Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells

Tiziana BISOGNO*, Nunzio SEPE*, Dominique MELCK*, Stefano MAURELLI*, Luciano De PETROCELLIS† and Vincenzo Di MARZO*‡

*Istituto per la Chimica di Molecole di Interesse Biologico and †Istituto di Cibernetica, C.N.R., Via Toiano 6, 80072 Arco Felice, Naples, Italy

The monoacylglycerol 2-arachidonoylglycerol (2-AG) has been recently suggested as a possible endogenous agonist at cannabinoid receptors both in brain and peripheral tissues. Here we report that a widely used model for neuronal cells, mouse N18TG2 neuroblastoma cells, which contain the CB1 cannabinoid receptor, also biosynthesize, release and degrade 2-AG. Stimulation with ionomycin (1–5 µM) of intact cells prelabelled with [3H]arachidonic acid ([3H]AA) led to the formation of high levels of a radioactive component with the same chromatographic behaviour as synthetic standards of 2-AG in TLC and HPLC analyses. The amounts of this metabolite were negligible in unstimulated cells, and greatly decreased in cells stimulated in the presence of the Ca²⁺-chelating agent EGTA. The purified component was further characterized as 2-AG by: (1) digestion with Rhizopus arrhizus lipase, which yielded radiolabelled AA; (2) gas chromatographic–MS analyses; and (3) TLC analyses on borate-impregnated plates. Approx. 20% of the 2-AG produced by stimulated cells was found to be released into the incubation medium when this contained 0.1% BSA. Subcellular fractions of N18TG2 cells were shown to contain enzymic activity or activities catalysing the hydrolysis of synthetic [3H]2-AG to [3H]AA. Cell homogenates were also found to convert synthetic [3H]-n-1-acyl-2-arachidonoylglycerols (AcAGs) into [3H]2-AG, suggesting that 2-AG might be derived from AcAG hydrolysis. When compared with ionomycin stimulation, treatment of cells with exogenous phospholipase C, but not with phospholipase D or A₂, led to a much higher formation of 2-AG and AcAGs. However, treatment of cells with phospholipase A₂ 10 min before ionomycin stimulation caused a 2.5–3-fold potentiation of 2-AG and AcAG levels. These results suggest that the Ca²⁺-induced formation of 2-AG proceeds through the intermediary of AcAGs but not necessarily through phospholipase C activation. By showing for the first time the existence of molecular mechanisms for the inactivation and the Ca²⁺-dependent biosynthesis and release of 2-AG in neuronal cells, the present paper supports the hypothesis that this cannabimimetic monoacylglycerol might be a physiological neuro-modulator.

INTRODUCTION

Two series of findings have opened new directions in the research on cannabinoids and the possible physiological implications of their effects in both central and peripheral mammalian tissues (reviewed in [1]): first the finding in nervous tissues of selective endogenous agonists for the brain CB1 cannabinoid receptor [2–4], and secondly the discovery of a peripheral cannabinoid receptor, designated CB2, in spleen marginal cells [5] and basophils/mast cells [6]. Among the endogenous cannabimimetic metabolites isolated so far, anandamide (arachidonylethanolamide, AnNH) is probably the most likely candidate as an ‘endocannabinoid’ in that it is capable of mimicking most of the pharmacological actions ascribed to cannabinoids in both the central nervous system and peripheral systems (reviewed in [7–10]), and enzymic activities responsible for its synthesis and/or degradation, respectively from and to arachidonic acid (AA) and ethanolamine, have been partly characterized [10–17]. In support of its proposed role as physiological ligand of the CB1 receptor, AnNH has been shown to be biosynthesized and released from rat central neurons stimulated with membrane-depolarizing stimuli such as ionomycin and high K⁺, through the phospholipase D (PLD)-catalysed hydrolysis of a membrane phospholipid precursor, N-arachidonoylphosphatidylethanolamine [18].

Since the discovery of AnNH and of the molecular mechanisms underlying its biosynthesis and degradation, another derivative of AA, 2-arachidonoylglycerol (2-AG), has been isolated from canine gut and rat brain [19,20] and shown to displace high-affinity cannabinoid ligands from their binding sites on synapticosomal preparations with a Kᵢ of 89 nM [20] and to bind to both CB1 and CB2 receptors with Kᵢ values in the high nanomolar–low micromolar range [19]. 2-AG was also found to exert some of the pharmacological and behavioural actions typical of cannabinoids, such as inhibition of forskolin-stimulated adenylate cyclase [19,21], modulation of lymphocyte proliferation [21] and induction of hypomotility, analgesia and hypothermy in rodents [19]. Previous studies [22–25] had reported chromatographic evidence (not supported by full chemical characterization) for the presence of a 2-AG-like metabolite in several

