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Inhibition by 2-Arachidonoylglycerol, a Novel Type of Possible Neuromodulator, of the Depolarization-Induced Increase in Intracellular Free Calcium in Neuroblastoma × Glioma Hybrid NG108-15 Cells

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2-Arachidonoylglycerol was found to inhibit the depolarization-induced increase in $[Ca^{2+}]_i$ in NG108-15 cells differentiated with prostaglandin E_1 and theophylline in a dose-dependent manner. Such an effect appears to be rather specific to polyunsaturated fatty acid-containing monoacylglycerols such as 2-arachidonoylglycerol. Neither 2-palmitoylglycerol nor free arachidonic acid exhibited appreciable inhibitory activity. These observations raise the possibility that 2-arachidonoylglycerol attenuates the increase in $[Ca^{2+}]_i$, thereby modulating several neural functions in this type of cell.

2-Arachidonoylglycerol is a novel type of bioactive lipid originally described as one of the putative endogenous cannabimimetic molecules in rat brain (1) and canine gut (2). This unique molecular species of monoacylglycerol is quite notable from various biochemical and pharmacological viewpoints. First of all, 2-arachidonoylglycerol is one of the major products of increased inositol phospholipid metabolism and can be released from cells (1,3,4). Secondly, 2-arachidonoylglycerol is abundant in brain (1). Thirdly, selective and effective synthetic pathways are present in brain, especially in synaptosomes (1,5-7). Fourthly, 2-arachidonoylglycerol has been shown to inhibit the specific binding of [^{3}H]-CP55940 to the cannabinoid receptor in rat brain synaptosomes (1), and the specific binding of [^{3}H]HU243 to those expressed on COS-7 cells (2), and to exhibit several cannabimimetic effects on mouse spleen cells (2,8), mouse vas deferens (2), and the behavior and body temperature of mice when administered intravenously (2). Nevertheless, not much is known concerning this novel type of bioactive lipid, especially in the nervous system.

Very recently, we found that low doses of 2-arachidonoylglycerol induce rapid transient elevation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in undifferentiated neuroblastoma × glioma hybrid NG108-15 cells through a cannabinoid receptor-dependent and Gi or Go-dependent mechanism (9). This observation strongly suggests that 2-arachidonoylglycerol actually plays some physiological role in the nervous system. In this study, we examined whether or not 2-arachidonoylglycerol affects $[Ca^{2+}]_i$ in differentiated NG108-15 cells, which are known to express voltage-gated Ca^{2+} channels (10). We found that 2-arachidonoylglycerol strongly inhibits the depolarization-induced increase in $[Ca^{2+}]_i$ in this type of cell.

MATERIALS AND METHODS

Chemicals. SR141716A was from Biomol (Plymouth Meeting, PA). Arachidonic acid, docosahexaenoic acid, linoleic acid, oleic acid, palmitic acid, prostaglandin E_1 (PGE_1), indomethacin, nordihydroguaiaretic acid and essentially fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Anandamide was prepared by a modification of the method of Devane et al. (11). Serinol (2-amino-1,3-propanediol), an amino group-containing analogue of glycerol, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). N-Arachidonoylserinol was prepared from serinol and arachidonoyl chloride, analogously to the synthesis of anandamide (9). Various types of triacylglycerols were synthesized from appropriate fatty acid anhydrides and glycerol (9). 2-Monoacylglycerols were prepared from the corresponding triacylglycerols by digestion with *Rhizopus delemar* lipase and purified as described elsewhere (9). Fura-2/AM, theophylline, ω-agatoxin IVA, ω-conotoxin GVIA and ω-conotoxin MVIIC were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). Hepes-Tyrode's solution consisted of 25 mM Hepes, 140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM

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Abbreviations: $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PGE_1, prostaglandin E_1.

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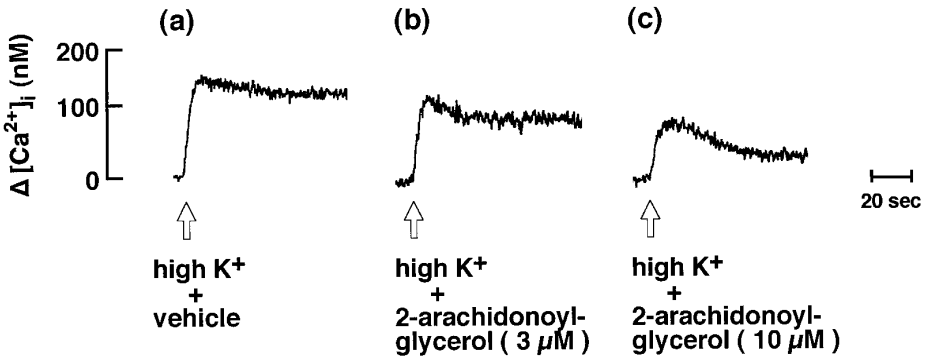

FIG. 1. Effect of 2-arachidonoylglycerol on the increase in $[Ca^{2+}]_i$ induced by high K^+ (50 mM). (a) high K^+ plus DMSO (0.2%); (b) high K^+ plus 2-arachidonoylglycerol (3 μM); (c) high K^+ plus 2-arachidonoylglycerol (10 μM).

