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2-Arachidonoylglycerol, a Putative Endogenous Cannabinoid Receptor Ligand, Induces Rapid, Transient Elevation of Intracellular Free Ca^{2+} in Neuroblastoma × Glioma Hybrid NG108-15 Cells

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Low concentrations of 2-arachidonoylglycerol were found to induce rapid, transient elevation of intracellular free Ca^{2+} in NG108-15 cells (EC50 was 150 nM). Free arachidonic acid, 2-palmitoylglycerol, 2-oleoylglycerol, 2-linoleoylglycerol and 2-docosahexaenoylglycerol were inactive. Anandamide acted as a partial agonist. Importantly, desensitization was observed upon sequential challenge with 2-arachidonoylglycerol. Furthermore, cross-desensitization was observed between 2-arachidonoylglycerol and WIN 55212-2, a cannabinoid receptor agonist. Pretreatment of the cells with SR141716A, a cannabinoid receptor antagonist, abolished the activities of both 2-arachidonoylglycerol and WIN 55212-2. These results strongly suggest that 2-arachidonoylglycerol and WIN 55212-2 bind to a common cannabinoid receptor to elicit cellular responses and that 2-arachidonoylglycerol has some physiological role in nervous tissues.

A cannabinoid receptor (CB1) was first identified in rat brain in 1988 (1), and was cloned from a rat brain cDNA library in 1990 (2). These findings strongly suggest the occurrence of endogenous cannabinoid receptor ligand(s) in this tissue. In 1992, Devane et al. (3) isolated N-arachidonoylethanolamine (anandamide) from porcine brain and demonstrated that it binds to the cannabinoid receptor and exhibits various cannabimimetic activities. Several studies have been directed toward the actions, biosynthesis and catabolism of anandamide in mammalian tissues (4-7), but the physiological roles of anandamide still remain to be established. The levels of anandamide in several tissues including brain were found to be usually very low (8,9), and further, anandamide was suggested to act as a partial agonist in N18 neuroblastoma cells (10). These observations prompted us to postulate the occurrence of other cannabimimetic materials in mammalian tissues.

Recently, we (11,12) and Mechoulam et al. (13) suggested that 2-arachidonoylglycerol, a product of increased inositol phospholipid metabolism, may function as another type of endogenous cannabinoid receptor ligand. 2-Arachidonoylglycerol inhibits the specific binding of a non-classical cannabinoid, [^{3}H]CP55940, to synaptosomal membranes (11,12) and the specific binding of [^{3}H]HU243 to COS-7 cells transfected with cannabinoid receptor genes (CB1 and CB2) (13). 2-Arachidonoylglycerol also exhibits several cannabimimetic activities on mouse spleen cells (13,14), on mouse isolated vasa deferentia (13) and on the behavior as well as the body temperature of mice when administered intravenously (13). Nevertheless, little information is available concerning the direct effects of 2-arachidonoylglycerol on neuronal

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Abbreviations: $[Ca^{2+}]_i$, intracellular level of free Ca^{2+}; PAF, platelet-activating factor; PTX, pertussis toxin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; fatty acids were designated in terms of number of carbon atoms:number of double bonds, e.g., 20:4 for arachidonic acid.

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tissues and cells. Such information is needed to establish whether 2-arachidonoylglycerol is actually implicated in the modulation of nervous system function.

In the present study, we examined the effects of 2-arachidonoylglycerol and related molecules, as well as WIN 55212-2, a well-known cannabimimetic alkylaminoindole, on the intracellular level of free Ca^{2+} ($[Ca^{2+}]_i$) in neuroblastoma × glioma hybrid NG108-15 cells. Our findings that low concentrations of both 2-arachidonoylglycerol and WIN 55212-2 induced a rapid and transient modest rise in $[Ca^{2+}]_i$, while anandamide acted as a weak agonist, imply that 2-arachidonoylglycerol may be an endogenous cannabinoid receptor agonist in nervous tissues.