Abbreviations used: AA, arachidonic acid; AACOCF₃, arachidonoyl trifluoromethylketone; AcAGs, 1-acyl-2-arachidonoylglycerols; 1-AG, 1-arachidonoylglycerol; 2-AG, 2-arachidonoylglycerol; AnNH, arachidonoylethanolamide; DAG, diacylglycerol; DMEM, Dulbecco’s modified Eagle’s medium; PI, phosphatidylinositol; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D.
‡ To whom correspondence should be addressed.
cell types, including mouse Swiss 3T3 cells and rat cortical and ganglion neurons. Also the cannabimimetic properties of 2-AG could be predicted from its structure, which contains two of the three chemical moieties reputed to be part of the cannabinoid pharmacophore, i.e. the hydroxy group and the n-pentyl alkyl chain [10,26], at almost the same distance as in AnNH. A possible structural and functional similarity between arachidonoylglycerols and AnNH had been suggested also by the finding that AnNH activates rat brain protein kinase C in vitro by acting as a partial agonist at the diacylglycerol (DAG)-binding site [27], even though an analogous effect has not been reported for 2-AG. Therefore the finding of high levels of 2-AG in cannabinoid receptor-containing mammalian tissues and the discovery of 2-AG cannabimimetic actions in these tissues, although being of great significance, should not be regarded as surprising, and are not by themselves sufficient to propose for the monoacylglycerol a role, along with AnNH, as an ‘endocannabinoid’. Hence there was a necessity to perform the present study in which we used mouse N18TG2 neuroblastoma cells, a tumoural line widely employed as a neuronal model for studies on the ‘cannabinergic system’ owing to its capability of responding to, biosynthesizing and inactivating AnNH ([10,17,28] and references cited therein). We show for the first time that N18TG2 cells (1) respond to treatment with the membrane depolarizing agent ionomycin by producing and releasing 2-AG in a Ca\(^{2+}\)-dependent fashion, and (2) possess enzymatic activities potentially responsible for the biosynthesis and degradation of 2-AG.

**MATERIALS AND METHODS**

N18TG2 cells were purchased from DSM (Braunschweig, Germany) and grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10\% (v/v) fetal calf serum at 37 °C and 5\% CO\(_2\). Synthetic 2-AG was kindly donated by Professor R. Mechoulam (The Hebrew University of Jerusalem, Jerusalem, Israel). Synthetic \([\text{H}]^2\)-2-AG was prepared from \([\text{H}]\text{AA}\) (230 mCi/mmol; NEN-Dupont), glycerol and benzaldehyde by the procedure previously described ([19], and references cited therein). Synthetic \(\text{sn}-1\)-stearoyl-2-\([\text{H}]\)arachidonoylglycerol and \(\text{sn}-1\)-arachidonoyl-2-\([\text{H}]\)arachidonoylglycerol were prepared by digestion of respectively \(\text{sn}-1\)-stearoyl-2-\([\text{H}]\)arachidonoylglycerol- \(\text{sn}-1\)-arachidonoyl-2-\([\text{H}]\)arachidonoylglycerol by pancreatic phospholipase A\(_2\) (Sigma) in Tris/HCl, pH 7.4, for 2 h at 37 °C, followed by lipid extraction and purification by open-bed silica chromatography (see below).

