Evidence That 2-Arachidonoylglycerol but Not N-Palmitoylethanolamine or Anandamide Is the Physiological Ligand for the Cannabinoid CB2 Receptor

We examined the effect of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, on the intracellular free Ca\(^{2+}\) concentrations in HL-60 cells that express the cannabinoid CB2 receptor. We found that 2-arachidonoylglycerol induces a rapid transient increase in intracellular free Ca\(^{2+}\) concentrations in HL-60 cells. The response was affected by neither cyclooxygenase inhibitors nor lipoxygenase inhibitors, suggesting that arachidonic acid metabolites are not involved. Consistent with this notion, free arachidonic acid was devoid of any agonistic activity. Importantly, the Ca\(^{2+}\) transient induced by 2-arachidonoylglycerol 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. G\(_i\) or G\(_s\) is also assumed to be involved, because pertussis toxin treatment of the cells abolished the response. We further examined the structure-activity relationship. We found that 2-arachidonoylglycerol is the most potent compound among a number of naturally occurring cannabimimetic molecules. Interestingly, anandamide and N-palmitoylethanolamine, other putative endogenous ligands, were found to be a weak partial agonist and an inactive ligand, respectively. These results strongly suggest that the CB2 receptor is originally a 2-arachidonoylglycerol receptor, and 2-arachidonoylglycerol is the intrinsic natural ligand for the CB2 receptor that is abundant in the immune system.

\(\Delta^9\)-Tetrahydrocannabinol (\(\Delta^9\)-THC), a major psychoactive constituent of marijuana, is known to exert a variety of pharmacological effects on laboratory animals and humans. When administered orally or intravenously, \(\Delta^9\)-THC induces reduced spontaneous motor activity, analgesia, heightened sensory awareness, euphoria, and impairment of short-term memory (1). \(\Delta^9\)-THC is also known to exert profound effects on several biological systems other than the central nervous system; for example, \(\Delta^9\)-THC exhibits potent immunosuppressive activities (2–12). The mechanism underlying such diverse actions of \(\Delta^9\)-THC remained obscure until the late 1980s. In 1988, Devane et al. (13) provided clear evidence that rat brain synaptosomes contain a specific binding site for cannabinoids using a radiolabeled synthetic cannabinoid \([^{1}H]CP55940\). Their finding strongly suggested that various cannabimimetic molecules including \(\Delta^9\)-THC itself bind to a specific receptor site(s) thereby eliciting a variety of responses. To date, two types of cannabinoid receptors have been identified as follows: the CB1 receptor expressed primarily in the nervous system (14), and the CB2 receptor expressed mainly in the immune system (15). Both are seven transmembrane, G protein-coupled receptors, and they share 44% overall identity (68% identity for transmembrane domains) (15).

The discovery of specific receptors for cannabinoids prompted the search for endogenous ligands. To date, two types of arachidonic acid-containing molecules, anandamide (16) and 2-arachidonoylglycerol (2-AG) (17, 18), have been reported as putative endogenous cannabinoid receptor ligands. Anandamide was isolated from porcine brain (16), and 2-AG was isolated from rat brain (17) and canine gut (18). So far, a number of studies have been directed toward anandamide (19, 20); yet, apparently, little attention has been paid to 2-AG. Previously, we investigated the level and the biosynthetic pathways for anandamide in the brain (21). We found that the level of anandamide in the fresh brain is very low. Similar results were obtained by several other investigators (22–24). We (21) and other investigators (25, 26) also provided evidence that anandamide is synthesized through the hydrolysis of N-arachidonylphosphatidylethanolamine which was formed from phosphatidylethanolamine and arachidonic acid esterified at the 1-position but not at the 2-position of phospholipids through the action of a transacylase, although selective or preferential synthesis of anandamide compared with other molecular species of N-acylethanolamines has not yet been observed. This observation together with a previous finding of a low level of anandamide in the brain raised the question of whether anandamide is actually of physiological importance, because usually only trace amounts of arachidonic acid are esterified at the 1-position of glycerophospholipids.

