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Is the Cannabinoid CB1 Receptor a 2-Arachidonoylglycerol Receptor? Structural Requirements for Triggering a Ca^{2+} Transient in NG108-15 Cells[1]

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The effects of Δ^9-tetrahydrocannabinol and 2-arachidonoylglycerol on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in NG108-15 cells were examined in detail. We found that Δ^9-tetrahydrocannabinol induces a rapid, modest increase in $[Ca^{2+}]_i$. The response was detectable with 3 nM Δ^9-tetrahydrocannabinol. We also found that very low concentrations of 2-arachidonoylglycerol elicit a rapid, more prominent increase in $[Ca^{2+}]_i$. Such a response was observed not only in NG108-15 cells but also in N18TG2 cells. The response induced by 2-arachidonoylglycerol in either NG108-15 cells or N18TG2 cells was abolished by pretreatment of the cells with a cannabinoid CB1 receptor-specific antagonist, SR141716A, suggesting that 2-arachidonoylglycerol interacts with the CB1 receptor to induce the response. The results of an experiment involving a phospholipase C inhibitor suggested that phospholipase C is involved in the rapid increase in $[Ca^{2+}]_i$ induced by 2-arachidonoylglycerol. We also found that 1(3)-arachidonoylglycerol exhibits similar activity to that of 2-arachidonoylglycerol, although its activity at low concentrations was somewhat weak compared with that of 2-arachidonoylglycerol. We further confirmed that several structural analogues of 2-arachidonoylglycerol were less active compared with 2-arachidonoylglycerol. These results suggest that the structure of 2-arachidonoylglycerol is strictly recognized by the CB1 receptor, which raises the possibility that the CB1 receptor is originally a 2-arachidonoylglycerol receptor.

Key words: anandamide, 2-arachidonoylglycerol, Ca^{2+} transient, cannabinoids, Δ^9-tetrahydrocannabinol.

The mechanism of action of Δ^9-tetrahydrocannabinol (Δ^9-THC), the psycoactive ingredient of marijuana, had long been unknown. The presence of a specific receptor site(s) for Δ^9-THC in mammalian tissues had been postulated by several investigators, although the details remained obscure until recently. Almost a decade ago, Devane *et al.* (*1*) demonstrated clearly that a cannabinoid receptor is present in rat brain using a radiolabeled ligand, [^{3}H]CP55940. The localization of a cannabinoid receptor in brain was also investigated intensively by Herkenham and co-workers (*2*). Finally, Matsuda *et al.* (*3*) cloned a cDNA encoding a cannabinoid receptor. Soon after this, Devane *et al.* (*4*) isolated *N*-arachidonoylethanolamine from porcine brain as the first endogenous cannabinoid receptor ligand, and termed it anandamide.

Recently, we investigated the levels of as well as the biosynthetic pathways for anandamide in brain and testis in detail (*5, 6*). We found that the levels of anandamide and its potential precursor [*N*-arachidonoylphosphatidylethanolamine (*N*-arachidonoylPE)] in the brain are very low (4.3 and 50.2 pmol/g wet tissue, respectively), and that both the enzyme activity involved in the synthesis of *N*-arachidonoylPE and the level of the substrate of this enzyme reaction, *i.e.*, 1-arachidonoyl-2-acyl-*sn*-glycero-3-phosphocholine, are also very low. These findings raise the question of whether anandamide actually has a physiologically important role in nervous tissues. Very recently, we (*7*), and Mechoulam *et al.* (*8*) proposed that 2-arachidonoylglycerol, a unique molecular species of monoacylglycerol, is another candidate for the endogenous cannabinoid receptor ligand. 2-Arachidonoylglycerol exhibits binding affinity toward the cannabinoid receptor in brain synaptosomes (*7, 9*), and cannabinoid receptor gene-transfected COS-7 cells (*8*), and exhibits several cannabimimetic activities on isolated mouse vas deferens and on the behavior as well as the body temperature of mice (*8*).

