J. Biochem. **132**, 7–12 (2002)

Cannabinoid Receptors and Their Endogenous Ligands

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Received April 1, 2002; accepted May 20, 2002

Δ^9-Tetrahydrocannabinol, a major psychoactive component of marijuana, has been shown to interact with specific cannabinoid receptors, thereby eliciting a variety of pharmacological responses in experimental animals and human. In 1990, the gene encoding a cannabinoid receptor (CB1) was cloned. This prompted the search for endogenous ligands. In 1992, *N*-arachidonoylethanolamine (anandamide) was isolated from pig brain as an endogenous ligand, and in 1995, 2-arachidonoylglycerol was isolated from rat brain and canine gut as another endogenous ligand. Both anandamide and 2-arachidonoylglycerol exhibit various cannabimimetic activities. The results of structure–activity relationship experiments, however, revealed that 2-arachidonoylglycerol, but not anandamide, is the intrinsic natural ligand for the cannabinoid receptor. 2-Arachidonoylglycerol is a degradation product of inositol phospholipids that links the function of cannabinoid receptors with the enhanced inositol phospholipid turnover in stimulated tissues and cells. The possible physiological roles of cannabinoid receptors and 2-arachidonoylglycerol in various mammalian tissues such as those of the nervous system are discussed.

Key words: anandamide, arachidonic acid, 2-arachidonoylglycerol, cannabinoid, neuromodulator.

Marijuana has been used as a traditional medicine and a pleasure-inducing drug for thousands of years in Central and South Asia. The major psychoactive component of marijuana is Δ^9-tetrahydrocannabinol (Δ^9-THC) (Fig. 1). Δ^9-THC exhibits diverse pharmacological activities *in vitro* and *in vivo* (*1*). For example, the administration of Δ^9-THC to experimental animals induces reduced spontaneous motor activity, immobility, analgesia, impairment of short-term memory and hypothermia. Δ^9-THC also exerts profound effects on several biological systems other than the central nervous system, such as the suppression of immune responses. The mechanisms of these actions of Δ^9-THC remained obscure until the 1980s.

Cannabinoid receptors

The occurrence of a specific binding site for cannabinoids in mammalian tissues, as in the cases of opioids, was argued by several investigators in the 1970s. However, binding experiments using Δ^9- THC or its analogs were not satisfactorily successful. In 1988, Howlett and co-workers finally succeded in providing evidence that a specific binding site for cannabinoids is present in rat brain synaptosomes using [^{3}H]CP55940 (Fig. 1), a radiolabeled synthetic cannabinoid, as a ligand (*2*). It thus became clear that Δ^9-THC binds to a specific receptor site(s), if not all, thereby eliciting a variety of pharmacological responses.

In 1990, there was a breakthrough. Matsuda *et al.* (*3*) reported the cloning of 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 (Table I). Later, Munro *et al.* (*4*) cloned another cDNA encoding a cannabinoid receptor (CB2) from a promyelocytic leukemia HL-60 cell cDNA library. The CB2 receptor is also a seven-transmembrane, G protein–coupled receptor, and consists of 360 amino acids (Table I). The CB1 and CB2 receptors share 44% overall identity (68% identity for the transmembrane domains). Both receptors are coupled to Gi/Go, and various biological responses mediated through them are abolished following treatment of tissues and cells with pertussis toxin. To date, a number of compounds acting on cannabinoid receptors have been synthesized. Several compounds were found to act as antagonists/inverse agonists: SR141716A (Fig. 1) is a selective antagonist/inverse agonist for the CB1 receptor, and SR144528 (Fig. 1) is a selective antagonist/inverse agonist for the CB2 receptor.

The CB1 receptor is expressed abundantly in the nervous system and in various peripheral tissues (Table I). It is noteworthy that the whole-brain cannabinoid receptor density is similar to whole-brain densities of receptors for glutamate and GABA (*5*). Among the various brain regions, the CB1 receptor is especially abundant in the substantia nigra, the globus pallidus, the molecular layer of the cerebellum, the hippocampus, and the cerebral cortex; the CB1 receptor is assumed to be involved in the regulation of cognition, memory and motor activity (*5*). On the other hand, the CB2 receptor is almost exclusively present in tissues of the immune system such as the spleen, tonsil and lymph nodes (Table I). The CB2 receptor is abundantly expressed in several types of leukocytes such as macrophages/monocytes, B lymphocytes, and natural killer cells, and is as-

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Abbreviations: 2-AG, 2-arachidonoylglycerol; DG, diacylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Δ^9-THC, Δ^9-tetrahydrocannabinol.