Recently, we investigated in detail the potencies of various cannabimimetic molecules to induce a Ca\(^{2+}\) transient in neuroblastoma x glioma hybrid NG108–15 cells that are known to express the CB1 receptor (27–29). We found that low nanomolar concentrations of 2-AG elicit Ca\(^{2+}\) transients through a CB1 receptor-dependent mechanism, the activity of 2-AG being more potent than those of other structurally related com-

*This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†To whom correspondence should be addressed. Fax: 81-426-85-1345.
‡The abbreviations used are: \(\Delta^2\)-THC, \(\Delta^2\)-tetrahydrocannabinol; 2-AG, 2-arachidonoylglycerol; [Ca\(^{2+}\)], intracellular free Ca\(^{2+}\) concentrations.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
pounds. In contrast to 2-AG, anandamide was found to act as a weak partial agonist at least in this assay system (27–29). Based on these observations, we proposed that the cannabinoid CB1 receptor is originally a 2-AG receptor (28, 29). The abundance (17, 30–32) and Ca\textsuperscript{2+}–induced selective and rapid formation (30) of 2-AG in the brain, neither of which is observed with anandamide, also support this hypothesis.

Despite recent rapid progress in cannabinoid receptor research, however, a number of important issues still remain unelucidated. One important question to be answered concerns the endogenous physiological ligand for the CB2 receptor. Previously, Facelli et al. (33) proposed that N-palmitoylthanolamine is an endogenous ligand for the CB2 receptor, yet conflicting experimental results have been reported by other investigators (34). Anandamide and 2-AG are also candidates for the endogenous ligand for the CB2 receptor, yet little information is available concerning the relative physiological importance of these molecules including N-palmitoylthanolamine as the CB2 receptor agonist. Because the CB2 receptor is assumed essential in the modulation of several immune cell functions (15, 35–42), it is important to clarify its intrinsic endogenous natural ligand. Here, we investigated the structure-activity relationship of various cannabimimetic molecules by comparing their potencies to induce Ca\textsuperscript{2+} transients using a promyelocytic leukemia cell line, HL-60, from which the CB2 receptor gene was cloned (15).

EXPERIMENTAL PROCEDURES

Chemicals—Arachidonic acid, palmitic acid, oleic acid, linoleic acid, \(\gamma\)-linolenic acid, eicosatrienoic acid (\(n - 3\) and \(n - 6\)), eicosapentaenoic acid (\(n - 3\)), docosatetraenoic acid (\(n - 6\)), docosahexaenoic acid (\(n - 3\)), and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). 2-Arachidonoylglycerol and the CB2 receptor antagonist, SR141716A, U73122, and U73343 were acquired from Biomol (Plymouth Meeting, PA). (\(\pm\))-\(\delta\)-Amino-1-propanol, \(\delta\)-(\(-\text{R})(\-\text{S})\)-2-amino-1-propanol, \(\delta\)-(\(+\text{S})(\-\text{R})\)-2-amino-1-propanol, \(\delta\)-(\(+\text{S})(\+\text{R})\)-2-amino-1-propanol, \(\delta\)-(\(+\text{R})(\+\text{S})\)-2-amino-1-propanol, \(\delta\)-(\(+\text{R})(\-\text{S})\)-2-amino-1-propanol, and \(\delta\)-(\(+\text{S})(\-\text{R})\)-2-amino-1-propanol, (\(+\text{R})(\+\text{S})\)-2-amino-1-propanol, (\(+\text{R})(\-\text{S})\)-2-amino-1-propanol, and (\(+\text{S})(\-\text{R})\)-2-amino-1-propanol, were from Tokyo Kasei Kogyo (Tokyo, Japan). WIN55212-2 and WIN55212-3 were from Research Biochemicals (Natick, MA). Per-actin, \(\alpha\)-fumaric acid-impregnated TLC using chloroform:methanol:acetic acid (98:24:1, \(v/v/v\)) was purchased from Amersham Pharmacia Biotech (Amersham, UK). Hybridization was performed at 65 °C for 2 h in QuickHyb solution (Strategene, Cambridge, UK). The filter was washed in 0.2× SSC (1× SSC = 0.15 mM NaCl and 0.015 mM sodium citrate) containing 0.1% SDS at 65 °C and subjected to autoradiography.