In a preceding study, we showed that low concentrations of 2-arachidonoylglycerol induce rapid, transient elevation of the intracellular free calcium concentration ($[Ca^{2+}]_i$) in neuroblastoma x glioma hybrid NG108-15 cells through a

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Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; $[Ca^{2+}]_i$, intracellular free calcium concentration; DADLE, [D-Ala^5,D-Leu^5]-enkephalin; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; AG, arachidonoylglycerol; PE, phosphatidylethanolamine.

cannabinoid CB1 receptor-dependent mechanism (*10*). This observation provided clear evidence that 2-arachidonoylglycerol is an endogenous CB1 receptor agonist, and that it plays some important role in the modulation of neural functions. However, the structural requirements of 2-arachidonoylglycerol have not yet been fully examined. For example, it is not known whether or not 1(3)-arachidonoylglycerol, an isomer of 2-arachidonoylglycerol, possesses similar biological activity. Furthermore, it is also not known whether Δ^9-THC, a psycoactive cannabinoid, induces a transient increase in $[Ca^{2+}]_i$ similar to 2-arachidonoylglycerol. In this study, we examined in detail the activity of Δ^9-THC as well as those of several arachidonic acid-containing compounds whose structures are closely related to that of 2-arachidonoylglycerol.

MATERIALS AND METHODS

Chemicals—SR141716A, U73122, and U73343 were obtained from Biomol (Plymouth Meeting, PA, USA). Arachidonic acid and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Serinol (2-amino-1,2-propanediol) was from Tokyo Kasei Kogyo (Tokyo). Fura-2/AM was from Wako Pure Chem. Ind. (Osaka). [D-Ala^5,D-Leu^5]-enkephalin (DADLE) was obtained from Peptide Institute (Osaka). Monoarachidonin was obtained from Nu-Check Prep (Elysian, MN, USA). We confirmed that the monoarachidonin used here mainly consisted of 1(3)-arachidonoylglycerol isomer (93%) by HPLC, as described below. Standard 1(3)-arachidonoylglycerol was chemically synthesized according to the method of Mattson and Volpenhein (*11*), and purified by TLC developed with petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v).

Cells—NG108-15 cells and N18TG2 cells were kindly donated by Dr. H. Higashida (Kanazawa University School of Medicine, Kanazawa). C6 cells were obtained from the American Type Cell Collection. NG108-15 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and HAT (hypoxantine, aminopterine and thymidine), and N18TG2 cells were grown in the same medium without HAT under an atmosphere of 90% air-10% CO_2. C6 cells were grown in Ham's F12 medium containing 15% horse serum and 2.5% FBS under an atmosphere of 95% air-5% CO_2.

Preparation of 2-Arachidonoylglycerol and Its Derivatives—Triarachidonoylglycerol was prepared as described previously (*7*). 2-Arachidonoylglycerol was obtained by digestion of triarachidonoylglycerol with *Rhizopus delemar* lipase (Seikagaku Kogyo, Tokyo), and purified by TLC developed with petroleum ether : diethyl ether : acetic acid (20 : 80 : 1, v/v)(*7*). 2-Arachidonoylglycerol was stored in chloroform : methanol (1 : 2, v/v) at −20°C and used at least within two weeks after its preparation. *N*-Arachidonoylserinol and monoarachidonoylethyleneglycol were prepared as described previously (*7, 10*). Arachidonoylisopropanol was synthesized from arachidonic anhydride and isopropanol, and purified by TLC developed with petroleum ether : diethyl ether : acetic acid (80 : 20 : 1, v/v).