MATERIALS AND METHODS

Chemicals. WIN 55212-2 and WIN 55212-3 were obtained from RBI (Natick, MA, USA). SR141716A was from Biomol (Plymouth Meeting, PA, USA). Arachidonic acid, docosahexaenoic acid, linoleic acid, oleic acid, palmitic acid and essentially fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Platelet-activating factor (PAF, C16:0) was from Bachem (Bubendorf, Switzerland). Serinol (2-amino-1,3-propanediol) was from Tokyo Kasei Kogyo (Tokyo, Japan). Anandamide was prepared by a modification of the method of Devane et al. (3). N-Arachidonoylserinol was prepared from serinol and arachidonoyl chloride, analogously to the synthesis of anandamide (3,9,12). Various types of triacylglycerols were synthesized from the appropriate fatty acid anhydrides and glycerol (12). 2-Monoacylglycerols were prepared from corresponding triacylglycerols by digestion with *Rhizopus delemar* lipase and purified as described earlier (12). Fura-2/AM was from Wako Pure Chem. Ind. (Osaka, Japan).

Cells. NG108-15 cells were kindly donated by Dr. H. Higashida (Kanazawa University, Kanazawa, Japan). Cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 5 % fetal bovine serum (FBS) and HAT (hypoxanthine, aminopterine and thymidine) in an atmosphere of 90 % air-10 % CO_2 (15).

Measurement of $[Ca^{2+}]_i$. Subconfluent cells grown in 100 mm polystyrene dishes were further incubated in fresh medium without FBS for 24 h. Then, the medium was removed and the cells were washed once with 25 mM Hepes-Tyrode's solution ($-Ca^{2+}$) (pH 7.4). The cells, suspended in Hepes-Tyrode's solution ($-Ca^{2+}$) containing 3 μM Fura-2/AM by gentle pipetting, were incubated at 37°C for 45 min. Following the incubation, the cells were spun down by centrifugation (100 × g for 5 min), washed twice with Hepes-Tyrode's solution ($-Ca^{2+}$) and resuspended in Hepes-Tyrode's solution ($-Ca^{2+}$) containing 0.025 % BSA. $[Ca^{2+}]_i$ was estimated as described elsewhere (16) using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo, Japan). $CaCl_2$ was added to the cells 4-5 min before the measurement (the final concentration of Ca^{2+} in the cuvette was 1 mM). 2-Arachidonoylglycerol and other analogues were dispersed in saline containing 0.25 % BSA by a brief sonication, and aliquots (10 μl each) were added to the cuvette (500 μl of cell suspension). WIN 55212-2, SR141716A, indomethacin and nordihydroguaiaretic acid were dissolved in dimethyl sulfoxide (DMSO), and aliquots (1 μl each) were added to the cuvette (the final concentration of DMSO was 0.2 %, or in some cases 0.4 %). DMSO *per se* did not affect $[Ca^{2+}]_i$, at least up to 0.4 %. The basal level of $[Ca^{2+}]_i$ in unstimulated cells was estimated to be 120-150 nM.

RESULTS AND DISCUSSION

First, we examined the effects of 2-arachidonoylglycerol on $[Ca^{2+}]_i$ in NG108-15 cells. As shown in Fig. 1 (a), we found that $[Ca^{2+}]_i$ in NG108-15 cells rapidly increased following the addition of 2-arachidonoylglycerol. The rise in $[Ca^{2+}]_i$ was transient: the peak was 15 sec after the stimulation and $[Ca^{2+}]_i$ returned to the basal level within 30 sec. The response was detectable with as little as 3-10 nM 2-arachidonoylglycerol and was augmented with increasing concentration of 2-arachidonoylglycerol. The EC50 value was 150 nM. We confirmed that the response was not markedly affected by indomethacin (5 μM) or nordihydroguaiaretic acid (10 μM) (added 4 min before the addition of 2-arachidonoylglycerol) (data not shown), suggesting that arachidonic acid metabolites are not implicated in the rise in $[Ca^{2+}]_i$ induced by 2-arachidonoylglycerol. The magnitude of the maximal response observed with 2-arachidonoylglycerol was about 40 % of that observed with PAF (Fig. 1 (d)), which is known to bind to PAF receptors on the cell surface and to elicit a rise in $[Ca^{2+}]_i$ in various types of cells, including neuronal cells (17). The EC50 value for PAF was 3 nM. We also found that WIN 55212-2, a synthetic cannabinoid receptor agonist, elicited a similar rise in $[Ca^{2+}]_i$ (Fig. 1 (b)). The response was detectable at the concentration of 10 nM WIN 55212-2. The EC50 value was 210 nM, and the magnitude of the maximal response induced by WIN 55212-2 was almost

