Sugiura et al., 1995). Mechoulam et al. also reported that 2-AG

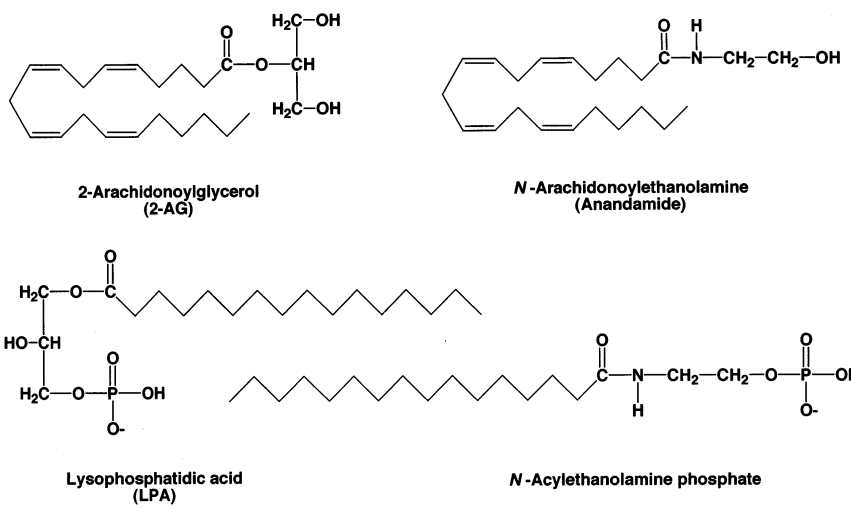

Fig. 1. Chemical structures of 2-AG, *N*-arachidonoylethanolamine (anandamide), LPA (1-palmitoyl) and *N*-acylethanolamine phosphate (*N*-palmitoyl).

is an endogenous cannabinoid receptor ligand at the same meeting and published their findings in 1995 (Mechoulam et al., 1995). They isolated 2-AG from canine gut and demonstrated that 2-AG possesses various activities, binding activity toward cannabinoid receptors expressed on COS-7 cells transfected with cannabinoid receptor genes, inhibition of adenylyl cyclase in mouse spleen cells and twitch response in mouse vas deferens, and induction of hypothermia, reduced spontaneous activity, analgesia and immobility in mice (Mechoulam et al., 1995). The above studies on 2-AG by us and by Mechoulam and co-workers were conducted independently and concurrently. At that time, however, 2-AG did not receive much attention, probably because another candidate, anandamide, was in the spotlight.

3. Biological activities of 2-AG

About 5 years have passed since the discovery of 2-AG as an endogenous cannabinoid receptor ligand. Nonetheless, available information concerning 2-AG is still limited as compared with the case of anandamide (Di Marzo, 1998a,b; Di Marzo and Deutsch, 1998; Di Marzo et al., 1998a, 1999a; Mechoulam et al., 1998a; Piomelli et al., 1998; Sugiura et al., 1998b). As mentioned above, 2-AG binds to the central and peripheral cannabinoid receptors (CB1 and CB2, Mechoulam et al., 1995; Sugiura et al., 1995), inhibits adenylyl cyclase and the twitch response in mouse vas deferens, and exerts several pharmacological effects on experimental animals (Mechoulam et al., 1995). 2-AG has also been shown to affect lymphocyte proliferation (Lee et al., 1995), although it remains uncertain whether this effect is due to 2-AG itself or arachidonic acid metabolites derived from 2-AG. We explored other possible biological activities of 2-AG intensively using cultured neural cells and synaptosomes, and found that 2-AG induces a rapid, transient elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neuroblastoma x glioma hybrid NG108-15 cells which express the cannabinoid CB1 receptor (Fig. 2, Sugiura et al., 1996b). This was surprising, because Felder et al. (1992) had found that cannabinoids did not induce cannabinoid receptor-mediated elevation of $[Ca^{2+}]_i$ in CHO cells transfected with the CB1 or the CB2 receptor. We also observed a similar elevation of $[Ca^{2+}]_i$ in N18TG2 neuroblastoma cells, but not in C6 glioma cells (Sugiura et al., 1997b). The effect in NG108-15 cells was detectable from low nanomolar concentrations of 2-AG. Free arachidonic acid did not induce such a response. We also confirmed that pretreatment of the cells with indomethacin, a cyclooxygenase inhibitor, or nordihydroguaiaretic acid, a lipoxygenase inhibitor, did not affect the response. Anandamide failed to exhibit potent agonistic activity in this assay system (Fig. 2). On the other hand, WIN55212-2, a well-known cannabinoid receptor agonist, was found to induce rapid transient increases in $[Ca^{2+}]_i$ similar to those seen with 2-AG, whereas its stereoisomer WIN55212-3, a compound lacking apparent cannabimimetic activity, did not. The response induced by 2-AG or WIN55212-2 was blocked by pretreatment of the cells with a cannabinoid CB1 receptor-specific antagonist SR141716A (Sugiura et al., 1996b). We found that the response induced by 2-AG was abolished by pretreatment of the cells with WIN55212-2 and vice versa. We also confirmed that pretreatment of the cells with several classical and synthetic cannabinoids such as Δ^9-THC, HU-210 and CP55940 nullified the response induced by 2-AG (Sugiura et al., 1997b, 1999b). These results clearly indicate that 2-AG interacts with

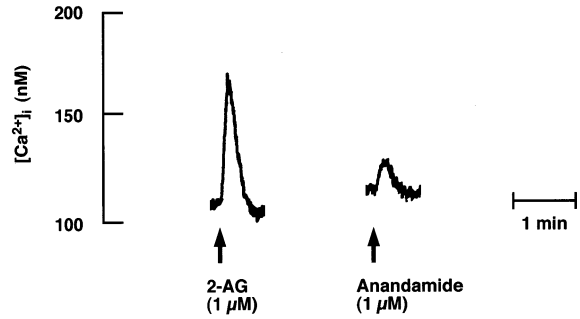

Fig. 2. Effects of 2-AG and anandamide on $[Ca^{2+}]_i$ in NG108-15 cells, which express the CB1 receptor. Cells were loaded with Fura-2/AM and the changes in $[Ca^{2+}]_i$ were determined using a CAF-100 Ca^{2+} analyzer.

the cannabinoid CB1 receptor, thereby eliciting the rapid elevation of $[Ca^{2+}]_i$.