$MgCl_2$, 0.4 mM NaH_2PO_4 and 5 mM glucose. The pH was adjusted to 7.4 with NaOH.

Cells. NG108-15 cells were kindly donated by Dr. Haruhiro Higashida (Kanazawa University School of Medicine, Kanazawa, Japan). The cells were grown at 37°C in DMEM containing 5 % fetal bovine serum (FBS) and HAT (hypoxanthine, aminopterine and thymidine) under an atmosphere of 90 % air-10 % CO_2 (9).

Measurement of $[Ca^{2+}]_i$ in differentiated NG108-15 cells. Subconfluent cells (approximately 80 %) grown in 100 mm polystyrene dishes were induced to differentiate by incubating them in DMEM (without FBS or aminopterine) containing PGE_1 (10 μM) and theophylline (1 mM) for 2-3 days (10). With this treatment, flat polygonal cells differentiated into rounded cells with extended neurites. Then, the medium was removed and the cells were washed with 25 mM Hepes-Tyrode's solution (without Ca^{2+}). The cells, resuspended in Hepes-Tyrode's solution (without Ca^{2+}) containing 5 μM Fura-2/AM by gentle pipetting, were incubated at 37°C for 2 h. Following the incubation, the cells were spun down by centrifugation (180 $\times$ g for 5 min), washed twice with Hepes-Tyrode's solution (without Ca^{2+}), and then resuspended in Hepes-Tyrode's solution (without Ca^{2+}) containing 0.025 % BSA. $[Ca^{2+}]_i$ was estimated as described elsewhere (9) using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo, Japan). $CaCl_2$ (final 1.8 mM) was added to the cells 3 min before the measurements. In some experiments, Ca^{2+} channel blockers were added 2 min prior to the addition of $CaCl_2$. Then, 170 μl of prewarmed Hepes-Tyrode's solution containing 143 mM KCl in place of NaCl and 1.8 mM $CaCl_2$ was added to 330 μl of the prewarmed cell suspension (final $5 \times 10^4 - 10^5$ cells/500 μl of Hepes-Tyrode's solution containing 50 mM KCl and 93 mM NaCl) to depolarize the cells. 2-Arachidonoylglycerol and analogues were dissolved in dimethyl sulfoxide (DMSO), and aliquots (1 μl each) were added to the cuvette. Indomethacin and nordihydroguaiaretic acid were also dissolved in DMSO. The final concentration of DMSO in the cuvette was 0.2 % (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 150-250 nM.

Statistical analysis. Statistical analysis was performed by means of Student's one-sample t test.

RESULTS

First, we examined the effects of 2-arachidonoylglycerol on the depolarization-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 1 (a), the addition of a high K^+ solution (final 50 mM) plus DMSO (0.2 %) elicited a rapid increase in $[Ca^{2+}]_i$, suggesting that voltage-gated Ca^{2+} channels were opened upon depolarization. The addition of DMSO (final 0.2 %) did not affect the response induced by high K^+ alone (data not shown). On the other hand, we found that the simultaneous addition of 2-arachidonoylglycerol with high K^+ resulted in marked reduction of the response induced by high K^+ (Fig. 1 (b) and (c)). For example, the maximal increase in $[Ca^{2+}]_i$ observed in the presence of 10 μM 2-arachidonoylglycerol was 50-70 % of that observed without 2-arachidonoylglycerol. In addition to the reduction of the maximal increase in $[Ca^{2+}]_i$, 2-arachidonoylglycerol enhanced the decrease in $[Ca^{2+}]_i$ after a plateau was reached (Fig. 1 (c)). The pretreatment of cells (5 min) with either indomethacin (3 μM) or nordihydroguaiaretic acid (3 μM) did not affect the inhibitory effect of 2-arachidonoylglycerol (data not shown), suggesting that arachidonic acid metabolites are not involved in such an effect.