Purities of 1(3)-Arachidonoylglycerol and 2-Arachidonoylglycerol—The purities of 1(3)-arachidonoylglycerol (commercially available as monoarachidonin) and 2-arachidonoylglycerol were checked using a HPLC system equipped with a reverse phase column and a fluorescence detector after converting them to dianthroyl derivatives. Briefly, 1(3)-arachidonoylglycerol or 2-arachidonoylglycerol (1 nmol each) dissolved in 100 μl of acetone (water-free) containing 0.2 mg 1-anthroyl cyanide and 0.16 mg of quinuclidine was kept at 45°C for 60 min. After the addition of 50 μl of methanol, the reaction products were separated by TLC developed with petroleum ether : diethyl ether : acetic acid (65 : 35 : 1, v/v). The band corresponding to dianthroylarachidonoylglycerol (R_f=0.46) was scraped off the TLC plates, and then extracted from the silica gel by the method of Bligh and Dyer (*12*). The extracted dianthroyl derivatives of monoarachidonoylglycerol were dissolved in acetonitrile and then analyzed with a HPLC system equipped with a reverse phase column (CAPCELL PAK C18 SG-120, 4.6 mm×250 mm; Shiseido, Tokyo) and a fluorescence detector (excitation, 370 nm; emission, 470 nm). The mobile phase was acetonitrile : isopropanol : water (90 : 4 : 6, v/v), and the flow rate was 1.4 ml/min. As shown in Fig. 1, the dianthroyl derivative of 1(3)-arachidonoylglycerol [1(3)-AG](retention time, 19.2 min) and the dianthroyl derivative of 2-arachidonoylglycerol (2-AG)(retention time, 20.4 min) were separated from each other. The purity of 1(3)-arachidonoylglycerol used in this experiment was 93% (the remainder was the 2-acyl species), and that of 2-arachidonoylglycerol was 95% [the remainder was the 1(3)-acyl species].

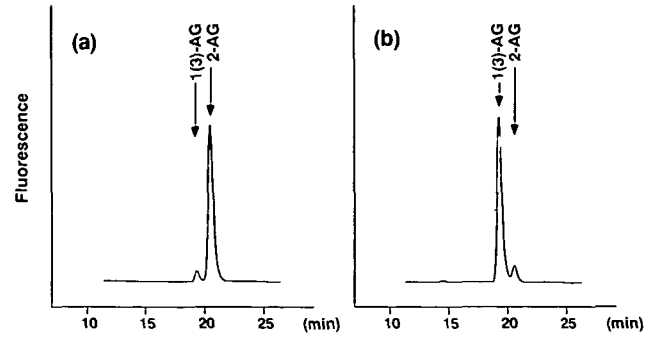

Fig. 1. **Separation of dianthroyl derivatives of 2-arachidonoylglycerol and 1(3)-arachidonoylglycerol by reverse-phase HPLC.** Dianthroyl derivatives of monoarachidonoylglycerols were prepared as described under "MATERIALS AND METHODS." Dianthroyl derivatives of 2-arachidonoylglycerol (a) and 1(3)-arachidonoylglycerol (b) were analyzed with a HPLC system equipped with a reverse-phase column and a fluorescence detector (excitation, 370 nm; emission, 470 nm) as described under "MATERIALS AND METHODS."

Measurement of $[Ca^{2+}]_i$—Subconfluent cells were further incubated in fresh medium without FBS for 24 h. Then, the cells were suspended in Hepes-Tyrode's solution ($-Ca^{2+}$) containing 3 μM Fura-2/AM by gentle pipetting, and further incubated at 37°C for 45 min. Then, the cells were centrifuged (180×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 previously (*10*) using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo). $CaCl_2$ was added 4-5 min before the measurement (final concentration of Ca^{2+} in the cuvette, 1 mM). 2-Arachidonoylglycerol and other analogues were dissolved in dimethyl sulfoxide (DMSO), and aliquots (1 μl each) were added to the cuvette

(final concentration of DMSO, 0.2%). DMSO (final 0.4%) *per se* did not affect $[Ca^{2+}]_i$ markedly.

RESULTS

Figure 2 shows the effects of 2-arachidonoylglycerol and related compounds on $[Ca^{2+}]_i$ in NG108-15 cells. We found that Δ^9-THC induces a transient, small increase in $[Ca^{2+}]_i$ (Fig. 2a). The response was detectable with 3 nM Δ^9-THC, and was augmented with increasing concentrations of Δ^9-THC. We also found that the addition of low doses of 2-arachidonoylglycerol to the cells elicited rapid, more prominent increase in $[Ca^{2+}]_i$ (Fig. 2b). The effect was detectable as low as 1 nM 2-arachidonoylglycerol. We confirmed that 1(3)-arachidonoylglycerol exhibits similar activity to that of 2-arachidonoylglycerol, although its activity at low concentrations was somewhat weaker than that of 2-arachidonoylglycerol (Fig. 2c). The response was detectable above 10 nM in the case of 1(3)-arachidonoylglycerol. We also found that monoarachidonoylethyleneglycol, which has only one free hydroxy group per molecule, possesses some agonistic activity (Fig. 2d). However, the