The action of 2-AG was blocked by pretreatment of the cells with pertussis toxin (Sugiura et al., 1996b), indicating that Gi/Go is involved in the response. Phospholipase C also appears to be involved, because the addition of U73122, a phospholipase C inhibitor, to the cells abolished the response induced by 2-AG, whereas the addition of an inactive analogue, U73343, did not (Sugiura et al., 1997b). The addition of EGTA did not totally abolish the response induced by 2-AG, implying that a part of Ca^{2+}, if not all, was released from intracellular store site(s). We hypothesized that the mechanism underlying the Ca^{2+} transient induced by 2-AG and other cannabimimetic molecules is as follows (Fig. 3). Cannabimimetic molecules bind to the cannabinoid CB1 receptor to stimulate Gi/Go. The liberated βγ subunits of Gi/Go then induce a variety of intracellular events, such as inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels and activation of K^+ channels, phospholipase Cβ and the mitogen-activated protein kinase (MAP kinase) cascade. Among them, the activation of phospholipase Cβ is assumed to be responsible for inducing the Ca^{2+} transient. Inositol 1,4,5-trisphosphate (IP_3), generated from phosphatidylinositol 4,5-bisphosphate through the action of phospholipase Cβ, would mobilize Ca^{2+} from the intracellular Ca^{2+} store site(s) to induce the elevation of $[Ca^{2+}]_i$. A similar mechanism has already been suggested to be involved in opioids-induced rapid elevation of $[Ca^{2+}]_i$ in NG108-15 cells (Jin et al., 1994). In this study, we employed undifferentiated NG108-15 cells, which do not express voltage-gated Ca^{2+} channels. This may be the reason why we were able to detect clearly the Ca^{2+} transient induced by 2-AG.

The effect of 2-AG on NG108-15 cells differentiated with prostaglandin E_1 and theophylline was then examined (Sugiura et al., 1997a). Differentiated NG108-15 cells are known to express voltage-gated Ca^{2+} channels. We found that the Ca^{2+} response induced by 2-AG was diminished in differentiated cells as compared with the case of undifferentiated cells. Indeed, 2-AG was found to suppress depolarization-induced elevation of $[Ca^{2+}]_i$ (Sugiura et al., 1997a). The magnitude of the inhibition induced by 3 μM 2-AG was comparable to that induced by 1 μM ω-conotoxin GVIA (N-type voltage-gated Ca^{2+} channel blocker) or 1 μM ω-conotoxin MVIIC (Q- or P-type voltage-gated Ca^{2+} channel blocker, Sugiura et al., 1997a). It seems very likely that 2-AG inhibits voltage-gated Ca^{2+} channels, thereby reducing depolarization-induced elevation of $[Ca^{2+}]_i$. In view of the fact that anandamide and other cannabimimetic molecules have been shown to inhibit voltage-gated Ca^{2+} channels through a cannabinoid CB1 receptor-dependent mechanism (Howlett, 1995), 2-AG may also exert its inhibitory effect through a CB1 receptor-dependent mechanism. It should be noted, however, that the specificity of the fatty acyl moiety of monoacylglycerols in the above response in differentiated cells (Sugiura et al., 1997a) was somewhat different from that observed in the case of CB1 recep-

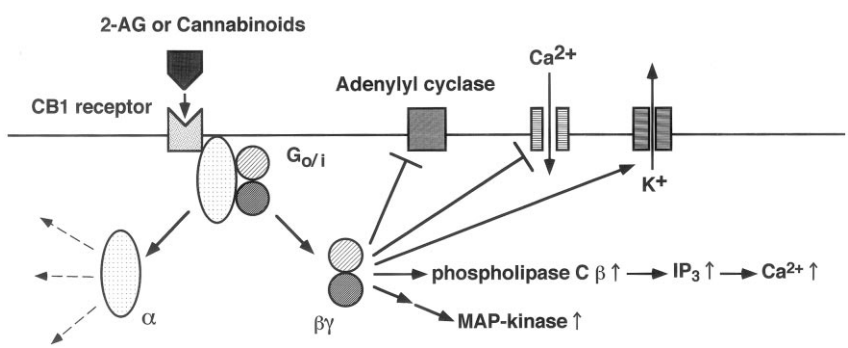

Fig. 3. Schematic illustration of the mechanism underlying the rapid elevation of $[Ca^{2+}]_i$ induced by 2-AG or other cannabinoids.

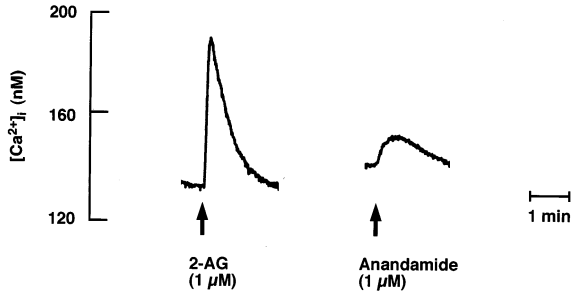

Fig. 4. Effects of 2-AG and anandamide on $[Ca^{2+}]_i$ in HL-60 cells, which express the CB2 receptor. Cells were loaded with Fura-2/AM and the changes in $[Ca^{2+}]_i$ were determined using a CAF-100 Ca^{2+} analyzer.

tor-mediated Ca^{2+} transients in undifferentiated cells (Sugiura et al., 1996b). Therefore, there remains the possibility that 2-AG also interacts with some sites other than the CB1 receptor to elicit biological responses (Sugiura et al., 1997a).