Quantitative Reverse Transcription (RT)-PCR—Quantitative RT-PCR was performed according to the method employed for the analysis of the CB1 receptor (\(\delta\)-agonist-activating for receptor gene and CB2 receptor (\(\delta\)-agonist-activating for receptor gene) (34). The primers for the CB2 receptor mRNA sequence were CB2-2-5 (sense, 5'-CTGCCAAGGT-GTTGTGTCCTG-3') and CB2-6 (antisense, 5'-ACCTCTACATCCAGCTTCCC-3'). The primers for the \(\beta\)-actin mRNA sequence were ACT1-2 (sense, 5'-TGCGCCAGGTCATGAGGCTAC-3') and ACT2 (antisense, 5'-ACTGATCTGTCAGGGTGGTT-3'). PCR was carried out using 2 units of Taq polymerase in 100 μl of a reaction mixture containing [\(\alpha\]-\(\text{P}\)]dCTP (111 TBq/mmol, 370 Bq in a reaction) under the following conditions: for the human CB1 receptor gene, denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 1 min; for the human CB2 receptor gene, denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 75 °C for 1 min; for the \(\beta\)-actin gene, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min. A 10-μl aliquot of the reaction mixture was sampled every cycle during the 24–33 cycles for amplification of either the CB1 receptor gene or the \(\beta\)-actin gene. When the amplification efficiencies were further incubated at 72 °C for 9 min and then electrophoresed on a 6% polyacrylamide gel. The relative radioactivity (photostimulated luminescence value) of the band corresponding to each PCR product was determined in a bioimaging analyzer BAS-1500 (Fuji Film, Tokyo, Japan). The results were plotted on a semilogarithmic scale versus the cycle number to obtain amplification curves, and the cycle difference between the logarithmic phase of the amplification curve for the CB1 receptor gene or the CB2 receptor gene was calculated. The ratio of CB1 receptor mRNA or CB2 receptor mRNA to \(\beta\)-actin mRNA was calculated using the following formula: ratio of CB1 receptor mRNA or CB2 receptor mRNA to \(\beta\)-actin mRNA = \(2^{-\Delta\Delta C_{T}}\).

Measurement of [\(\text{Ca}^{2+}\)]\(\text{i}\)—Subconfluent cells were further incubated in fresh medium without fetal bovine serum for 24 h. The cells were then suspended by gentle pipetting in 25 ml Hepes-buffered Tyrode’s solution (\(\Delta\text{[Ca}^{2+}\]) \(\text{i}\) (pH 7.4) containing 3 mM Fura-2/AM and further incubated at 37 °C for 45 min. The cells were then centrifuged (180 × 1000 g for 5 min), washed twice with Hepes-Tyrode’s solution (\(\Delta\text{[Ca}^{2+}\]) \(\text{i}\)), and resuspended in Hepes-Tyrode’s solution (\(\Delta\text{[Ca}^{2+}\]) \(\text{i}\)) containing 0.1% BSA. [\(\text{Ca}^{2+}\]) \(\text{i}\) was estimated using a CAF-100 \(\text{Ca}^{2+}\) analyzer (JASCO, Tokyo, Japan) as described previously (27–29). Ca\textsubscript{184} was added 4–5 min before the measurement (final Ca\textsubscript{184} concentration in the cuvette, 1 mM). 2-AG and other related compounds were dissolved in dimethyl sulfoxide (Me\textsubscript{2}SO), and aliquots (1 μl each) were added to the cuvette to the desired final Me\textsubscript{2}SO concentration, 0.2%. Me\textsubscript{2}SO (final concentration, 0.4%) per se did not markedly affect the [\(\text{Ca}^{2+}\]) \(\text{i}\). In some experiments, cells suspended in 500 μl of Hepes-Tyrode’s solution (\(\Delta\text{[Ca}^{2+}\]) \(\text{i}\)) containing 0.1% BSA were pretreated with CP55940 (final concentration, 10 μM) or 2-arachidonoylglycerol (final concentration, 10 μM) or vehicle alone (1 μl of Me\textsubscript{2}SO) at 37 °C for 1 min. Cells were then seated by centrifugation and resuspended in Hepes-Tyrode’s solution (\(\Delta\text{[Ca}^{2+}\]) \(\text{i}\)) containing 0.1% BSA. After the addition of Ca\textsubscript{184} (final concentration, 1 mM), 2-AG (final concentration, 1 μM) was added to the cuvette, and the changes in [\(\text{Ca}^{2+}\]) \(\text{i}\), were analyzed. To examine the effect of the removal of extracellular free Ca\textsubscript{184}, cells were incubated in Hepes-Tyrode’s solution containing 1 mM CaCl\textsubscript{2} at 37 °C for 3 min. The cell suspension was then centrifuged, and the supernatant was removed. The sediments cells were resuspended in Hepes-Tyrode’s solution containing 0.1 mM EGTA. The effect of 2-AG (final concentration, 1 μM) on [\(\text{Ca}^{2+}\]) \(\text{i}\), was analyzed as described above.
**RESULTS**