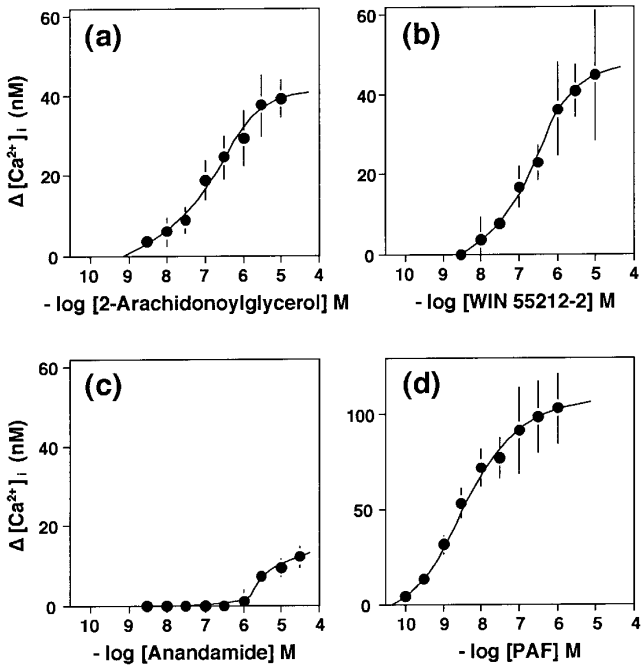

FIG. 1. Effects of 2-arachidonoylglycerol, WIN 55212-2, anandamide and PAF on $[Ca^{2+}]_i$ in NG108-15 cells. Cells loaded with Fura-2/AM were stimulated with various concentrations of 2-arachidonoylglycerol (a), WIN 55212-2 (b), anandamide (c) and PAF (d). Changes in $[Ca^{2+}]_i$ were measured using a Ca^{2+} analyzer (CAF-100) and expressed as $\triangle$ $[Ca^{2+}]_i$ (nM). The mean and SD were calculated from the results of three separate experiments.

the same as that observed for 2-arachidonoylglycerol. We confirmed that a pharmacologically less active stereoisomer, WIN 55212-3 (10 μM), failed to induce the elevation of $[Ca^{2+}]_i$ (data not shown). In contrast to 2-arachidonoylglycerol and WIN 55212-2, anandamide elicited only a weak response (Fig. 1 (c)). The response was detectable at above 1 μM anandamide, its magnitude being still very small even at 30 μM.

Next we examined the abilities of various structural analogues of 2-arachidonoylglycerol and related compounds to induce the elevation of $[Ca^{2+}]_i$. As shown in Fig. 2, a weak activity was observed with N-arachidonoylserinol (10 μM). On the other hand, 2-palmitoylglycerol, 2-oleoylglycerol, 2-linoleoylglycerol and 2-docosahexaenoylglycerol did not induce any appreciable rise in $[Ca^{2+}]_i$, even at 10 μM. Interestingly, free arachidonic acid (10 μM and 30 μM) also failed to induce a rise in $[Ca^{2+}]_i$. These results indicate that 2-arachidonoylglycerol is by far the most potent agonist among the various lipid analogues examined here, and suggest that the structures of 2-arachidonoylglycerol and WIN 55212-2 are rather strictly recognized by NG108-15 cells.

In order to examine whether 2-arachidonoylglycerol exerts its effects through a putative receptor on the cell surface, we conducted desensitization experiments. As shown in Fig. 3 (a), cells challenged with 2-arachidonoylglycerol did not respond to a subsequent challenge with 2-arachidonoylglycerol. Similarly, cells challenged with WIN55212-2 failed to respond to a second challenge with the same compound (Fig. 3 (b)). On the other hand, cells challenged with 2-arachidonoylglycerol responded to a subsequent challenge with PAF (Fig. 3 (d)). These results suggest the existence of a specific binding site for 2-arachidonoylglycerol on the cell surface. Importantly, cells challenged with 2-arachidonoylglycerol did not respond well to WIN 55212-2 and *vice versa* (Fig. 3 (e) and (f)), indicating that 2-arachidonoylglycerol and