We next examined the effect of 2-AG on HL-60 cells, which are known to express the cannabinoid CB2 receptor. We found that 2-AG induces a rapid transient increase in $[Ca^{2+}]_i$ in HL-60 cells similar to that seen in the case of NG108-15 cells, which express the CB1 receptor (Fig. 4, Sugiura et al., 2000b). The response was not affected by either cyclooxygenase inhibitors or lipoxygenase inhibitors, suggesting that arachidonic acid metabolites are not involved. Importantly, the Ca^{2+} transient induced by 2-AG was blocked by pretreatment of the cells with SR144528, a CB2 receptor-specific antagonist, but not with SR141716A, a CB1 receptor-specific antagonist, indicating the involvement of the CB2 receptor but not the CB1 receptor in this cellular response. Gi/Go is also assumed to be involved, because pertussis toxin treatment of the cells abolished the response, as in the case of NG108-15 cells. In contrast to 2-AG, anandamide was found to act as a weak partial agonist (Fig. 4). Very recently, Gonsiorek et al. (2000) also reported that 2-AG acts as a full agonist at the CB2 receptor. We also found that 2-AG induces the activation of p42/44 MAP kinases in addition to rapid transient elevation of $[Ca^{2+}]_i$ in HL-60 cells (Sugiura, T., unpublished result) and that 2-AG induces a Ca^{2+} transient in RBL-2H3 cells (Sugiura, T., unpublished result). 2-AG may play physiologically important roles in various types of immune tissues and cells, though the details remain unclear. In relation to the possible roles of 2-AG in immune cells, Ouyang et al. (1998) demonstrated that 2-AG suppresses interleukin-2 gene expression in murine T lymphocytes through down-regulation of the nuclear factor. It remains to be determined, however, whether the effect of 2-AG is mediated through the cannabinoid receptor and whether 2-AG itself, rather than its metabolites, is implicated in the response.

Other biological activities of 2-AG have been reported by several investigators. 2-AG, as well as anandamide, has been shown to inhibit the proliferation of breast cancer cells (De Petrocellis et al., 1998; Melck et al., 2000) and prostate cancer cells (Melck et al., 2000). Paria et al. (1998) demonstrated that 2-AG arrests mouse embryo development in vitro. Stella et al. (1997) reported that 2-AG inhibits long-term potentiation in rat hippocampal slices. This effect of 2-AG was abolished in the presence of SR141716A, suggesting that the CB1 receptor is involved. Sinor et al. (2000) reported that 2-AG exerts neuroprotective effect in cerebral cortical neuron cultures subjected to hypoxia and glucose deprivation, although such an effect was not mediated through either the CB1 or the CB2 receptor. Varga et al. (1998) reported that 2-AG induces hypotension in rats. Mechoulam et al. (1998b) also found that the administration of 2-AG or its ether-linked analogue (HU-310) to rats induces hypotension. Jarai et al. (2000) also demonstrated that 2-AG and its ether-linked analogue (2-AG ether) induce hypotension in mice and reported that a part of the action of 2-AG is attributed to arachidonic acid metabolite(s) derived from 2-AG and a part is mediated through the cannabinoid CB1 receptor. Ben-Shabat et al. (1998) demonstrated that simultaneous administration of 2-linoleoylglycerol, which does not bind to the cannabinoid receptors, with 2-AG to mice potentiates the biological activities of 2-AG in vivo, possibly by modulating the catabolism of 2-AG.

4. Structure-activity relationship of 2-AG and related compunds as CB1 receptor agonists

Various cannabinoid receptor ligands including 2-AG induce Ca^{2+} transients in NG108-15 cells through a cannabinoid receptor-dependent mechanism (Sugiura et al., 1996b). Because such a cellular response can be detected immediately after the addition of the ligands to the cells, and because the method employed is sensitive enough to detect even small responses induced by low concentrations of cannabinoid receptor agonists such as 2-AG, measurement of $[Ca^{2+}]_i$ is a useful tool in evaluating the agonistic activities of various CB1 receptor ligands. We examined the structure-activity relationship of cannabinoid CB1 receptor ligands in detail employing this assay system (Sugiura et al., 1997b, 1999b).

We confirmed that low concentrations of 2-AG elicit rapid transient increases in $[Ca^{2+}]_i$. The response was detectable at 0.3 nM and was augmented with increasing concentration of 2-AG. The EC_{50} was around 30 nM. 1-AG and 3-AG, positional isomers of 2-AG, also exhibited substantial agonistic activities, although their activities were apparently lower than that of 2-AG. We next examined the activities of metabolically stable ether-linked analogues of AG and found that 2-eicosatetraenylglycerol, an ether-linked analogue of 2-AG (2-AG ether), possesses some agonistic activity, although its activity was markedly lower than that of 2-AG (Sugiura et al., 1999b; Suhara et al., 2000). The response was detectable from 30 nM, and the response induced by 10 μM 2-AG ether was about 50% of that induced by 10 μM 2-AG. On the other hand, the activity of 1(3)-eicosatetraenylglycerol, an ether-linked analogue of 1(3)-AG (1(3)-AG ether), was very weak, compared with that of 2-AG ether, even at high doses such as 10 μM. Such preference of the 2-isomer over the 1(3)-isomer is consistent with the results for the ester-linked compounds described above. Another metabolically stable analogue of 2-AG, 2-hydroxymethyl-7,10,13,16-docosatetraen-1-ol (a methylene-linked analogue of 2-AG) was devoid of appreciable activity, suggesting that an oxygen atom in the linkage is important for agonistic activity.