First, we examined the subtype of cannabinoid receptor mRNA present in HL-60 cells by Northern blot analysis and by quantitative RT-PCR analysis. Fig. 1 shows the result of Northern blot analysis. HL-60 cells were found to possess the CB2 receptor mRNA but not the CB1 receptor mRNA. This is in contrast to the case in the brain where the CB1 receptor mRNA is predominant. We detected two major transcripts (4.4 and 2.5 kb) for CB2 receptor mRNA in either HL-60 cells or the spleen, the result being consistent with previous reports (15, 45). The abundance of the CB2 receptor mRNA compared with the CB1 receptor mRNA in HL-60 cells was further confirmed by quantitative RT-PCR analysis (Fig. 2).

Next, we explored the biological activities of putative endogenous ligands for the cannabinoid receptor such as 2-AG using HL-60 cells. Previously, we have found that 2-AG induces a rapid, transient increase in [Ca^{2+}] in NG108–15 cells through a cannabinoid CB1 receptor-dependent mechanism (27–29), although nothing is so far known about the CB2 receptor. In the present study, we first examined whether or not 2-AG induces a Ca^{2+} transient in HL-60 cells which express the CB2 receptor. We found that 1 μM 2-AG induces a rapid transient increase in [Ca^{2+}] (Fig. 3). The response was not affected by pretreatment of the cells with either 10 μM indomethacin (a cyclooxygenase inhibitor) or 10 μM nordihydroguaiaretic acid (a lipooxygenase inhibitor) (Fig. 3). We further confirmed that the response was not influenced by pretreatment of the cells with 10 μM eicosatetraynoic acid which blocks both cyclooxygenase and lipooxygenase (data not shown). These results clearly indicate that the response induced by 2-AG is not due to arachidonic acid metabolites that may be produced during the incubation of the cells with 2-AG.

Next, we examined the effects of pretreatment with cannabinoid receptor antagonists on the response induced by 2-AG. As shown in Fig. 4, pretreatment of the cells with SR141716A, a CB1 receptor-specific antagonist (or inverse agonist), did not affect the response induced by 2-AG, whereas pretreatment of the cells with SR144528, a CB2 receptor-specific antagonist (or inverse agonist), reduced the response markedly. It is evident from these results that the response induced by 2-AG is mediated mostly through the cannabinoid CB2 receptor.

We then examined whether or not CP55940 induces a similar response. As shown in Fig. 5, CP55940 induces a rapid, transient increase in [Ca^{2+}], similar to 2-AG. We further examined whether or not the desensitization is observed in 2-AG- or CP55940-treated cells. We found that pretreatment of the cells with 2-AG abolished the response induced by either 2-AG or CP55940, indicating that desensitization took place in those cells following the treatment with 2-AG. Similarly, pretreatment of the cells with CP55940 nullified the response induced by either 2-AG or CP55940. These results strongly suggest that 2-AG and CP55940 interact with a common receptor site to elicit Ca^{2+} transients in these cells.

Next, we examined the mechanism underlying rapid transient increases in [Ca^{2+}]. As shown in Fig. 6, pretreatment of the cells with pertussis toxin (100 ng/ml) reduced the response induced by either 2-AG (1 μM) or CP55940 (1 μM) markedly. It is apparent, therefore, that the response induced by either 2-AG or CP55940 is mediated in a large part by G_{i} or G_{o}. The effect of U73122, a phospholipase C inhibitor, on the response induced by 2-AG was examined next. We found that pretreatment of the cells with U73122 abolished the response induced by 2-AG, whereas pretreatment of the cells with U73343, an inactive analogue, did not affect the response (data not shown). These results suggest that phospholipase C is involved in 2-AG-induced transient increases in [Ca^{2+}]. We also found that the removal of extracellular free Ca^{2+} by EGTA did not abolish the response induced by 1 μM 2-AG (50–60% of the response still remained) (data not shown), suggesting that the rapid transient increase in [Ca^{2+}] is due, at least in part, to the mobilization of Ca^{2+} from intracellular Ca^{2+} store site(s).