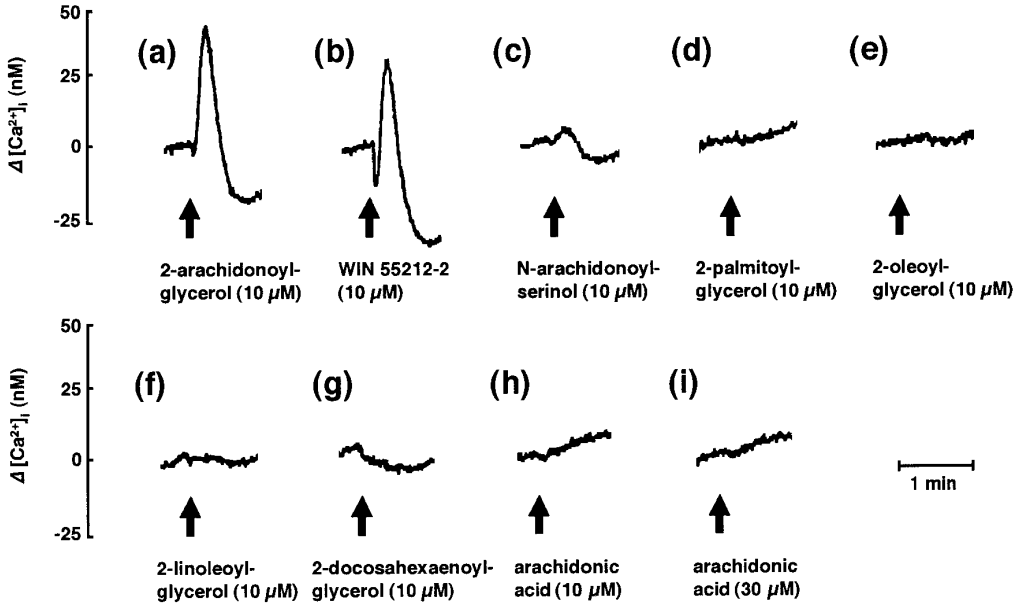

FIG. 2. Effects of 2-arachidonoylglycerol and its structural analogues on $[Ca^{2+}]_i$ in NG108-15 cells. Cells loaded with Fura-2/AM were stimulated with 2-arachidonoylglycerol and its analogues. The results are representative of those in five separate experiments which gave similar results.

WIN 55212-2 bind to a common receptor site, i.e., a cannabinoid receptor. To confirm this, we examined the effect of a specific cannabinoid receptor (CB1) antagonist, SR141716A on 2-arachidonoylglycerol- and WIN 55212-2-induced elevation of $[Ca^{2+}]_i$. We found that cells treated with 100 nM SR141716A failed to respond to either 2-arachidonoylglycerol or WIN 55212-2 (Fig. 3 (g) and (h)), indicating that 2-arachidonoylglycerol and WIN 55212-2 acted on a common cannabinoid receptor (CB1) to induce the transient rise in $[Ca^{2+}]_i$.

Finally, we examined the effects of pertussis toxin. As shown in Fig. 4, the pretreatment of cells with pertussis toxin abolished the responsiveness to both 2-arachidonoylglycerol and WIN 55212-2, suggesting that pertussis toxin-sensitive G protein(s), such as Gi or Go, is involved in the 2-arachidonoylglycerol- and WIN 55212-2-induced elevation of $[Ca^{2+}]_i$ shown here.

It is clear from the above results that there is a specific functional receptor site for 2-arachidonoylglycerol in NG108-15 cells. This receptor appears to be coupled with pertussis toxin-sensitive G protein(s), as has already been suggested for cannabinoid receptor (5). Previously, Felder et al. (18) demonstrated that relatively high concentrations (1-10 μM) of a synthetic cannabinoid receptor agonist, HU210, induce a cannabinoid receptor (CB1)-independent gradual rise in $[Ca^{2+}]_i$ in CHO cells. They also reported that high concentrations of anandamide (above 10 μM) induced a similar cannabinoid receptor (CB1)-independent rise in $[Ca^{2+}]_i$ in CHO cells (19). In this study, we showed that low concentrations of both 2-arachidonoylglycerol and WIN 55212-2 acted on a common cannabinoid receptor to elicit rapid cellular responses. Therefore, the mechanisms are apparently different.