For 2-AG biosynthesis experiments, confluent cells were prelabelled overnight with \([\text{H}]\text{AA}\) (0.5 \(\mu\)Ci/ml culturing medium), and then washed three times with serum-free DMEM before stimulation with 1–5 \(\mu\)M ionomycin (Sigma) in 3 ml of serum-free DMEM per dish. In some experiments EGTA (1 mM; Sigma) was added to the incubation medium. Stimulation was also performed with either \(C. \text{perfringens}\) PLC (10 units/ml of DMEM), Streptomyces chromofuscus PLD (Sigma; 10 units/ml of DMEM) or pig pancreas phospholipase \(A_2\) (PLA\(_2\); Sigma; 10 units/ml of DMEM). Finally, cells were also stimulated with 5 \(\mu\)M ionomycin after a 15 min preincubation with PLC (10 units/ml), PLA\(_2\) (10 units/ml) or the PLC inhibitor neomycin sulphate (1 mM). In this case the phospholipases and the inhibitor were also present during the subsequent stimulation with ionomycin. In all cases, cells from four to six 100 mm Petri dishes (0.9 \(\times\) 10\(^{10}\) cells per dish) were used for stimulation with each of the above agents, which was performed for 15 min at 37 °C and terminated by the addition of 3 ml of ice-cold methanol. Cells were then transferred into 50 ml Falcon tubes and an equal volume of chloroform was added. In experiments on the release of 2-AG from cells, stimulation was performed in DMEM containing 0.1\% BSA, and cells and incubation media were extracted separately with chloroform/methanol (2:1, v/v). After sonication for 3 min at 4 °C, the organic phase, separated from the aqueous phase by centrifugation, was dried down under decreased pressure, redissolved in chloroform and pre-purified by open-bed chromatography carried out in mini-columns packed with 2 ml of silica gel (Merck) in chloroform/methanol (9:1, v/v). The columns were eluted first with 4 ml of chloroform/methanol (9:1, v/v) and then with 4 ml of chloroform/methanol (1:1, v/v), to separate neutral lipids from phosphoacylglycerols. The first eluate was then analysed by TLC with silica gel-coated polyethylene TLC plates (Merck) developed with the organic phase of a mixture of iso-octane/ethyl acetate/water/acetic acid (50:110:100:20, by vol.) (solvent system A). Radioactivity was then measured by a one-dimensional TLC radioscanner (Packard). Radioactive bands with an \(R_p\) of 0.75, identical with that of synthetic 2-AG, were scraped off the plates and analysed by reverse-phase HPLC, performed as described previously [28] on a Spherisorb ODS-2 analytical column with an elution gradient of methanol in water. The radioactivity contained in aliquots of the 1 ml HPLC fractions was measured by a liquid-scintillation \(\beta\)-counter (Packard) after the addition of 5 ml of scintillation fluid (Ultima Gold, Packard). Radioactive TLC bands with \(0.9 < R_p < 1.0\), containing free fatty acids and acylglycerols, were also scraped from TLC plates and re-analysed by TLC developed with a mixture of diethyl ether/light petroleum (boiling range 40–60 °C)/NH\(_4\)OH (50:50.1, by vol.), which permits the separation of AA (\(R_p = 0\)), DAGs (\(R_p = 0.5\)) and triacylglycerols (\(R_p = 0.9\)). To ascertaining that the metabolite produced on stimulation of cells with ionomycin was 2-AG, radioactive HPLC fractions with the same retention time as synthetic 2-AG (27 min) obtained from several experiments (corresponding to approx. 1.8 \(\times\) 10\(^{10}\) cells) were pooled and aliquots were: (1) digested for 1 h at 37 °C with \(R. \text{arrhizus}\) lipase (Sigma; 8000 units/ml) in 0.5 ml of 50 mM sodium acetate/acetate buffer, pH 5.6, containing 0.1 M NaCl and 10 mM CaCl\(_2\); (2) analysed by TLC on borate-treated silica gel-coated plates developed with chloroform/acetone (96:4, v/v), to separate 1-AG from 2-AG; and (3) acetylated by reaction with 100 \(\mu\)l of acetic anhydride in 400 \(\mu\)l of anhydrous pyridine, to analyse the compound by gas chromatography–MS (GC–MS). \(R. \text{arrhizus}\) digests were extracted with chloroform/methanol (2:1, v/v) and the extracts analysed by TLC developed with solvent system A. Borate-impregnated plates were prepared by a quick immersion of silica gel-coated glass plates (Merck) in 0.4 M boric acid solution, followed by heating at 110 °C for 2 h. Radioactivity profiles of these TLC analyses were obtained by scraping the silica off TLC plates at 0.5 cm intervals and measuring its \(\beta\)-emission in scintillation vials containing 1 ml of methanol and 10 ml of scintillation liquid. GC–MS was performed on a Hewlett-Packard instrument consisting of an HP-GC 5890 series II apparatus equipped with an HP-5MS capillary column (30 m \(\times\) 0.25 mm, 0.25 \(\mu\)m film thickness; cross-linked 5\% HP ME siloxane), and of an HP-MS 5989B quadrupole mass analyser equipped with an electron impact source operating at 70 eV and 250 °C. To improve sensitivity, acquisition was performed in the Selected Ion Monitoring mode, which consists of monitoring the presence in the GC eluate of only few selected ion fragments. GC was conducted by using a 3 min isothermal step at 120 °C followed by a temperature gradient from 120 to 300 °C at a rate of 10 °C/min (helium flow 1 ml/min, injector and transfer line.
temperature 260 °C. Under these conditions synthetic standards of either diacetoxy-1-AG or diacetoxy-2-AG are eluted after 21.1 min.