We further explored the effects of 2-arachidonoylglycerol and related molecules on the depolarization-induced increase in $[Ca^{2+}]_i$ precisely. As shown in Fig. 2 (a), inhibition by 2-arachidonoylglycerol was detectable at least from the concentration of 1 μM, and was augmented with increasing concentrations of 2-arachidonoylglycerol. We also found that N-arachidonoylserinol, an amide bond-containing analogue of 2-arachidonoylglycerol, and anandamide, a putative endogenous cannabinoid receptor ligand, had some inhibitory effects on the increase in $[Ca^{2+}]_i$ induced by high K^+ (Fig. 2 (b) and (c)), while their activities were considerably weak compared with that of 2-arachidonoylglycerol (Fig. 2 (a)). We confirmed that free arachidonic acid itself did not affect the response induced by high K^+ (Fig. 2 (d)). As for other species of 2-monoacylglycerols, 2-palmitoylglycerol did not exhibit any appreciable activity (Fig. 2 (e)). The inhibition by 2-oleoylglycerol was also rather low (Fig. 2 (f)). On the other hand, 2-linoleoylglycerol and 2-docosahexaenoylglycerol were found to exhibit appreciable inhibitory effects on the increase in $[Ca^{2+}]_i$ induced by high K^+ (Fig. 2 (g) and (h)).

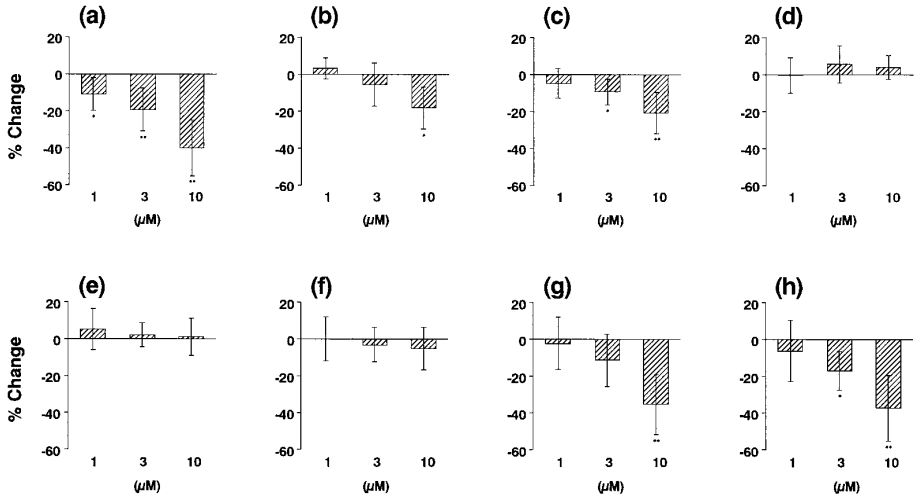

FIG. 2. Effects of 2-arachidonoylglycerol and related compounds on the maximal increase in $[Ca^{2+}]_i$ induced by high K^+. (a) 2-Arachidonoylglycerol; (b), N-arachidonoylserinol; (c) anandamide; (d) free arachidonic acid; (e) 2-palmitoylglycerol; (f) 2-oleoylglycerol; (g) 2-linoleoylglycerol; (h) 2-docosahexaenoylglycerol. The data are the means ± SD for nine separate experiments. * $P<0.01$, ** $P<0.001$.

Then, we examined the effects of several Ca^{2+} channel blockers on the depolarization-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 3 (b) and (c), the pretreatment of cells with ω-conotoxin GVIA (N-type channel blocker) (1 μM) and ω-conotoxin MVIIC (Q-, P-, and N-type channel blocker) (1 μM) reduced the response induced by high K^+. The inhibitory effects of these toxins (1 μM) were almost comparable to that observed with 3 μM 2-arachidonoylglycerol. In contrast to these two toxins, ω-agatoxin IVA (P-type channel blocker) (1 μM) failed to inhibit the response induced by high K^+ (Fig. 3 (a)).

DISCUSSION

It is clear from the above results that 2-arachidonoylglycerol, the most abundant monoacylglycerol in rat brain (1), has a profound effect on the increase in $[Ca^{2+}]_i$ induced by depolarization. Such an effect appears to be rather specific to polyunsaturated fatty acid-containing monoacylglycerols such as 2-arachidonoylglycerol. Because the proportion of arachidonoylglycerol in polyunsaturated fatty acid-containing monoacylglycerols in brain (67.3 %) is much higher than those of linoleoylglycerol (1.2 %) and docosahexaenoylglycerol (24.8 %) (1), here we focused on arachidonoylglycerol. To our knowledge, this is the first report showing antagonizing effects of polyunsaturated fatty acid-containing monoacylglycerols such as 2-arachidonoylglycerol on the increase in $[Ca^{2+}]_i$.