The activities of isopropanol-type, ethyleneglycol-type and propanediol-type analogues of AG were next examined. The isopropanol-type analogue of 2-AG did not exhibit any agonistic activity, whereas the ethyleneglycol-type analogue of 2-AG and 2-hydroxypropyl arachidonate showed substantial agonistic activities. Interestingly, the activity of 3-hydroxypropyl arachidonate, another analogue of 1(3)-AG, was much weaker than that of 2-hydroxypropyl arachidonate, suggesting that the presence of a free hydroxy group adjacent to the ester linkage is essential for strong agonistic activity.

We next examined the activities of anandamide and its analogues. We confirmed that anandamide induces the elevation of $[Ca^{2+}]_i$. The response was detectable from 3 nM, although the maximal response induced by anandamide was rather small. (*R*)-1-methanandamide and *N*-arachidonoylserinol, an acid amide bond-containing analogue of 2-AG, also exhibited only weak agonistic activities.

As for 2-monoacylglycerols containing various species of fatty acids, saturated or monoenoic 2-monoacylglycerols, such as 2-palmitoylglycerol and 2-oleoylglycerol, lacked appreciable agonistic activity. The activities of 2-linoleoylglycerol and 2-γ-linolenoylglycerol were also very low. Thus, the chemical structure of the acyl moiety is crucially important for strong agonistic activity. Interestingly, there was a marked difference in the activities of four species of 2-eicosatrienoylglycerols (Sugiura et al., 1999b; Nakane et al., 2000). 2-Eicosa-11′,14′,17′-trienoylglycerol did not exhibit appreciable agonistic activity. The activity of 2-eicosa-8′,11′,14′-trienoylglycerol was greater than that of 2-eicosa-11′,14′,17′-trienoylglycerol, although its activity was still weaker than that of 2-AG. On the other hand, 2-eicosa-5′,11′,14′-trienoylglycerol and 2-eicosa-5′,8′,11′-trienoylglycerol possessed strong agonistic activities. In particular, the activity of 2-eicosa-5′,8′,11′-trienoylglycerol was comparable to that of 2-AG (2-eicosa-5′,8′,11′,14′-tetraenoylglycerol). These results strongly suggest that the presence of the double bond at the Δ5 position of the C20 fatty chain is essential. In agreement with this, we found that 2-eicosa-5′,8′,11′,14′,17′-pentaenoyl-

glycerol exhibits substantial agonistic activity. In contrast to these C20 polyunsaturated fatty acid-containing 2-monoacylglycerols, C22 polyunsaturated fatty acid-containing 2-monoacylglycerols, such as 2-docosa-7′,10′,13′,16′-tetraenoylglycerol and 2-docosa-4′,7′,10′,13′,16′,19′-hexaenoylglycerol, failed to exhibit strong agonistic activities.

We then examined the activities of several classical and synthetic cannabinoids and compared their activities to that of 2-AG. We found that Δ^9-THC induces rapid transient elevation of $[Ca^{2+}]_i$: its activity was detectable from low nanomolar concentrations. The maximal response induced by Δ^9-THC was, however, small, suggesting that Δ^9-THC acted as a partial agonist toward the CB1 receptor. We also found that HU-210 and CP55940 exhibited appreciable agonistic activities. The response was detectable from 0.3 nM, with a plateau at around 100 nM in each case, but the maximal responses induced by these cannabinoids were also somewhat smaller than that induced by 2-AG.

In summary, 2-AG appears to be the optimum ligand among the compounds examined, and the structure of 2-AG is strictly recognized by the CB1 receptor (Table 1). Based on this, we proposed that the cannabinoid CB1 receptor is primarily a 2-AG receptor and 2-AG is the intrinsic natural ligand of the CB1 receptor (Sugiura et al., 1997b, 1999b).

Table 1
Preferred structures of monoacylglycerols and their structural analogues as cannabinoid CB1 receptor agonists

Backbone	Glycerol > ethyleneglycol, propanediol > > isopropanol
Position	2-Isomer > 1-isomer, 3-isomer
Bond	Ester > ether > methylene, amide
Acyl moiety	20:4 (*n*-6) > 20:3 (*n*-9) > 20:3 (*n*-6) ($\Delta^{5,11,14}$), 20:5 (*n*-3) > 20:3 (*n*-6) ($\Delta^{5,8,14}$) > 20:3 (*n*-3), 22:4 (*n*-6), 22:6 (*n*-3), 18:3 (*n*-6), 18:2 (*n*-6), 18:1 (*n*-9), 16:0

5. Structure-activity relationship of 2-AG and related compounds as CB2 receptor agonists

We then investigated the structure–activity relationship of the CB2 receptor ligands employing HL-60 cells, which express the CB2 receptor (Sugiura et al., 2000b). As described before, we found that 2-AG induces rapid transient elevation of $[Ca^{2+}]_i$ in HL-60 cells through a cannabinoid CB2 receptor-dependent mechanism. The response was detectable at 1 nM and was augmented with increasing concentration of 2-AG. Two types of positional isomers of 2-AG, 1-AG and 3-AG, also exhibited appreciable agonistic activities, although their activities were lower than that of 2-AG. This was also the case for an ethyleneglycol-type analogue of 2-AG. We confirmed that free arachidonic acid is devoid of any agonistic activity, indicating that free arachidonic acid, which may be generated from 2-AG, is not involved in the 2-AG-induced increases in $[Ca^{2+}]_i$ described above. This was confirmed by the fact that 2-AG ether is capable of eliciting rapid increases in $[Ca^{2+}]_i$ to some extent, although the magnitude of the response induced by 2-AG ether was weak compared with that of 2-AG. *N*-Arachidonoylserinol also exhibited some agonistic activity, though it was far less pronounced than that of 2-AG. These results suggest that an ester linkage is needed for strong agonistic activity. In keeping with this, anandamide, another type of acid-amide bond-containing analogue of 2-AG, was found to possess only weak agonistic activity.