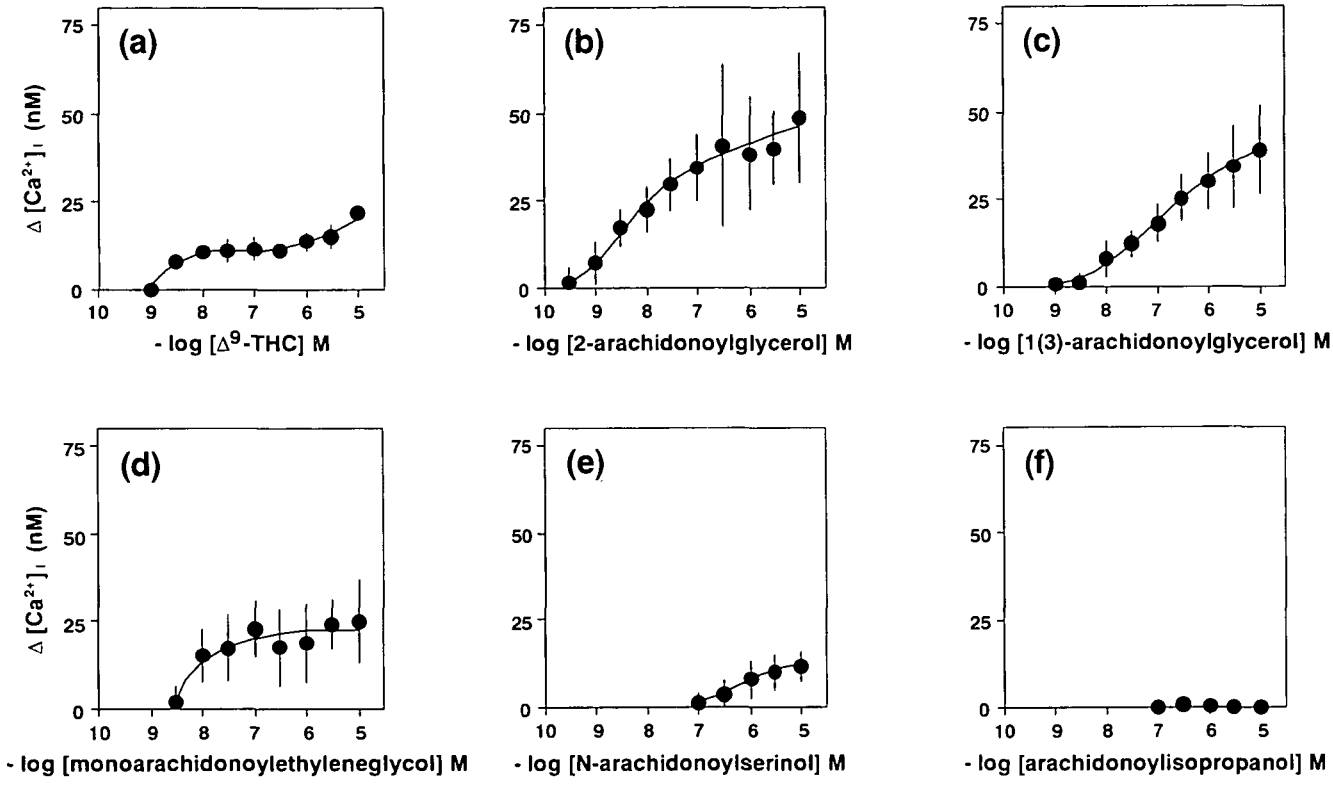

Fig. 2. **Effects of Δ^9-THC, 2-arachidonoylglycerol, and related compounds on $[Ca^{2+}]_i$ in NG108-15 cells.** Cells loaded with Fura-2/AM were stimulated with various concentrations of Δ^9-THC (a), 2-arachidonoylglycerol (b), 1(3)-arachidonoylglycerol (c), monoarachidonoylethyleneglycol (d), *N*-arachidonoylserinol (e), or arachidonoylisopropanol (f). Changes in $[Ca^{2+}]_i$ were measured using a Ca^{2+} analyzer (CAF-100) and expressed as $\Delta[Ca^{2+}]_i$ (nM). The mean and SD were calculated from the results of six to eight separate experiments.