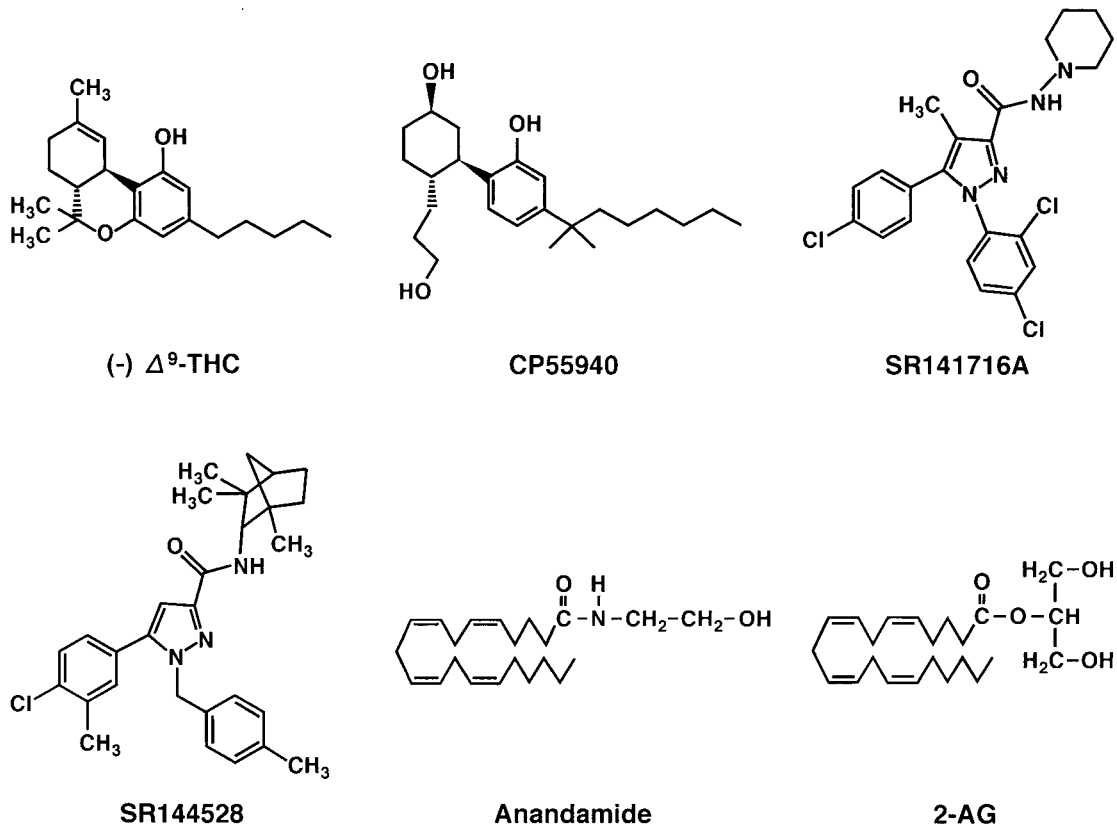

Fig. 1. **Chemical structures of cannabinoid receptor ligands.**

TABLE I. **Comparison of the CB receptor and the CB2 receptor.**

	CB1	CB2
Amino acids (human)	472	360
G protein	Gi/Go	Gi/Go
Adenylyl cyclase	↓	↓
MAP kinases	↑	↑
Votage-gated Ca^{2+} channels	↓	
Inwardly rectifying K^+ current	↑	
Endogenous natural ligand	2-AG, anandamide (?)	2-AG
Antagonists	SR141716A, AM251, AM281	SR144528, JTE-907, AM630
Distribution	Brain (globus pallidus, striatum, substantia nigra, cerebellum, hippocampus, cerebral cortex), lung, small intestine, uterus, testis, vas deferens, urinary bladder *etc.*	Spleen, tonsil, lymph nodes, (macrphage/monocytes, B lymphocytes, natural killer cells)

sumed to participate in the regulation of immune responses and/or inflammatory reactions, although the details remain to be clarified.