Among the 2-monoacylglycerols, 2-palmitoylglycerol did not exhibit any agonistic activity. The activity of 2-oleoylglycerol was also negligible. On the other hand, 2-linoleoylglycerol showed weak agonistic activity. Similar weak agonistic activities were observed with 2-γ-linolenoylglycerol, 2-eicosa-11′,14′,17′-trienoylglycerol and 2-eicosa-8′,11′,14′-trienoylglycerol. A striking observation is that 2-eicosa-5′,8′,11′-trienoylglycerol and 2-eicosa-5′,8′,11′,14′,17′-pentaenoylglycerol exhibited rather strong agonistic activities. In particular, the activity of 2-eicosa-5′,8′,11′-trienoylglycerol was comparable to that of 2-AG, as in the case of NG108-15 cells. In addition to these C20 polyunsaturated fatty acid-containing 2-monoacylglyc-

Table 2
Preferred structures of monoacylglyceols and their structral analogues as cannabinoid CB2 receptor agonists

Backbone	Glycerol > ethyleneglycol
Positon	2-Isomer > 1-isomer, 3-isomer
Bond	Ester > ether > amide
Acyl moiety	20:4 (*n*-6) > 20:3 (*n*-9) > 20:5 (*n*-3) > 22:4 (*n*-6), 22:6 (*n*-3) > 20:3 (*n*-6), 20:3 (*n*-3), 18:3 (*n*-6), 18:2 (*n*-6) > 18:1 (*n*-9), 16:0

erols, two types of C22 polyunsaturated fatty acid-containing 2-monoacylglycerol, 2-docosa-7′,10′,13′,16′-tetraenoylglycerol and 2-docosa-4′,7′,10′,13′,16′,19′-hexaenoylglycerol, also showed appreciable agonistic activities, although their activities were weaker than those of 2-eicosa-5′,8′,11′-trienoylglycerol and 2-eicosa-5′,8′,11′,14′,17′-pentaenoylglycerol.

The activities of several classical and synthetic cannabinoids and related compounds were next examined. Interestingly, Δ^9-THC exhibited only weak agonistic activity. In contrast, either CP55940 and HU-210 exhibited strong agonistic activity. WIN55212-2, a cannabimimetic aminoalkylindole, possessed substantial agonistic activity, whereas its inactive isomer WIN55212-3 did not. We also found that *N*-palmitoylethanolamine, a possible endogenous CB2 receptor ligand (Facci et al., 1995), did not act as an agonist.

These results concerning the structure-activity relationship of the CB2 receptor ligands are in general agreement with those of the CB1 receptor ligands described before. A difference is that CP55940 and HU-210 acted as partial agonists in the case of the CB1 receptor, whereas these compounds acted as potent full agonists in the case of the CB2 receptor. In any case, 2-AG was the most active among these structurally related compounds (Table 2). Based on these experimental data, we concluded that not only the cannabinoid CB1 receptor, but also the CB2 receptor is essentially a 2-AG receptor and 2-AG is the endogenous physiological ligand for the CB2 receptor (Sugiura et al., 2000b).

6. Tissue levels of 2-AG

Not much information was available concerning the tissue levels of 2-AG until recently. Mechoulam et al. (1995) detected 2-AG in canine gut, although they did not estimate its level. We detected 3.25 nmol/g tissue of AG (Sugiura et al., 1995) or 3.36 nmol/g tissue of 2-AG (Kondo et al., 1998), and Stella et al. (1997) detected 4.0 nmol/g tissue of 2-AG in rat brain. Recently, we further estimated the levels of 2-AG in several rat tissues. The levels of 2-AG in rat liver, spleen, lung, kidney and plasma were 1.15 nmol/g tissue, 1.17 nmol/g tissue, 0.78 nmol/g tissue, 0.98 nmol/g tissue and 0.012 nmol/ml, respectively (Kondo et al., 1998). Substantial amounts of 1(3)-AG always co-existed with 2-AG. The levels of 1(3)-AG were 1.39 nmol/g tissue (brain), 0.55 nmol/g tissue (liver), 0.63 nmol/g tissue (spleen), 0.59 nmol/g tissue (lung), 0.48 nmol/g tissue (kidney) and 0.004 nmol/ml (plasma, Kondo et al., 1998). Tissue levels of 2-AG have also been reported by Schmid et al. (2000), 13.04–6.19 pmol/μmol lipid P (kidney), 3.21–3.05 pmol/μmol lipid P (testis), 10.94 pmol/μmol lipid P (heart), 29.03 pmol/μmol lipid P (spleen) and 5.05 pmol/μmol lipid P (liver).

Bisogno et al. (1999c) reported that 2-AG is distributed among various brain regions, 10.5 nmol/g tissue for medulla, 4.3 nmol/g tissue for cortex, 10.0 nmol/g tissue for limbic forebrain, 14.0 nmol/g tissue for brainstem, 10.7 nmol/g tissue for striatum, 12.6 nmol/g tissue for hippocampus, 2.0 nmol/g tissue for diencephalon, 3.5 nmol/g tissue for cerebellum and 4.0 nmol/g tissue for mesencephalon. 2-AG was also detected in the anterior pituitary (3.7 nmol/g tissue) and the hypothalamus (3.1 nmol/g tissue, Gonzalez et al., 1999). Huang et al. (1999) reported that 2-AG is present in rat sciatic nerve (0.052 nmol/g tissue), lumbar spinal cord (0.432 nmol/g tissue) and lumbar dorsal root ganglion (0.370 nmol/g tissue). On the other hand, Giuffrida et al. (1999) could not detect 2-AG in the microdyalisates from dorsal striatum of freely moving rats. 2-AG was also detected in rat retina (2.97 nmol/g tissue, Straiker et al., 1999) and bovine retina (1.63 nmol/g tissue, Bisogno et al., 1999b). Berrendero et al. (1999) reported that the level of 2-AG as well as that of

anandamide in rat brain varied in a different way during the course of development.