Details of the mechanism underlying the rapid, receptor-mediated transient rise in $[Ca^{2+}]_i$ are unclear. We found that the response was decreased to 20 % when the extracellular Ca^{2+} concentration was decreased by adding 1.5 mM EGTA (data not shown); the presence of extracellular Ca^{2+} appears to be required to elicit the full response. This observation, however, may also suggest the possibility that a part of the Ca^{2+} was released from intracel-

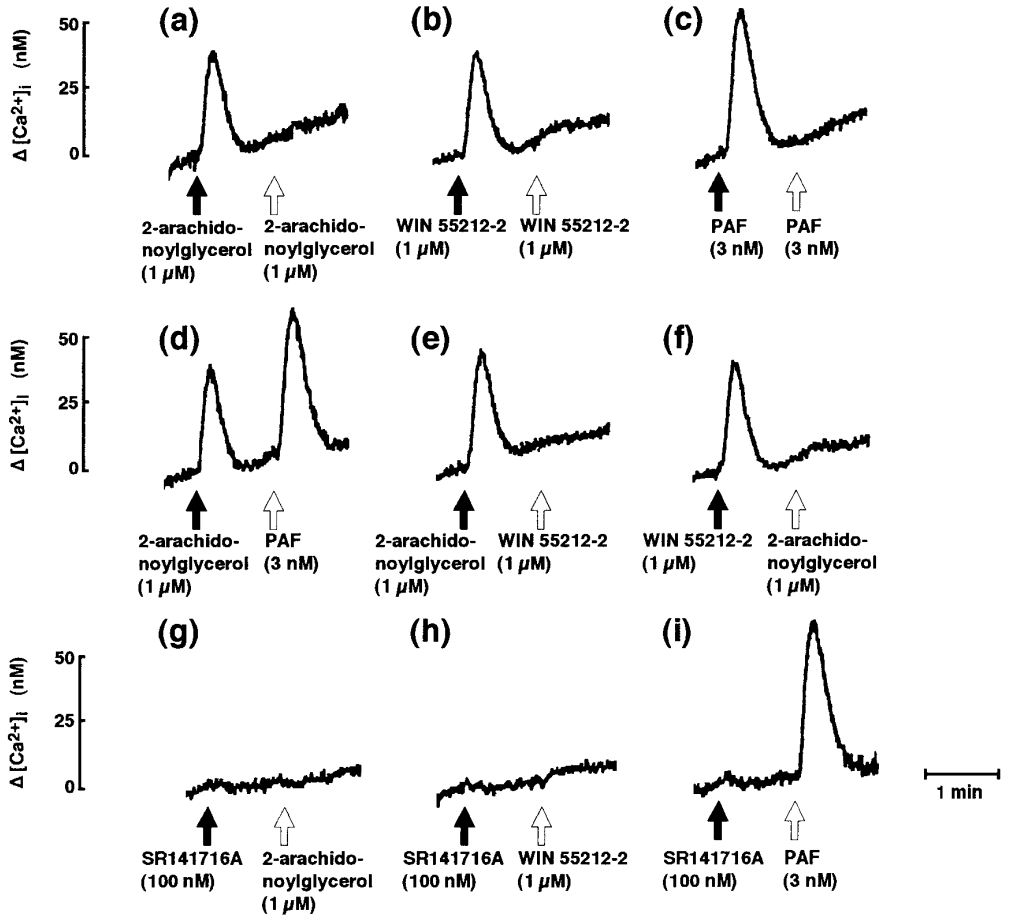

FIG. 3. Effects of sequential challenge and a receptor antagonist on the rise in $[Ca^{2+}]_i$ in NG108-15 cells induced by 2-arachidonoylglycerol, WIN 55212-2 and PAF. First, cells loaded with Fura-2/AM were challenged with an individual stimulant or a receptor antagonist (closed arrows). After 1 min, cells were challenged with the second stimulant (open arrows). The results are representative of four separate experiments which gave similar results.

lular store site(s) upon 2-arachidonoylglycerol stimulation. Further detailed studies are required to clarify the mechanism by which 2-arachidonoylglycerol triggers the elevation of $[Ca^{2+}]_i$ in neuronal cells.