Finally, we examined the structure-activity relationship of 2-AG and related compounds in detail. Fig. 7 illustrates the chemical structures of the various cannabinoid receptor ligands and their analogues examined here, and Fig. 8 summarizes their activities in inducing rapid transient increases in [Ca^{2+}] in HL-60 cells. First, we examined the dose dependence of the 2-AG-induced elevation of [Ca^{2+}] as shown in Fig. 8A. 2-AG was found to exhibit strong agonistic activity even at low nanomolar concentrations. The response was detectable from as low as 1 nM and was augmented with increasing concentrations 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 apparently lower than that of 2-AG (Fig. 8, B and C). This is also the case for monoarachidonoylxyethylene glycol (Fig. 8D), an analogue of 2-AG lacking one of two hydroxy methylene groups. We confirmed that free arachidonic acid is devoid of any agonistic activity (Fig. 8E), indicating that free arachidonic acid, which is possibly generated from 2-AG during the incubation, is not involved in the 2-AG-induced increases in [Ca^{2+}], described above. This was confirmed by the fact that an ether-linked analogue of 2-AG (2-AG ether) is capable of eliciting rapid increases in [Ca^{2+}] to some extent (Fig. 8F), yet the magnitude of the response induced by 2-AG ether was weak compared with that of 2-AG.

Next, we examined the activities of N-arachidonoylserinol, an acid-amide bond-containing analogue of 2-AG. As shown in Fig. 8G, N-arachidonoylserinol exhibited some agonistic activity, yet its activity was far less pronounced than that of 2-AG as in the case of 2-AG ether. It seems very likely that an ester linkage is needed in the molecule to render strong agonistic activity. In keeping with this, anandamide (N-arachidonoylthanolamine), another type of acid-amide bond-containing analogue of 2-AG, was found to possess only weak agonistic activity (Fig. 8H).

The activities of various species of 2-monoacylglycerols were next examined. As shown in Fig. 8I, 2-palmitoylglycerol did not exhibit any agonistic activity. The activity of 2-oleoylglycerol was also negligible (Fig. 8I). On the other hand, 2-linoleoylglycerol was found to exhibit weak agonistic activity (Fig. 8K),...
its activity being comparable to that of N-arachidonoylserinol. Similar weak agonistic activities were observed with 2-γ-linolenoylglycerol (Fig. 8L), 2-eicosatrienoyl-(n − 3)-glycerol (Fig. 8M), and 2-eicosatrienoyl-(n − 6)-glycerol (Fig. 8N). A striking observation shown here is that 2-eicosatrienoyl-(n − 9)-glycerol (Fig. 8O) and 2-eicosapentaenoyl-(n − 3)-glycerol (Fig. 8P) exhibited rather strong agonistic activities. In particular, the activity of 2-eicosatrienoyl-(n − 9)-glycerol was comparable to that of 2-AG, indicating that the entire structure of the arachidonyl moiety is not necessarily required for strong agonistic activity. In addition to these C20 polyunsaturated fatty acid-containing 2-monoacylglycerols, two types of C22 polyunsaturated fatty acid-containing 2-monoacylglycerol, 2-docosatetraenoyl-(n − 6)-glycerol and 2-docosahexaenoyl-(n − 9)-glycerol, were also found to exhibit appreciable agonistic activities (Fig. 8, Q and R), although their activities were apparently lower than those of 2-eicosatrienoyl-(n − 9)-glycerol and 2-eicosapentaenoyl-(n − 3)-glycerol.