Endogenous cannabinoid receptor ligands

Identification of anandamide as an endogenous cannabinoid receptor ligand. The discovery of the specific receptors for cannabinoids prompted the search for endogenous ligand(s). In 1992, Devane *et al.* (*6*) isolated *N*-arachidonoylethanolamine (anandamide) (Fig. 1) from pig brain as an endogenous cannabinoid receptor ligand. They demonstrated that anandamide binds to the brain cannabinoid receptor with high affinity (K_i = 52 nM) and induces inhibition of the mouse twitch response (*6*). They also showed that anandamide elicits reduced spontaneous motor activities, immobility, hypothermia, and analgesia when administered to mice. Anandamide has also been shown to exhibit the inhibition of voltage-gated Ca^{2+} channels, the activation of an inwardly rectifying K^+ current, the stimulation of [^{35}S]GTP γS binding to G proteins, Ca^{2+} transients, the activation of MAP kinases, and the neural form of focal adhesion kinase, the inhibition of neurotransmitter release, the impairment of memory, the inhibition of long-term potentiation in hippocampal slices, the inhibition of nociception, vasodilation, hypotension, bradycardia, the stimulation of constitutive nitric oxide synthase, the inhibition of inducible nitric oxide synthase, the inhibition of the growth of human breast and prostate cancer cells and the inhibition of sperm acrosome reaction (*7–9*). It should be noted, however, that anandamide was found to act as a partial agonist in some cases (*7*).

In addition to acting as an endogenous cannabinoid receptor ligand, anandamide was shown to act as an endogenous ligand for an ion channel–type vanilloid receptor

(VR1 receptor). Furthermore, anandamide can also bind to several other types of ion channels. Therefore, it seems possible that some of the effects of anandamide mentioned above are mediated through receptors or binding sites other than the cannabinoid receptors. One should keep this in mind when interpreting the experimental results concerning the biological activities of anandamide.

Biosynthesis and degradation of anandamide. Anandamide can be formed enzymatically *via* two independent synthetic pathways (*7–10*). One is the direct *N*-acylation of ethanolamine, and the other is transacylase-phosphodiesterase-mediated synthesis. The first pathway is catalyzed by the reverse reaction of an anandamide amidohydrolase/fatty acid amide hydrolase (*7–11*), suggesting that the formation of anandamide *via* this pathway may not be physiologically relevant, yet there remains the possibility that a significant amount of anandamide can be formed via this pathway if high concentrations of arachidonic acid and ethanolamine are co-localized at certain sites within the cell. The second pathway is the formation of anandamide from pre-existing *N*-arachidonoyl phosphatidylethanolamine (PE) through the action of a phosphodiesterase. This enzyme reaction has been assumed to be the major synthetic route for various *N*-acylethanolamines such as *N*-palmitoyl- and *N*-stearoyl-ethanolamine (*12*). Several investigators have provided evidence that anandamide can be formed from *N*-arachidonoyl PE through the action of a phosphodiesterase (*7–10*). This is probably the main synthetic pathway for anandamide in various mammalian tissues. However, this pathway does not appear to be able to generate a large amount of anandamide, because the tissue level of *N*-arachidonyl PE is usually very low. The reason for this is that *N*-arachidonoyl PE is synthesized from PE and arachidonic acid, and esterified at the 1-position of glycerophospholipids through the action of a transacylase (*7–10*). Importantly, the level of arachidonic acid esterified at the 1-position of glycerophospholipids is usually very low (*7*). The absence of efficient synthetic pathways for anandamide is in agreement with the observation that the tissue levels of anandamide are generally low (the order of pmol/g tissue) except in a few cases (*7, 13*).

The degradation of anandamide is catalyzed by two separate types of anandamide amidohydrolase/fatty acid amide hydrolase: the optimal pH of one isoform is 8.5–10 (*11*) and that of the other is 5 (*14*). The gene encoding the former enzyme protein has been cloned (*15*).