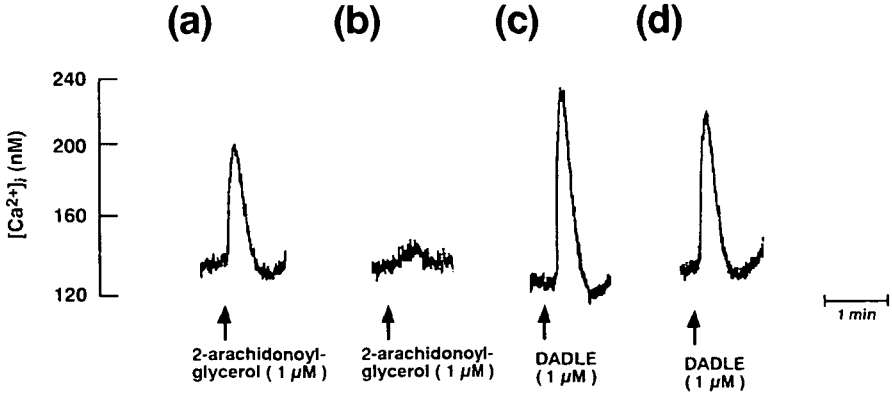

Fig. 3. **Effects of pretreatment of cells with Δ^9-THC on the 2-arachidonoylglycerol-induced or DADLE-induced rapid increase in $[Ca^{2+}]_i$.** NG108-15 cells loaded with Fura-2/AM were stimulated with Δ^9-THC (10 μM) [(b) and (d)] or the vehicle alone (DMSO) (final, 0.2%) [(a) and (c)] in Hepes-Tyrode's solution ($-Ca^{2+}$) containing 0.025% BSA at 37°C for 2 min. Then, the cells were sedimented by centrifugation and resuspended in Hepes-Tyrode's solution ($-Ca^{2+}$) containing 0.025% BSA. After the addition of $CaCl_2$ (final, 1 mM), 2-arachidonoylglycerol (1 μM) [(a) and (b)], or DADLE (1 μM) [(c) and (d)] was added to the cuvette. Changes in $[Ca^{2+}]_i$ were analyzed as described under "MATERIALS AND METHODS.

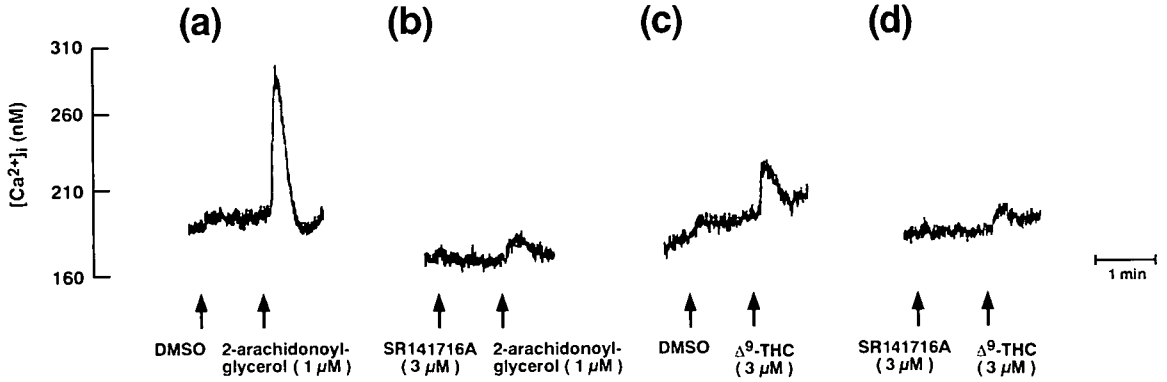

Fig. 4. **Effects of the CB1 receptor-specific antagonist, SR141716A, on the 2-arachidonoylglycerol or Δ^9-THC-induced increase in $[Ca^{2+}]_i$.** NG108-15 cells loaded with Fura-2/AM were challenged first with SR141716A (3 μM) [(b) and (d)] or the vehicle alone (DMSO) (final, 0.2%) [(a) and (c)], and then with 2-arachidonoylglycerol (1 μM) [(a) and (b)] or Δ^9-THC (3 μM) [(c) and (d)].