Recently, we found that the amount of 2-AG in the brains obtained from rats killed by soaking in liquid nitrogen was 0.23 nmol/g tissue (Sugiura, T., unpublished result), which was about one-fifteenth of the amount of 2-AG in the brains obtained from rats by decapitation without freezing (Kondo et al., 1998). This strongly suggests that a substantial amount of 2-AG was rapidly produced in the brain during the post-mortem period. In fact, we confirmed that rapid (within 30 s) generation of 2-AG occurs in rat brain after decapitation (Sugiura, T., unpublished result). It is essential, therefore, to take special care to avoid post-mortem changes in the level of 2-AG in order to estimate the exact tissue level of 2-AG under physiological conditions. It is also worth mentioning that only a part of the 2-AG found in tissues is able to act as a receptor agonist, because, usually, not all the 2-AG produced in cells was found to be released into the extracellular fluid.

7. Biosynthesis and degradation of 2-AG

It was 17 years ago that the generation of AG in thrombin-stimulated platelets was mentioned in the literature (Prescott and Majerus, 1983). Later, the generation of AG in platelet-derived growth factor-stimulated Swiss 3T3 cells (Hasegawa-Sasaki, 1985) and in bradykinin-stimulated rat dorsal ganglion neurons (Gammon et al., 1989) was reported. However, at that time, physiological significance of AG or 2-AG as an endogenous cannabinoid receptor ligand was unknown. Stimulus-induced generation of 2-AG as an endogenous cannabinoid receptor ligand was first described in ionomycin-stimulated N18TG2 cells (Bisogno et al., 1997) and in electrically stimulated rat hippocampal slices and ionomycin-stimulated neurons (Stella et al., 1997). We also investigated the generation of 2-AG and found that rapid generation of 2-AG occurs in rat brain homogenate following the addition of $CaCl_2$ (Kondo et al., 1998) and in thrombin- or A23187-stimulated human umbilical vein endothelial cells (Sugiura et al., 1998b). Furthermore, very recently, we found that the level of 2-AG in rat brain was augmented dramatically (6-fold) following the intraperitoneal injection of picrotoxinin (Sugiura et al., 2000a). As for blood cells or inflammatory cells, the generation of 2-AG has recently been reported in LPS-stimulated rat platelets (Varga et al., 1998) and LPS-stimulated rat macrophages and LPS- or ionomycin-stimulated J774 macrophage-like cells (Di Marzo et al., 1999b). It is noteworthy that substantial amounts of 2-AG were released from cells upon stimulation (Hasegawa-Sasaki, 1985; Bisogno et al., 1997; Sugiura et al., 1998b).

When we published our experimental data concerning a possible role of 2-AG as an endogenous cannabinoid receptor ligand (Sugiura et al., 1995), we pointed out that 2-AG can be formed from arachidonic acid-enriched membrane phospholipids such as inositol phospholipids through the

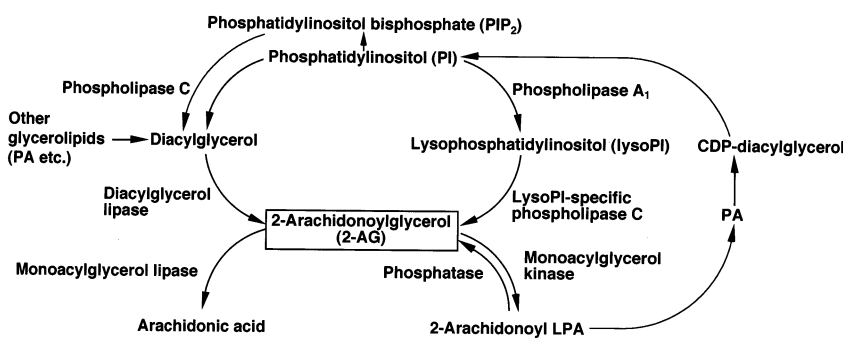

Fig. 5. Metabolic pathways of 2-AG.

combined actions of phospholipase C and diacylglycerol lipase or through the combined actions of phospholipase A1 and phospholipase C (Fig. 5). We also suggested that 2-AG can also be formed from arachidonic acid-containing lysophosphatidic acid through the action of a phosphatase (Fig. 5, Sugiura et al., 1995). The first pathway, involving rapid hydrolysis of inositol phospholipids by phospholipase C and subsequent hydrolysis of the resultant diacylglycerol by diacylglycerol lipase, was described by Prescott and Majerus (1983) as a degradation pathway for arachidonic acid-containing diacylglycerols in platelets. Stella et al. (1997) demonstrated that these enzyme activities are involved in ionomycin-induced generation of 2-AG in cultured neurons using metabolic inhibitors. Recently, we presented evidence that this pathway is crucially important for Ca^{2+}-induced generation of 2-AG in rat brain homogenate (Sugiura, T., unpublished result). Structures and functions of the enzymes involved in this pathway, i.e. phospholipase C (Rhee and Bae, 1997) and diacylglycerol lipase (Bell et al., 1979; Farooqui et al., 1990, 1993; Moriyama et al., 1999), have already been studied by a number of investigators. Evidence is accumulating that this pathway can operate in a variety of tissues and cells under conditions of stimulation.