Experiments on the presence of 2-AG-hydrolysing activity in N\textsubscript{2}T\textsubscript{2} cells were performed by homogenizing confluent cells in 50 mM Tris/HCl, pH 7.4, with a Dounce homogenizer. The homogenate was centrifuged sequentially at 800 g (5 min), 10000 g (15 min) and 100000 g (30 min). The pellets as well as the supernatant from the last centrifugation (0.3–0.5 mg of total proteins) were incubated in 0.5 ml of 50 mM Tris/HCl, pH 7.4, at 37 °C for 30 min in the presence of 5000 c.p.m. (0.9 nmol) of synthetic \textsuperscript{3}H[2]-AG (5 mM/cm). Incubations were also conducted in the presence of either 0.5 mM PMSF (Sigma), 250 \mu M arachidonoyltrifluoromethylketone (AACOCF\textsubscript{3}; Cascade) or 100 \mu M AnNH (Sigma). Incubations were terminated by adding 1 ml of chloroform/methanol (1:1, v/v) and lowering the temperature to 4 °C. The organic phase was then analysed by TLC on polyethylene silica-coated plates developed with solvent system A, and the radioactivity due to \textsuperscript{3}H[2]-AA (R\textsubscript{p} = 0.9), produced from the hydrolysis of \textsuperscript{3}H[2]-AG, was measured by a one-dimensional TLC scanner.

Experiments on the conversion of either synthetic sn-1-stearoyl-2-[\textsuperscript{3}H]arachidonoylglycerol or sn-1-arachidonoyl-2-[\textsuperscript{3}H]arachidonoylglycerol into \textsuperscript{3}H[2]-AG were performed by incubating 50000 c.p.m. of either DAG species (1.3 pmol and 9 nmol respectively) with 1 ml of whole N\textsubscript{2}T\textsubscript{2} cell homogenates (0.5 mg of total proteins), obtained by homogenizing confluent cells in 20 mM Hepes buffer, pH 7.4, containing 2 mM CaCl\textsubscript{2} (0.5 mg of total proteins) were incubated in 0.5 ml of 50 mM Tris/HCl, pH 7.4, for 15 min and 30 min at 37 °C. The incubation was stopped by adding 2 ml of chloroform/methanol (2:1, v/v) and lowering the temperature to 4 °C. The organic phase was then dried down and analysed by TLC developed with solvent system A. Radioactivity TLC profiles were obtained by scanning the TLC plate with a one-dimensional TLC scanner. The chemical nature of the TLC peak at the same position of acylglycerols and phosphoglycerols, strongly support the structure of the sn-2 isomer of monoarachidonoylglycerol for the radioactive component produced on the stimulation of N\textsubscript{2}T\textsubscript{2} cells with ionomycin. Definitive evidence for this conclusion was obtained from TLC analyses of the purified radioactive component on borate-impregnated TLC silica plates, commonly used [22,25] to separate 1- from 2-monoacylglycerols [in fact, neither normal-phase TLC nor reverse-phase HPLC conditions used for the analyses shown in Figures 1(A) and 1(B) permit a separation of the two isomers]. This showed that the arachidonoylglycerol produced by stimulated cells was composed of 47.2 ± 9.9 % of the 1-isomer and of 52.8 ± 9.9 % of the 2-isomer (means ± S.D., n = 2) (Figure 1F). The presence of the former isomer might be due to the migration of the arachidonoyl moiety from the sn-2 to the sn-1 position, a phenomenon likely to occur particularly during chromatographic purification of acylglycerols on silica (whereas acyl migration from the sn-1 to the more sterically hindered sn-2 position is less likely to occur) ([22,25] and references cited therein).

After having provided unprecedented chemical evidence for the stimulus-induced formation of 2-AG, we next wished to assess whether ionomycin-induced 2-AG biosynthesis was Ca\textsuperscript{2+}-dependent, and therefore stimulated the cells in the presence of the Ca\textsuperscript{2+}-chelating agent EGTA (1 mM). This substance inhibited significantly the effect of 5 \mu M ionomycin on 2-AG levels (Figure 2A), suggesting that, in neurons as in fibroblasts [22], the formation of 2-AG might be triggered by an increase in cytoplasmic Ca\textsuperscript{2+} concentration.