Identification of 2-arachidonoylglycerol (2-AG) as an endogenous cannabinoid receptor ligand. 2-AG (Fig. 1) is a unique molecular species of monoacylglycerol, having esterified arachidonic acid at the 2-position of the glycerol backbone. Its chemical structure somewhat resembles that of anandamide. Hence, we examined whether 2-AG possesses 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 (*16*). We also found that arachidonoylglycerols are present in rat brain at concentrations in the order of nmol/g tissue. Based on these experimental results, we assumed that arachidonic acid-containing monoacylglycerol may act as an endogenous cannabinoid receptor ligand at certain sites in the brain (*16*). The reason for the relatively high apparent K_i value of 2-AG in that study may be due, at least in part, to the possible hydrolysis of 2-AG during the incubation. We then examined the effect of the addition of diisopropyl fluorophosphate, an esterase inhibitor. The K_i value of 2-AG estimated in the presence of diisopropyl fluorophosphates 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 hypothesis 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 and published them in 1995 (*17*). Mechoulam *et al.* (*18*) also reported that 2-AG is an endogenous cannabinoid receptor ligand at the same meeting and published their findings in 1995. They isolated 2-AG from canine gut and demonstrated that 2-AG possesses various cannabimimetic activities including a binding activity toward the cannabinoid receptors expressed on COS-7 cells transfected with cannabinoid receptor genes (*18*).

2-AG exhibits various cannabimimetic activities similar to those of anandamide described before (*7–9, 19*). Noticeably, 2-AG was found to act as a full agonist at either the CB1 receptor or the CB2 receptor in many cases (*7, 19*). Based on the results of structure–activity relationship experiments using neuroblastoma x glioma hybrid NG108-15 cells, which express the CB1 receptor, we deduced that the CB1 receptor is originally and primarily a 2-AG receptor. Also, based on the results of structure–activity relationship experiments using HL-60 cells, which express the CB2 receptor, we concluded that the CB2 receptor is a 2-AG receptor (*7, 19*).

Biosynthesis and degradation of 2-AG. 2-AG is present in relatively large amounts in various mammalian tissues (in the order of nmol/g tissue) (*7, 19*). Importantly, the levels of 2-AG were elevated rapidly in tissues and cells upon stimulation (*7, 19*). Furthermore, a significant portion of newly formed 2-AG can be released from stimulated cells (*7, 19*). These characteristics appear to be favorable in the action of 2-AG as an intercellular mediator derived from stimulated cells.

Then, how is 2-AG formed in living cells? Previously, we mentioned that 2-AG can be formed from arachidonic acid–enriched membrane phospholipids, such as inositol phospholipids, through the combined actions of phospholipase C and diacylglycerol (DG) lipase or the combined actions of phospholipase A_1 and phospholipase C (*17*). The first pathway, involving rapid hydrolysis of inositol phospholipids by phospholipase C and subsequent hydrolysis of the resultant DG by DG lipase, was described by Prescott and Majerus two decades ago as a degradation pathway for arachidonic acid-containing DG in platelets (*20*). Stella *et al.* (*21*) demonstrated that these enzyme activities are involved in ionomycin-induced generation of 2-AG in cultured neurons using metabolic inhibitors. Recently, we confirmed that this pathway is important for the depolarization-induced generation of 2-AG in rat brain synaptosomes (T. Sugiura, unpublished results).

The second pathway involves hydrolysis of phosphatidylinositol (PI) by phospholipase A_1 and hydrolysis of the resultant lysoPI by a specific phospholipase C. Noticeably,

lysoPI-specific phospholipase C is distinct from various types of phospholipase C which act on other inositol phospholipids, and is localized in the synaptosomes (*22*). It is possible, therefore, that this unique enzyme may also be involved in the generation of 2-AG in synapses. In addition to these two pathways, there are several possible routes for the generation of 2-AG, for example, from 2-arachidonoyl LPA (*23*) or 2-arachidonoyl PA (*24*). The biosynthetic pathways for 2-AG appear to differ, depending on the types of tissues and cells, and the types of stimuli. Further detailed studies are necessary for a full understanding of the mechanism and regulation of the biosynthesis of 2-AG.

Like other monoacylglycerols, 2-AG is metabolized by monoacylglycerol lipase. In addition, several investigators have demonstrated that anandamide amidohydrolase/fatty acid amide amidohydrolase is also able to metabolize 2-AG (*7, 19*). 2-AG may be degraded by anandamide amidohydrolase/fatty acid amide amidohydrolase in addition to monoacylglycerol lipase under some circumstances.