The enzyme activities involved in the second pathway, i.e. hydrolysis of phosphatidylinositol by phospholipase A_1 and hydrolysis of the resultant lysophosphatidylinositol by a specific phospholipase C, were investigated intensively by Okuyama and co-workers (Ueda et al., 1993; Tsutsumi et al., 1994, 1995), although they were unaware that these enzyme activities might be implicated in the generation of an endogenous cannabinoid receptor ligand. Interestingly, lysophosphatidylinositol-specific phospholipase C is distinct from the phospholipase C which acts on phosphatidylinositol, and is localized in synaptosomes (Tsutsumi et al., 1994, 1995); it is possible, therefore, that this unique enzyme is involved in the metabolism of lysophosphatidylinositol and the generation of lysophosphatidylinositol-derived lipid mediators such as 2-AG in synapses. The relative importance of the two pathways in the generation of 2-AG remains to be established.

Further pathways for the generation of 2-AG are the conversion of 2-arachidonoyl LPA to 2-AG and the conversion of 2-arachidonoyl PA to 2-AG. We have detected a substantial amount of arachidonic acid-containing LPA in rat brain (Sugiura et al., 1999a). Although the positional distribution was not examined, arachidonic acid is assumed to be esterified at the 2-position. We have also detected a phosphatase activity which hydrolyzes LPA to yield a monoacylglycerol in a rat brain homogenate (Nakane, S. and Sugiura, T., unpublished result); there is a possibility, therefore, that 2-arachidonoyl LPA is utilized for the synthesis of 2-AG under conditions of stimulation in the brain. On the other hand, Bisogno et al. (1999a) demonstrated that 2-AG is formed from PA in ionomycin-stimulated N18TG2 neuroblastoma cells using several metabolic inhibitors. In this case, 2-arachidonoyl PA was first converted to 1-acyl-2-arachidonoylglycerol and then to 2-AG. They concluded that inositol phospholipid breakdown is not involved in the generation of 2-AG. A part of 2-AG may also be derived from other arachidonic acid-containing phospholipids such as 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Di Marzo et al., 1996). Thus, the biosynthetic pathways for 2-AG appear to differ, depending on the types of tissues and cells and the types of stimuli.

As for the degradation of 2-AG, the most plausible mechanism is that 2-AG is metabolized by a monoacylglycerol lipase, like other monoacylglycerols. Goparaju et al. (1999) have demonstrated that 2-AG can be hydrolyzed by a monoacylglycerol lipase-like activity present in porcine brain cytosol and particulate fractions. An enzyme activity catalyzing the hydrolysis of 2-AG was also found and partially characterized in macrophages (Di Marzo et al., 1999b). Properties of monoacylglycerol lipase activity in the brain have been studied by Horrockes and co-workers (Farooqui et al., 1990, 1993). On the other hand, Goparaju et al. (1998), Di Marzo et al. (1998b) presented evidence that anandamide amidohydrolase is able to metabolize 2-AG as well. 2-AG may be degraded by anandamide amidohydrolase in addition to monoacylglycerol lipase under some circumstances.

In addition to being metabolized by hydrolyzing enzymes, 2-AG can be metabolized to 2-arachidonoyl LPA through the action of a kinase(s). The enzyme activity involved in the formation of 2-acyl LPA from the corresponding 2-monoacylglycerol has already been studied by several investigators (Kanoh et al., 1986; Shim et al., 1989). This pathway is probably important to reconstitute phosphatidylinositol from 2-AG. Simpson et al. (1991) presented evidence that [^{3}H]glycerol-labeled 2-AG, but not free [^{3}H]glycerol, was gradually incorporated into phosphatidylinositol when added to Swiss 3T3 cells. They demonstrated that these cells contain a monoacylglycerol kinase activity and the resultant 2-arachidonoyl LPA can be metabolized to 1-stearoyl-2-arachidonoyl PA through a CoA-dependent transacylation reaction. The newly formed 1-stearoyl-2-arachidonoyl PA then enters the so-called 'phosphatidylinositol cycle' to form phosphatidylinositol. Di Marzo et al. (1998b, 1999b) also demonstrated that [^{3}H]arachidonic acid-containing 2-AG was gradually converted to phospholipids prior to its hydrolysis to arachidonic acid in N18TG2 cells and in RBL-2H3 cells and in murine macrophages; it is conceivable that a part of 2-AG was converted to phospholipids after being converted to 2-[^{3}H]arachidonoyl LPA, as found in Swiss 3T3 cells.

8. Possible physiological roles of 2-AG

What, then, is the physiological role(s) of 2-AG in the nervous system? It seems unlikely that the physiological compound 2-AG induces psychedelic reactions such as heightened sensory awareness, dissociation of ideas, errors in judgement of time and space, illusions and hallucinations in living animals, such as are very often observed with Δ^9-THC. Δ^9-THC is a partial agonist toward the CB1 receptor and is metabolically rather stable, in contrast to 2-AG; Δ^9-THC may interfere with the action of the physiological ligand 2-AG thereby inducing such pharmacological effects. In relation to this, it should be noted that the well-known hallucinogen lysergic acid diethylamide (LSD) acts as a partial agonist to several types of serotonin receptors, such as the 5-HT$_{2A}$ receptor. Presumably LSD interferes with the action(s) of serotonin at some sites in the brain, thereby causing hallucinations in man, as might be the case for Δ^9-THC versus 2-AG.