maximal response induced by monoarachidonoylethyleneglycol was considerably weak compared with that of 2-arachidonoylglycerol. Noticeably, *N*-arachidonoylserinol, an amide bond-containing analogue of 2-arachidonoylglycerol, elicited only a weak response even at high doses (Fig. 2e), suggesting that the presence of an ester linkage at the *sn*-2 position is crucially important for induction of the transient increase in $[Ca^{2+}]_i$. In keeping with this, the response induced by 10 μM anandamide was about 25% of that induced by 10 μM 2-arachidonoylglycerol (data not shown). We also confirmed that arachidonoylisopropanol, a free hydroxy group-lacking analogue of 2-arachidonoylglycerol (Fig. 2f), or free arachidonic acid itself (data not shown) did not exhibit appreciable activity even at 10 μM.

To examine the possibility that 2-arachidonoylglycerol and Δ^9-THC act on a common receptor molecule, we investigated whether or not cross-desensitization is observed when cells are stimulated sequentially with Δ^9-THC and 2-arachidonoylglycerol. As shown in Fig. 3, the pretreatment of cells with Δ^9-THC (10 μM) abolished the response induced by 2-arachidonoylglycerol (1 μM). On the other hand, the pretreatment of cells with Δ^9-THC (10 μM) did not affect the response induced by DADLE (1 μM) markedly (Fig. 3). These observations strongly suggest that 2-arachidonoylglycerol and Δ^9-THC interact with a common receptor site on the cell surface.

Then, we checked the effect of SR141716A, a specific CB1 receptor antagonist, on the 2-arachidonoylglycerol- or Δ^9-THC-induced increase in $[Ca^{2+}]_i$. We confirmed that the response induced by 1 μM 2-arachidonoylglycerol was abolished by the pretreatment of cells (1 min) with 3 μM SR141716A (Fig. 4). We also observed that the response induced by 3 μM Δ^9-THC was diminished on pretreatment of the cells with 3 μM SR141716A (Fig. 4).

Next, we examined whether or not a 2-arachidonoylglycerol-induced rapid increase in $[Ca^{2+}]_i$ can also be observed in cells other than NG108-15. Here, we focused on N18TG2 neuroblastoma cells and C6 glioma cells, because NG108-15 cell is a daughter cell line of N18TG2 cells and C6BU-1 cells, a thymidine kinase-deficient clone of C6 cells. We found that 2-arachidonoylglycerol elicits a rapid increase in $[Ca^{2+}]_i$ in N18TG2 cells similar to in NG108-15 cells, while such a response was not observed in C6 cells (Fig. 5). We confirmed that the response induced

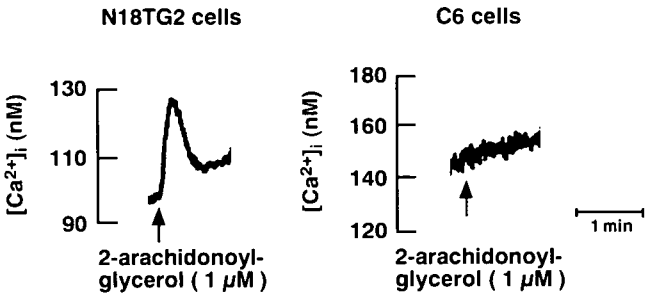

Fig. 5. **Effects of 2-arachidonoylglycerol on $[Ca^{2+}]_i$ in N18TG2 cells and C6 cells.** Cells were loaded with Fura-2/AM, and then stimulated with 2-arachidonoylglycerol (1 μM). Changes in $[Ca^{2+}]_i$ were analyzed as described under "MATERIALS AND METHODS."

by 2-arachidonoylglycerol (1 μM) in N18TG2 cells was abolished on pretreatment of the cells with SR141716A (1 μM)(data not shown), as in the case of NG108-15 cells.