An ether-linked analog of 2-AG. Among the various 2-AG analogs, an ether-linked analog of 2-AG (2-AG ether or HU310) (*25–27*), is a useful tool in exploring the possible biological activities of 2-AG, especially *in vivo*, because this compound is quite stable against hydrolyzing enzymes. Recently, Hanus *et al.* (*28*) reported that 2-AG ether is present in pig brain (they re-named it "noladin ether"). However, we did not detect 2-AG ether in the rat brain or pig brain (S. Oka and T. Sugiura, unpublished results). The ether bond is known to be located exclusively at the 1-position of the glycerol backbone in mammalian tissues (*29*); whether 2-AG ether is a naturally-occurring molecule should be re-examined.

Physiological roles of endogenous ligands

Physiological significance of anandamide. Anandamide is the first endogenous cannabinoid receptor ligand to be identified and has been shown to exhibit a variety of cannabimimetic activities *in vitro* and *in vivo*. Nonetheless, there are several enigmatic issues. (i) The levels of anandamide in the tissues are usually very low. (ii) No selective or efficient synthetic pathway for anandamide has hitherto been found. In addition, (iii) anandamide, as well as Δ^9-THC, act as a partial agonist at least in some cases. Indeed, anandamide is a very weak partial agonist of the CB2 receptor. It seems unlikely that an endogenous natural ligand acts as a partial agonist of its own receptor. It is thus questionable whether anandamide actually acts as an endogenous cannabinoid receptor ligand with significant physiological importance (*7, 19*). The physiological significance of anandamide may lie mainly in it being an endogenous ligand of a receptor other than the cannabinoid receptors or as a modulator of ion channels.

Physiological roles of 2-AG. In contrast to anandamide, 2-AG was found to act as a full agonist at either the cannabinoid CB1 receptor or the CB2 receptor. Moreover, 2-AG can be produced rapidly and selectively from a variety of cells upon stimulation. Unlike anandamide, 2-AG does not bind to the vanilloid receptor. Recently, we proposed that 2-AG is the endogenous natural ligand of the cannabinoid receptors (*7, 19*).

What are the physiological roles of 2-AG? It does not seem possible that the physiological compound 2-AG induces psychedelic reactions such as heightened sensory awareness, dissociation of ideas, errors in judgment of time and space and hallucinations in normal living animals. The cannabinoid CB1 receptor is mainly present in the presynapses and is assumed to be involved in the attenuation of neurotransmission (*5*). We hypothesized that the physiological role of 2-AG, the natural ligand of the CB1 receptor, in the synapse is as follows: 2-AG generated through increased phospholipid metabolism, especially inositol phospholipid breakdown, in neurons (presynapses and/or postsynapses) during accelerated synaptic transmission, plays an important role in calming the excitation of neuronal cells by acting at the cannabinoid CB1 receptor, thereby diminishing any subsequent neurotransmitter release (Fig. 2) (*7, 19, 30*).

Previously, we found that 2-AG suppresses depolarization-induced rapid elevation of the intracellular free Ca^{2+} concentrations in differentiated NG108-15 cells (*31*). Furthermore, recently, we obtained evidence that a substantial amount of 2-AG was released from rat brain synaptosomes upon depolarization (T. Sugiura, unpublished results). It is thus conceivable that 2-AG, generated from stimulated neurons, attenuates several neuronal cell functions such as neurotransmitter release by reducing the intracellular free Ca^{2+} concentrations. Such a negative feedback regulation mechanism should be effective in calming stimulated neurons after excitation (*7, 19, 30*). In any case, the above 2-AG– and cannabinoid CB1 receptor–dependent negative feedback regulation of neurotransmission could be of great physiological significance, because sustained activation of neuronal cells is known to cause cell exhaustion and may lead to neuronal cell death. It is noteworthy that several investigators have recently provided evidence that endogenous cannabinoid receptor ligands derived from the postsynapse as retrograde messenger molecules play important roles in the attenuation of neurotransmission (*32–34*). Moreover, Stella *et al.* (*21*) have reported that 2-AG suppresses long-term potentiation in rat hippocampal slices. Ameri and Simmet (*35*) also reported that 2-AG reduces neuronal excitability in rat hippocampal slices in a cannabinoid CB1 receptor-dependent manner. Further intensive studies on the physiological roles of 2-AG in the synapses are essential to better understand the regulatory mechanisms of neurotransmission in the mammalian nervous system.