The cannabinoid CB1 receptor is present mainly in presynapses and is assumed to be involved in the attenuation of neurotransmission. We hypothesized that the physiological role of 2-AG, the natural ligand for the CB1 receptor, in the synapse is as follows: 2-AG generated through increased phospholipid metabolism, especially inositol phospholipid breakdown, at presynapses and/or postsynapses during accelerated synaptic transmission, plays an important role in calming the excitation of neuronal cells through acting at the cannabinoid CB1 receptor, thereby diminishing subsequent neurotransmitter release (Fig. 6). Recently, we obtained evidence that a substantial amount of 2-AG was released from rat brain synaptosomes upon depolarization (Sugiura, T., unpublished result). Such a negative feedback regulation mechanism should be effective for calming stimulated neurons after excitation. A similar negative feedback regulation mechanism may operate in the case of adenosine, a degradation product of ATP. Such 2-AG- and cannabinoid CB1 receptor-dependent negative feedback regulation of neurotransmission would be of great physiological significance, because sustained activation of neuronal cells is known to cause cell exhaustion and may lead to neuronal cell death. Further work on the physiological roles of 2-AG in synapses may help us to understand in more detail the regulatory mechanisms of neurotransmission in mammals.

Another important issue concerning 2-AG is its possible involvement as an intercellular mediator in the cardiovascular system. We have demonstrated that human endothelial cells produce and release 2-AG upon stimulation with thrombin or A23187 (Sugiura et al., 1998a). We proposed that 2-AG, possibly derived from endothelial cells or peripheral nerve terminals, may play an essential role(s) in the vascular system through acting on the CB1 receptor, because (1) the CB1 receptor is present in human vascular smooth muscle cells (Sugiura et al., 1998a) and (2) the CB1 receptor

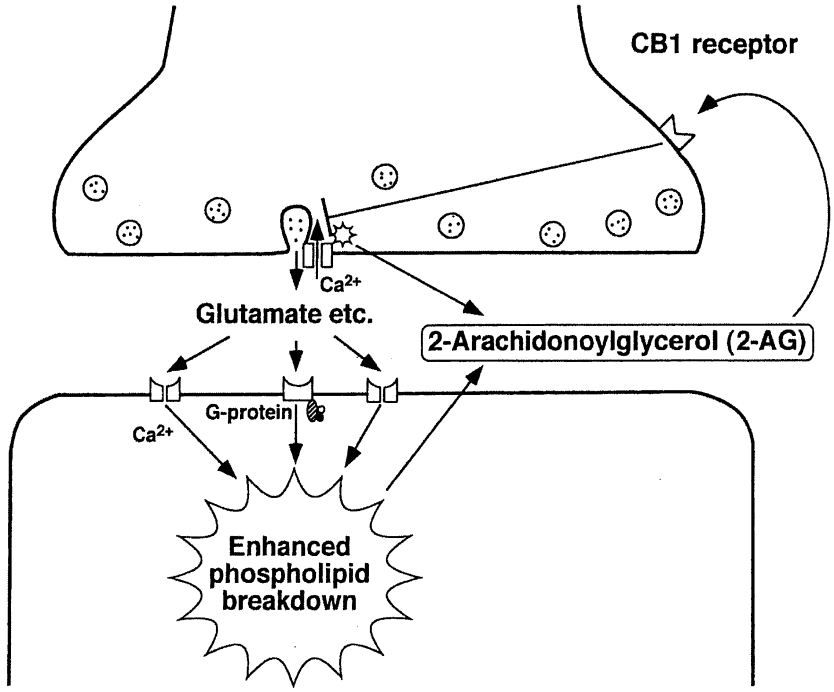

Fig. 6. Schematic illustration of the possible role of 2-AG in neurotransmission.

has been reported to participate in the attenuation of the release of norepinephrine from peripheral sympathetic nerves (Ishac et al., 1996; Deutsch et al., 1997; Malinowska et al., 1997). As noted above, several investigators have demonstrated that the administration of 2-AG induces hypotension in experimental animals (Varga et al., 1998; Mechoulam et al., 1998b; Jarai et al., 2000). Randall et al. (1996) suggested that anandamide is a candidate for endothelium-derived hyperpolarizing factor (EDHF), although there are conflicting reports as to this (Plane et al., 1997; Fulton and Quilley, 1998). Whether or not 2-AG actually acts as an endogenous vasodilator in living animals, and if so, whether or not 2-AG is an EDHF-like molecule should be clarified in the future.

In addition to its role as the natural ligand for the CB1 receptor, 2-AG is the natural ligand for the CB2 receptor. This strongly suggests that 2-AG has some physiological role(s) during the course of inflammatory reactions and/or immune responses, because several lines of evidence strongly suggest that the CB2 receptor is involved in such physiological or pathophysiological responses (Munro et al., 1993; Derocq et al., 1995; Carayon et al., 1998). Pioneering work on 2-AG and the function of murine lymphocytes has already been conducted by Kaminski and co-workers (Lee et al., 1995; Ouyang et al., 1998). Nevertheless, precise physiological function of 2-AG and its receptor site, i.e. the CB2 receptor, in acute and chronic inflammation and/or immune responses remain obscure.

Several years ago, Hecht et al. (1996), An et al. (1997) showed that the Vzg-1/Edg2 gene product, which has about 30% homology with the cannabinoid receptors, is a specific receptor for the bioactive lipid LPA. It is now known that the Edg gene family, encoding LPA receptors and sphingosine-1-phosphate receptors, and the cannabinoid receptor gene family are very close receptor gene families. This is not surprising, because the chemical structure of LPA resembles that of 2-AG (Fig. 1). In addition, LPAs and monoacylglycerols are metabolically closely related (Fig. 5). Therefore, it is reasonable to postu-

late that 2-AG and LPAs, as well as sphingosine-1-phosphate, compose a novel bioactive lyso(phospho)lipid superfamily. More than 20 years have passed since the discovery of LPA as a bioactive lipid, and evidence is accumulating that LPA is involved in a variety of physiological and pathophysiological processes (Tokumura, 1995). On the other hand, only about 5 years have passed since the discovery of the role of 2-AG as a lipid mediator. Thus, further intensive studies are essential for a full understanding of the metabolism, mode of action and physiological and pathophysiological roles of 2-AG in various mammalian tissues and cells.

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