Finally, we examined the effect of a phospholipase C inhibitor, U73122 (*13*), on the 2-arachidonoylglycerol-induced increase in $[Ca^{2+}]_i$ in NG108-15 cells. We found that the rapid increase in $[Ca^{2+}]_i$ induced by 2-arachidonoylglycerol (1 μM) was diminished in the presence of U73122 (1 μM), whereas such inhibition was not observed with U73343 (1 μM), an inactive analogue of U73122 (Fig. 6), suggesting that phospholipase C is involved in the 2-arachidonoylglycerol-induced rapid increase in $[Ca^{2+}]_i$ in this type of cells.

DISCUSSION

To our knowledge, this is the first report that Δ^9-THC induces a transient, modest increase in $[Ca^{2+}]_i$ in cultured neuronal cells. Previously, we demonstrated that WIN-55212-2 and 2-arachidonoylglycerol induce rapid, more prominent increases in $[Ca^{2+}]_i$ through a similar CB1 receptor-dependent mechanism. Therefore, the induction of a Ca^{2+} transient may be a common feature, more or less, of cannabinoid receptor agonists, at least in NG108-15 cells. We confirmed that such a response was observed not only in NG108-15 cells but also in N18TG2 cells, which are known to express the CB1 receptor on their surface and respond to cannabimimetic drugs, in contrast to C6 cells

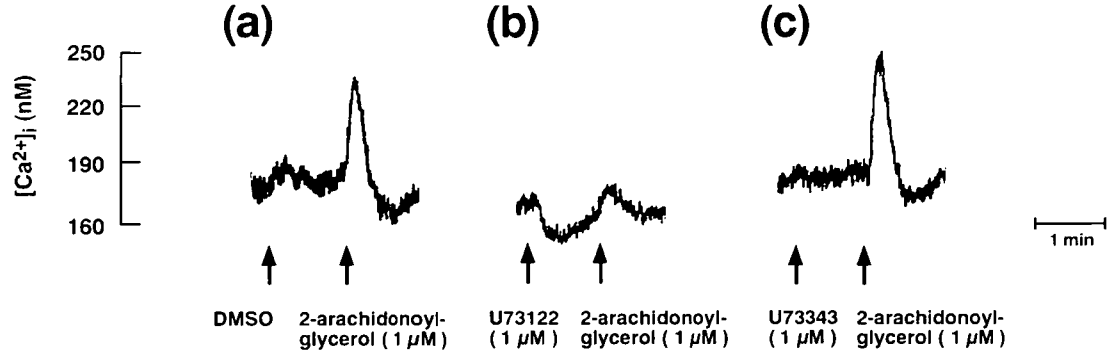

Fig. 6. **Effects of the phospholipase C inhibitor, U73122, and its inactive analogue, U73343, on the 2-arachidonoylglycerol-induced increase in $[Ca^{2+}]_i$.** NG108-15 cells loaded with Fura-2/AM were treated with U73122 (1 μM) (b), U73343 (1 μM) (c), or the vehicle alone (DMSO) (final, 0.2%) (a). Then, the cells were challenged with 2-arachidonoylglycerol (1 μM).

(*14*) (Fig. 5).