The physiological meaning of the transient rise in $[Ca^{2+}]_i$ induced by 2-arachidonoylglycerol remains to be clarified. Intracellular Ca^{2+} is known to play crucial roles in neural transmission; thus, there is a possibility that the transient rise in $[Ca^{2+}]_i$ is directly or indirectly implicated in the cannabimimetic actions of 2-arachidonoylglycerol observed *in vitro* or *in vivo* (13). Several investigators have already suggested that changes in intracellular Ca^{2+} are responsible for cannabinoid-induced antinociception (20). Recently, Connor et al. (21) reported that nociceptin, a putative endogenous ligand for the 'orphan' opioid receptor (ORL1), induces both a transient rise in $[Ca^{2+}]_i$ and inhibition of N-type Ca^{2+} channels. Cannabinoid receptor agonists such as WIN 55212-2 also inhibit N-type Ca^{2+} channels (5,10,20). Thus, it seems very likely that both the endogenous cannabinoid receptor (CB1) agonist(s) and several types of opioids play important roles in the regulation of Ca^{2+}-mobilization in neuronal cells. In relation to this, we observed that the addition of relatively high concentrations of WIN 55212-2 (10 μM)

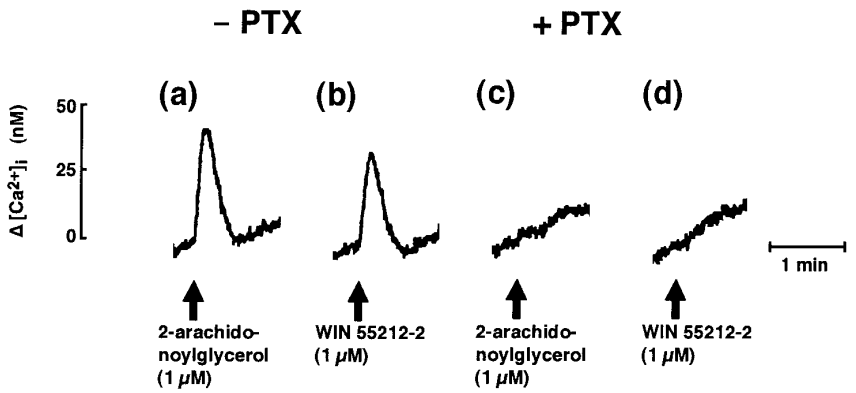

FIG. 4. Effects of pertussis toxin treatment on the rise in $[Ca^{2+}]_i$ in NG108-15 cells induced by 2-arachidonoylglycerol and WIN 55212-2. Cells were incubated with or without pertussis toxin (PTX) (100 ng/ml) at 37°C for 16 h. Then, the medium was replaced with fresh medium containing Fura-2/AM. Fura-2/AM-loaded cells were then stimulated with 2-arachidonoylglycerol (1 μM) ((a) and (c)) or WIN 55212-2 (1 μM) ((b) and (d)). (a) and (b), without pertussis toxin treatment; (c) and (d), with pertussis toxin treatment. The results are representative of three separate experiments which gave similar results.

and 2-arachidonoylglycerol (10 μM) induced a rapid, modest drop in basal $[Ca^{2+}]_i$ in addition to the simultaneous transient rise in $[Ca^{2+}]_i$ (Fig. 2). The mechanism of this phenomenon is also unclear. A drop in the basal $[Ca^{2+}]_i$ was also observed to some extent with 2-docosahexaenoylglycerol (Fig. 2), though this compound lacks the ability to induce the transient rise in $[Ca^{2+}]_i$. 2-Docosahexaenoylglycerol may interact weakly with the receptor site. In any case, it will be of great value to explore further whether the binding of 2-arachidonoylglycerol to its own receptor elicits various physiological responses, including the regulation of Ca^{2+} current, in neuronal cells.

In conclusion, we have found that there is a specific receptor for 2-arachidonoylglycerol in NG108-15 cells and that 2-arachidonoylglycerol induces a rapid, transient rise in $[Ca^{2+}]_i$. These findings, together with our and others' previous findings of 1) the abundance of 2-arachidonoylglycerol, rather than anandamide, in brain (9,12), 2) the binding ability of 2-arachidonoylglycerol to the cannabinoid receptor in synaptosomes (12) and to the receptor expressed on COS-7 cells (13), 3) cannabimimetic activities when administered intravenously (13) and 4) the occurrence of selective synthetic pathways in brain (12,22-24), strongly support the hypothesis that 2-arachidonoylglycerol is an endogenous cannabinoid receptor ligand and plays important roles in the modulation of neural functions.

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