In addition to its roles in the central nervous system, 2-AG may also play important physiological roles in other biological systems such as the reproductive system and the cardiovascular system. We have demonstrated that human endothelial cells produce and release 2-AG upon stimulation with thrombin or A23187 (*36*). We proposed that 2-AG derived from endothelial cells or peripheral nerve terminals may play an essential role in the vascular system through acting on the CB1 receptor (*36*). Several investigators have demonstrated that the administration of 2-AG induces hypotension in experimental animals and relaxation of blood vessels *in vitro* (*7, 19*); it seems possible that 2-AG acts as an endogenous vasodilator in living animals.

As mentioned before, 2-AG is the intrinsic natural ligand not only for the CB1 receptor but also for the CB2 receptor. This raised the possibility that 2-AG plays a physiological role 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

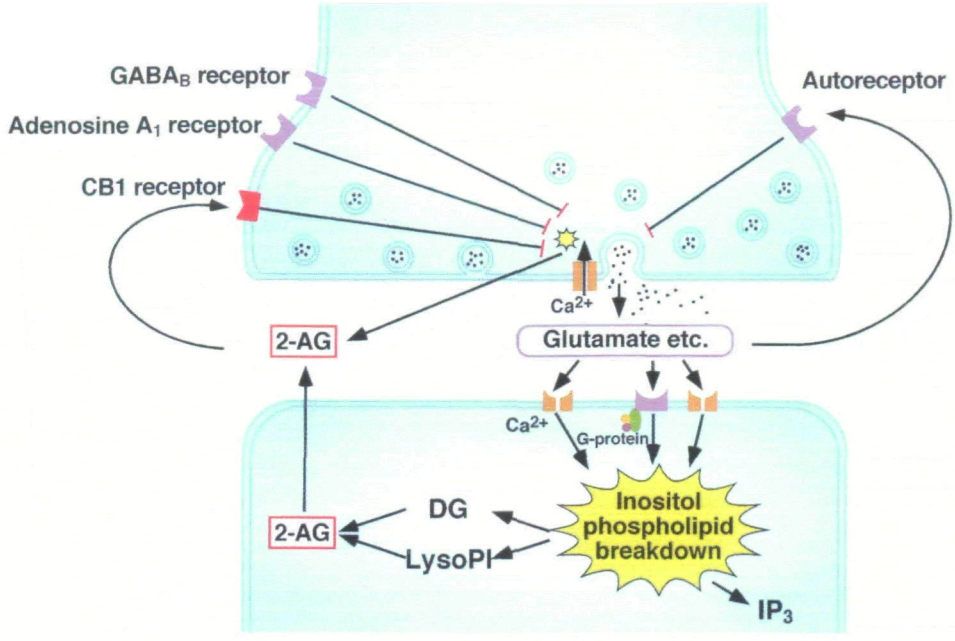

Fig. 2. **A possible regulatory role of 2-AG in neurotransmission.**

physiological or pathophysiological responses. Kaminski and co-workers (*37, 38*) have conducted pioneering studies on 2-AG and the function of murine lymphocytes. However, the precise physiological function of 2-AG and its receptor, *i.e.*, the CB2 receptor, in acute and chronic inflammation and/or immune responses still remains uncertain. Recently, we have found that 2-AG induces the activation of MAP kinases and accelerates the generation of chemokines such as IL-8 and MCP-1 in HL-60 cells (*7*). On the other hand, Iwamura *et al.* (*39*) have recently demonstrated that JTE-907, a CB2 receptor antagonist/inverse agonist, suppresses inflammatory reactions *in vivo*. These results together suggest that 2-AG acts as a proinflammatory molecule under certain circumstances.

Concluding remarks

Cannabinoid receptors are widely distributed in various mammalian organ systems. Evidence is accumulating that endogenous cannabinoid receptor ligands are physiologically essential molecules in various biological systems such as the nervous, cardiovascular and immune systems. It is apparent that cannabinoid receptors and their endogenous ligands are important attractive targets for new drugs in the future. Despite their potential physiological and pharmacological importance, however, the available information concerning these molecules (cannabinoid receptors and their endogenous ligands) is not yet sufficient as compared with the cases of other receptor systems such as the opioid system. Further detailed studies are necessary for a full understanding of the physiological roles of cannabinoid receptors and their endogenous ligands in diverse mammalian tissues and cells under various physiological and pathophysiological conditions. Such studies would be helpful in clarifying the precise regulatory mechanisms of several important physiological processes such as synaptic neurotransmission, control of vascular tone, inflammation and immune response.

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