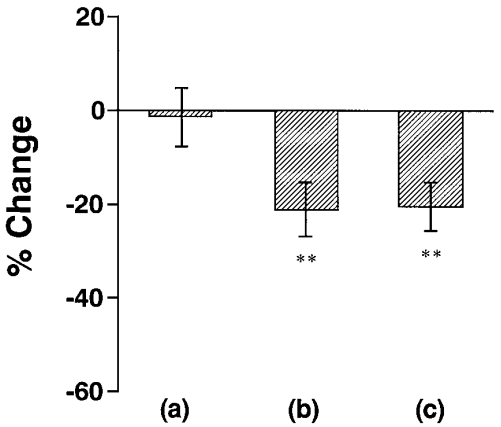

FIG. 3. Effects of several Ca^{2+} channel blockers on the maximal increase in $[Ca^{2+}]_i$ induced by high K^+. (a) ω-Agatoxin IVA (1 μM); (b) ω-conotoxin GVIA (1 μM); (c) ω-conotoxin MVIIC (1 μM). The data are the means ± SD for seven separate experiments. ** $P<0.001$.

In the preceding study, we demonstrated that low doses (nM order) of 2-arachidonoylglycerol induce a transient, but modest increase in $[Ca^{2+}]_i$ in undifferentiated NG108-15 cells in a cannabinoid receptor (CB1)-dependent manner (9). The response in differentiated cells was, however, considerably low compared with that in undifferentiated cells (data not shown). In any case, it is apparent that 2-arachidonoylglycerol has both stimulating (nM order) and inhibitory (μM order) effects on $[Ca^{2+}]_i$ in this type of cell. Such dual effects on $[Ca^{2+}]_i$ have also been reported for opioids (12).

The mechanism underlying the inhibition by 2-arachidonoylglycerol of the depolarization-induced increase in $[Ca^{2+}]_i$ is not yet fully understood. One of the possible explanations is that the entry of Ca^{2+} through voltage-gated Ca^{2+} channels is blocked, similar to in the cases of Ca^{2+} channel blocking toxins (Fig. 3). Several types of cannabinoids, including anandamide, have already been reported to inhibit voltage-gated Ca^{2+} channels (13–15). Because SR141716A, a specific cannabinoid receptor (CB1) antagonist, *per se* had some inhibitory effect on the increase in $[Ca^{2+}]_i$ in the differentiated cells used here (data not shown), we could not

determine whether or not CB1 receptor is involved in 2-arachidonoylglycerol-induced inhibition of the increase in $[Ca^{2+}]_i$, at least with the present experimental system. Further detailed studies are needed to clarify this important issue, and to answer the question as to which type of Ca^{2+} channel and/or other Ca^{2+}-mobilizing systems is affected by 2-arachidonoylglycerol. In relation to this, it should be noted that anandamide has been reported to bind directly to L-type Ca^{2+} channels (16,17). Furthermore, 1-arachidonoylglycerol is known to stimulate purified Na^+-K^+ ATPase (18), which may also affect $[Ca^{2+}]_i$ in combination with the action of a Na^+/Ca^{2+} exchanger. In addition, anandamide was shown to inhibit gap junction and intercellular signalling in astrocytes via a CB1 receptor-independent pathway (19). It may be possible, therefore, that 2-arachidonoylglycerol interacts with some sites other than cannabinoid receptors as well to elicit some biological responses. Whether or not such a possibility is the case will be determined in the future.

Whatever the mechanism, the fact that 2-arachidonoylglycerol strongly inhibits the depolarization-induced increase in $[Ca^{2+}]_i$ should have some physiological implications. Ca^{2+} is known to play crucial roles in the regulation of various functions of the nervous system. For example, an increase in $[Ca^{2+}]_i$ in presynaptic terminals is essential for the release of neurotransmitter (20). Ca^{2+} is also known to be a key molecule in the induction of long term potentiation (21). Furthermore, sustained elevation of $[Ca^{2+}]_i$ is known to lead to neuronal cell death (22). It is tempting, therefore, to speculate that 2-arachidonoylglycerol modulates several neuronal cell functions such as the triggering of neurotransmitter release by regulating $[Ca^{2+}]_i$.

Interestingly, 2-arachidonoylglycerol is one of the major degradation products of inositol phospholipids in several types of cells (1,3,4). In brain, 2-arachidonoylglycerol can be formed from inositol phospholipids through the combined actions of phospholipase C and diacylglycerol lipase or the combined actions of phosphatidylinositol-specific phospholipase A_1 and lysophosphatidylinositol-specific phospholipase C (1,5-7). A portion of 2-arachidonoylglycerol may be derived from phosphatidylcholine (23) or from lysophosphatidic acid (1). In view of the fact that inositol phospholipids are common precursors of several important bioactive molecules such as inositol phosphates and diacylglycerols in various tissues and cells, it would not be so surprising if 2-arachidonoylglycerol also exerts some biological activities in several types of cells including neuronal cells. Possible regulation or modulation of neurotransmission by 2-arachidonoylglycerol would be an interesting issue to be examined in the future.

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