The activities of several classical and synthetic cannabinoids and related compounds were examined next. As shown in Fig. 8S, Δ9-THC was found to exhibit only weak agonistic activity. In contrast to this, either CP55940 or HU-210 exhibited strong agonistic activity (Fig. 8, T and U). We also found that WIN55212-2, a cannabinimimetic aminoalkylindole, possessed substantial agonistic activity (Fig. 8V), whereas its inactive isomer WIN55212-3 did not exhibit appreciable agonistic activity (Fig. 8W). We also confirmed that N-palmitoylethanolamine, which has been reported as a possible endogenous CB2 receptor ligand, did not act as an agonist (Fig. 8X).

**DISCUSSION**

Δ9-THC, the active constituent of marijuana, is known to suppress a variety of immune cell functions in vitro and in vivo (2–12), although the mechanism of action is not yet fully elucidated. Several lines of evidence strongly suggest that at least a part of this cannabinoid-induced immune suppression is mediated through specific receptor sites for cannabinoids expressed on immune cells. Several years ago, Kaminski and co-workers (46) provided evidence that the cannabinoid receptors are actually present and function in splenocytes. Furthermore, Casellas and co-workers (45, 47) demonstrated that the cannabinoid CB2 receptor mRNA is abundant in various types of leukocytes as well as in several types of leukemia cells such as HL-60 cells. It has generally been assumed that the CB2 receptor is a physiologically important and functionally relevant cannabinoid receptor in immune cells (15, 35–42, 45, 47).

What then is the endogenous natural ligand for the cannabinoid CB2 receptor? There are several possibilities. Anandamide is the first endogenous cannabinoid receptor ligand (16) and has been shown to exhibit strong binding activity toward the CB2 receptor in addition to the CB1 receptor (18), yet its activity as a CB2 receptor agonist was found to be very weak (36, 38, 48). 2-AG is the second endogenous cannabinoid receptor ligand (17, 18) and has been shown to enhance or suppress...
lymphocyte proliferation in vitro (39). However, it is not known whether such effects are due to 2-AG itself or its metabolites possibly formed during prolonged incubation of the cells with 2-AG. *N*-Palmitoylethanolamine is also suggested to act as an endogenous ligand for the CB2 receptor (33), yet there is a conflicting report concerning *N*-palmitoylethanolamine (34). Thus, until very recently, the principal physiologically relevant natural ligand for the cannabinoid CB2 receptor remained quite elusive.

As for the CB1 receptor ligands, previously, we found that 2-AG and other cannabinoid receptor ligands such as anandamide and Δ⁹-THC induce rapid transient increases in \([Ca^{2+}]_i\) in NG108–15 cells through a cannabinoid CB1 receptor-dependent mechanism (27–29). To our knowledge, this is the first report showing that a cannabinoid receptor ligand induces a Ca²⁺ transient through a cannabinoid receptor-dependent mechanism. Importantly, the cellular response can be detected immediately after the addition of the ligands to the cells. Furthermore, the method employed is sensitive enough to detect even small responses observed with low concentrations of cannabinoid receptor agonists such as 2-AG; measurement of \([Ca^{2+}]_i\) is a useful tool in evaluating agonistic activities of various CB1 receptor ligands. Based on the results of structure-activity relationship experiments, we proposed that 2-AG but not anandamide is the intrinsic physiological ligand for the CB1 receptor (28, 29).