The mechanism underlying the increase in $[Ca^{2+}]_i$ induced by 2-arachidonoylglycerol in this type of cells is not yet fully understood. Previously, we demonstrated that pertussis toxin-treatment of the cells abolished the 2-arachidonoylglycerol- or WIN55212-2-induced increase in $[Ca^{2+}]_i$ (*10*). This observation clearly indicates that G_i or G_o is involved in such a cellular response. Interestingly, the $\beta\gamma$ subunit, derived from G_i or G_o, is assumed to stimulate phospholipase Cβ thereby inducing a rapid increase in $[Ca^{2+}]_i$ in opioid-stimulated NG108-15 cells (*15*). It is possible that a similar molecular mechanism operates in the cases of cannabinoid CB1 receptor agonists. We confirmed that phospholipase C is possibly involved in the 2-arachidonoylglycerol-induced rapid increase in $[Ca^{2+}]_i$ (Fig. 6), as in the case of opioid-stimulated NG108-15 cells (*15*). In relation to the possible involvement of the $\beta\gamma$ subunit in the response observed here, we note that the $\beta\gamma$ subunit of G_i or G_o inhibits voltage-gated Ca^{2+} channels and adenylate cyclase, and activates the MAP-kinase cascade (*16*); all these responses are known to be induced by cannabinoid CB1 receptor agonists as well (*17, 18*). In any case, further detailed studies are required to elucidate the exact molecular mechanism underlying the 2-arachidonoylglycerol-induced increase in $[Ca^{2+}]_i$. Whatever the mechanism and whatever the physiological implications, estimation of the Ca^{2+} transient in NG108-15 cells should be of practical value in the screening of CB1 receptor agonists and antagonists.

One of the noticeable observations here is that the activity of 1(3)-arachidonoylglycerol, an isomer of 2-arachidonoylglycerol, is somewhat weak compared with that of 2-arachidonoylglycerol at low concentrations. Because a small amount of 2-arachidonoylglycerol (7%) was present in the 1(3)-arachidonoylglycerol used here, the actual activity of 1(3)-arachidonoylglycerol may be even weaker than observed in this study. The finding that 1(3)-arachidonoylglycerol is less active compared with 2-arachidonoylglycerol at low concentrations suggests that the CB1 receptor strictly distinguishes the structure of 2-arachidonoylglycerol. This was further supported by the observation that structurally related analogues, monoarachidonoylethyleneglycol, *N*-arachidonoylserinol, and arachidonoylisopropanol, exhibited only weak agonistic activity or negligible activity. Previously, we confirmed that the activities of free arachidonic acid, 2-palmitoylglycerol, 2-oleoylglycerol, 2-linoleoylglycerol, and 2-docosahexaenoylglycerol are negligible. Furthermore, anandamide was shown to act as a partial agonist, at least in the present assay system. These observations, together with those presented here, strongly suggest that the CB1 receptor is originally a 2-arachidonoylglycerol receptor.

As for the source of 2-arachidonoylglycerol, we have already suggested that inositol phospholipids, which usually contain large amounts of arachidonic acid at the 2-position of the glycerol backbone, or choline glycerophospholipids are putative sources of 2-arachidonoylglycerol in stimulated tissues and cells (*7*). The generation of arachidonoylglycerol has actually been demonstrated in platelet-derived growth factor (PDGF)-stimulated Swiss 3T3 cells (*19*), bradykinin-stimulated rat dorsal root ganglion neurons (*20*), thrombin-stimulated platelets (*21*), and A23187-stimulated N18TG2 cells (*22*). Di Marzo and coworkers (*22*) obtained evidence that 2-[^{3}H]arachidonoylglycerol is actually present in the [^{3}H]arachidonoylglycerol fraction. We also found that a considerable amount of 2-arachidonoylglycerol, together with a small amount of 1(3)-arachidonoylglycerol, is present in rat brain (Kondo, S. and Sugiura, T., unpublished data). Thus, it appears that 2-arachidonoylglycerol is a rather common molecule, and can be produced in several mammalian tissues and cells.

Noticeably, the cannabinoid CB1 receptor is present in presynapses and is assumed to play an important role in the regulation of neurotransmitter release (*17*). Hence, the idea that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor may indicate the following hypothesis, *i.e.* that 2-arachidonoylglycerol, generated upon neuronal cell stimulation through increased phospholipid metabolism, especially increased inositol phospholipid breakdown, plays a role by modulating or attenuating neurotransmitter release through the presynaptic cannabinoid CB1 receptor. Such a mechanism should be effective in establishing negative feedback control of neurotransmission in some synapses where the CB1 receptor is present, and neurotransmitters causing increased inositol phospholipid metabolism are involved in the neurotransmission. Further detailed studies are necessary to determine whether or not 2-arachidonoylglycerol actually plays such an important physiological role in the central nervous system of mammals.

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