The question then arises whether or not cannabinoid receptor ligands including 2-AG induce Ca²⁺ transients in the case of the CB2 receptor. In this study, we examined this point in detail. We found that various cannabinoid receptor ligands including 2-AG induce rapid transient increases in \([Ca^{2+}]_i\) in HL-60 cells. It is evident that the response was mediated through the cannabinoid CB2 receptor but not the CB1 receptor, because SR144528, a CB2 receptor-specific antagonist (or inverse agonist) (49), but not SR141716A, a CB1 receptor-specific antagonist (or inverse agonist) (50), blocked the response (Fig. 4). Gi or Go and phospholipase C are also assumed to be involved as in the cases of the CB1 receptor (27, 28) and the opioid receptor (51). We confirmed that the response induced by 2-AG (0.1 µM) was greater than that induced by the same concentration of platelet-activating factor (1-O-hexadecyl) or lysophosphatidic acid (1-palmitoyl); \([Ca^{2+}]_i\) was 31 nM for platelet-activating factor and 18 nM for lysophosphatidic acid (the means obtained from two separate experiments). Noticeably, the activity of 2-AG was most potent and prominent among those of various species of monoacylglycerols and naturally occurring cannabimimetic molecules (Fig. 8). Various species of 2-monoacylglycerols containing saturated, monoenic, dienoic, and trienoic fatty acids other than eicosatrienoic acid \((n=9)\) were found to exhibit negligible or only weak agonistic activities. Even the positional isomers of 2-AG, i.e. 1-AG and 3-AG, and monoarachidonylethylene glycol, whose chemical structure is closely related to that of 2-AG, exhibited apparently lower agonistic activities than 2-AG. As for other
candidates for endogenous ligand, anandamide was found to act as a weak partial agonist toward the CB2 receptor, which is consistent with the observations by several investigators using other assay systems (36, 38, 48). We also found that N-palmitoylethanolamine did not exhibit any appreciable agonistic activity. We confirmed that pretreatment of the cells with N-palmitoylethanolamine did not affect the response induced by 2-AG or CP55940 (data not shown), indicating that N-palmitoylethanolamine acts as neither an agonist nor an antagonist toward the CB2 receptor. All these results strongly suggest that the structure of 2-AG is strictly recognized by the CB2 receptor and that 2-AG but not N-palmitoylethanolamine or anandamide is the intrinsic physiological ligand for the cannabinoid CB2 receptor.

The results of structure-activity relationship experiments concerning the CB2 receptor ligands shown here resemble in general those for the CB1 receptor ligands reported earlier (27–29). This is noteworthy, because there is only 44% homology between the CB1 and CB2 receptors. Marked differences between the present experimental results concerning the CB2 receptor and previous results concerning the CB1 receptor are as follows: 1) CP55940 and HU-210 were partial agonists for the CB1 receptor (29) but potent full agonists for the CB2 receptor (Fig. 8). 2) Δ⁹-THC exhibited substantial agonistic activity toward the CB1 receptor (28) but weak agonistic activity for the CB2 receptor (Fig. 8). Weak agonistic activity of Δ⁹-THC toward the CB2 receptor has been reported by several investigators (36, 38, 48).

It was four to five years ago that the role of 2-AG as an endogenous cannabinoid receptor ligand was first suggested and described in the literature (17, 18, 52). Yet, surprisingly, little attention has been paid toward 2-AG as compared with anandamide. The reason for this may be that anandamide appeared to exhibit much more potent binding activity toward the cannabinoid receptors than 2-AG (17, 18, 52). Despite its strong binding activity toward the cannabinoid receptors, however, it is becoming evident that anandamide is a rather weak partial agonist toward the cannabinoid CB1 receptor (53). In contrast to anandamide, 2-AG was found to act as a potent full agonist toward the CB1 receptor (27–29). Moreover, the binding activity of 2-AG reported in preceding studies may have been underestimated because of possible degradation by hydrolyzing enzyme(s) during prolonged incubation (17). In fact, 2-AG was metabolized rapidly by a monoacylglycerol lipase and/or an amidohydrolase in cultured cells (54). Thus, recently, 2-AG has received much attention, and evidence is gradually accumulating that 2-AG is a physiologically important cannabinoid CB1 receptor agonist in the nervous system.
Here, we provided further evidence that not only the cannabinoid CB1 receptor but also the CB2 receptor abundant in the immune system was originally a 2-AG receptor, which lead us to postulate that 2-AG plays some important physiological role in the immune system as well as in the nervous system.

What is the physiological role of 2-AG in the immune system? Further studies are required to answer this question. Several years ago, Lee et al. (39) reported that 2-AG exerts both stimulative and inhibitory effects on the proliferation of splenocytes. The IgM antibody-forming cell response of splenocytes was also enhanced in the presence of 2-AG (39). Furthermore, Ouyang et al. (55) have recently demonstrated that 2-AG suppressed interleukin-2 gene expression in activated T cells. However, it is not clear whether these effects are attributable to 2-AG itself or arachidonic acid metabolites derived from 2-AG, and whether these cellular responses are mediated through the cannabinoid receptors. On the other hand, Casellas and co-workers (36, 37) demonstrated that low nanomolar concentrations of CP55940 actually enhance the proliferation of B cells. In contrast to CP55940, anandamide did not stimulate cell proliferation. Such an effect of CP55940 was mediated through the cannabinoid CB2 receptor, because pretreatment of the cells with SR144528 but not SR141716A abolished the response (37). These authors proposed that the cannabinoid CB2 receptor is involved in the differentiation of B cells (37); it is possible that 2-AG, the intrinsic natural ligand for the cannabinoid CB2 receptor, plays some important role in the regulation of growth and differentiation of B cells by acting on the CB2 receptor.

Previously, we provided evidence that 2-AG is actually present in the immune tissues such as the spleen (30). The following questions are then raised: which types of cells generate 2-AG, and which types of stimuli are required or are sufficient to synthesize and release 2-AG in the immune system? Recently, Di Marzo and co-workers (56) demonstrated that lipopolysaccharide-stimulated macrophages generate 2-AG. Prescott and Majerus (57) and Varga et al. (58) have also demonstrated that platelets generate 2-AG upon stimulation. Nonetheless, further detailed studies are still required to clarify the cellular

**Fig. 8.** Comparison of the effects of 2-AG, its structural analogues, and related compounds on $[Ca^{2+}]_i$ in HL-60 cells. Cells, loaded with Fura-2/AM, were challenged with 2-AG and other compounds, and changes in $[Ca^{2+}]_i$ were analyzed. The mean and S.D. were calculated from the results of five separate experiments.
origin of 2-AG as well as the mechanism of synthesis and release of 2-AG in the immune system. Such information should be essential to understand better the physiological significance of 2-AG in the immune tissues.

Acknowledgments—We thank Dr. Ikuo Yamamoto for providing Δ9-THC and Keiko Kimoto for technical assistance.

REFERENCES

1. Dewey, W. L. (1986) Pharmacol. Rev. 38, 151–178
2. Hallister, L. E. (1992) J. Psychoact. Drugs 24, 159–164
3. El-Sohemy, A., Hasse, H., Klein, T. W., Stewart, W. E. II, and Friedman, H. (1986) Int. J. Immunopharmacol. 8, 819–824
4. Specter, S., Lanetz, G., and Hazelde, J. (1990) Int. J. Immunopharmacol. 12, 261–267
5. Specter, S., Lanetz, G., and Goodfellow, D. (1991) J. Leukocyte Biol. 50, 423–426
6. Klein, T. W., Kawakami, Y., Newton, C., and Friedman, H. (1991) J. Toxicol. Environ. Health 13, 1445–1452
7. Zheng, Z.-M., Boichot, E., Germain, N., Allain, N., Anger, J.-P., and Lagente, V. (1997) Eur. J. Pharmacol. 330, 231–249
8. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Rtinger, A., and Mechoulam, R. (1992) Nature 356, 65–67
9. Kaminski, N. E., Koh, W. S., Yang, K. H., Lee, M., and Kessler, F. K. (1994) J. Pharmacol. Exp. Ther. 267, 1558–1565
10. Kusher, D. I., Dawson, L. O., Taylor, A. C., and Djeu, J. Y. (1994) Biochem. Pharmacol. 47, 676–683
11. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) Nature 346, 561–564
12. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Rtinger, A., and Mechoulam, R. (1992) Nature 356, 65–67
13. Devane, W. A., Dysarz, F. A., III, Johnson, M. R., Melvin, L. S., and Howlett, A. B. (1988) Mol. Pharmacol. 34, 695–613
14. Matsuda, L. A., Loai, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) Nature 346, 561–564
15. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) Eur. J. Pharmacol. 267, 442–447
16. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Rtinger, A., and Mechoulam, R. (1992) Nature 356, 65–67
17. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1996) Biochem. Pharmacol. 52, 1920–1929
18. Mechoulam, R., Ben-Shabat, S., Meiri, U., and Horowitz, M. (1998) Eur. J. Pharmacol. 362, R1–R3
19. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
20. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
21. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
22. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
23. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
24. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
25. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
26. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
27. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
28. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
29. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
30. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
31. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
32. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
33. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
34. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
35. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
36. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
37. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
38. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96
39. Sugiura, T., Kondo, S., Nakane, S., Mutsuka, A., Kato, H., and Waku, K. (1995) Biochem. Pharmacol. 50, 83–96