To propose a role for a metabolite as a neuromodulator, its release from and inactivation by neurons must also be demonstrated. When N\textsubscript{2}T\textsubscript{2} cells labelled with \textsuperscript{3}H[2]-AA were stimulated with 5 \mu M ionomycin in DMEM containing 0.1 % BSA, 20 ± 8 % of total \textsuperscript{3}H[2]-AG produced (mean ± S.D., n = 3) was found in the medium, suggesting that a significant portion of 2-AG biosynthesized in a Ca\textsuperscript{2+}-dependent manner is released outside neurons. This value for 2-AG release is comparable to analogous data obtained in platelet-derived growth factor-stimulated Swiss 3T3 cells [22]. The presence of an enzymic mechanism for 2-AG degradation was also shown in the present study in separate cytoplasmic, microsomal or 10000 \mu g pellet fractions of N\textsubscript{2}T\textsubscript{2} cells, which rapidly catalysed the hydrolysis of synthetic \textsuperscript{3}H[2]-AG to \textsuperscript{3}H[2]-AA (respectively 51.3 ± 0.1, 70.0 ± 0.7 and 70.4 ± 5.8 pmol/min per mg of protein using 900 pmol of \textsuperscript{3}H[2]-AG in a 0.5 ml assay volume containing 0.3–0.5 mg of total proteins; means ± S.E.M. for four independent determinations). A purified preparation of the PMSF-sensitive brain enzyme responsible for AnNH hydrolysis to ethanolamine, ‘anandamide amidohydrolase’, was recently shown to catalyse the hydrolysis of arachidonate methyl ester [29]. However, N\textsubscript{2}T\textsubscript{2} cell 2-AG-hydrolysing activity or activities were only slightly inhibited by a high concentration (0.5 mM) of PMSF, and significantly inhibited neither by the more selective ‘anandamide amidohydrolase’ inhibitor AACOCF\textsubscript{3} (200 \mu M) nor by AnNH (100 \mu M) (Table 1). Accordingly, purified fractions of ‘anandamide amidohydrolase’ from N\textsubscript{2}T\textsubscript{2} cells [17] did not catalyse the hydrolysis of \textsuperscript{3}H[2]-AG (results not shown). In contrast, the wide distribution of 2-AG-hydrolysing activity or activities in N\textsubscript{2}T\textsubscript{2} cell fractions suggests that 2-AG might be the substrate for more than one lipase. Therefore the possibility of the existence of a specific lipase for 2-AG hydrolysis, responsible for the physiological inactivation of this putative mediator, should be investigated in future studies.

Returning to the biosynthesis of 2-AG, several pathways have been suggested to contribute to this in the central nervous system (see also [20], and references therein), although the one that has found most experimental support is that involving 1-acyl-2-
Cells prelabelled with \( ^{3}H \)AA were stimulated with 5 \( \mu \)M ionomycin and the lipids extracted and purified as described in the Experimental section: (A) radioactive profile of a typical TLC analysis (development in solvent system A; 1-AG and 2-AG have the same \( R_f \) under these conditions) of pre-purified lipid extracts; (B) radioactive profile of a typical reverse-phase HPLC analysis of the 2-AG-like component from the TLC chromatographic step (1-AG and 2-AG have the same retention time under these conditions); (C) radioactive profile of a typical TLC analysis (development in solvent system A) of the 2-AG-like peak from the HPLC chromatographic step after digestion with \( R. \) arrhizus lipase; (D) electron impact fragmentogram obtained by Selected Ion Monitoring GC–MS of an acetylated aliquot of pooled 2-AG-like peaks from HPLC analyses of samples from three separate stimulation experiments (1.8 \( \times \) 10^8 cells); (E) electron-impact mass spectrum of the peak at 21.1 min of the GC–MS analysis of acetylated 2-AG synthetic standard; (F) radioactive profile of a typical TLC analysis, performed on borate-impregnated TLC plates developed with chloroform/acetone (96:4, v/v), of an aliquot of the pooled 2-AG-like peaks from HPLC [see (D)]. Lines indicate the chromatographic behaviour of synthetic standards of 1-AG, 2-AG, AcAG and AA. 1-AG and 2-AG have the same chromatographic behaviour except in (F).

arachidonoylglycerols (AcAGs) as direct precursors for 2-AG through the action of an \( sn-1 \)-specific DAG lipase [22–24]. AcAGs are derived either by the action of PLC enzymes on phosphatidylycholine or phosphatidylinositol (PI), or from the hydrolysis of phosphatidic acid catalysed by phosphatidic acid phosphatase. Phosphatidic acid, in turn, can be produced either through the activation of PLD, or from synthesis de novo, using monoacylglycerol 3-phosphate and AA (as AA-CoA) as precursors. Alternatively, 2-AG might be derived from PI through the sequential activation of PI-specific phospholipase \( A_1 \) and lyso-PI-specific PLC, two enzymes recently characterized in nervous tissues [25]. This latter pathway, however, is not likely to participate in the Ca^{2+}-induced formation of 2-AG described here, because the activity of the only type of PI-specific phospholipase \( A_1 \) reported so far is not dependent on Ca^{2+} [25]. When we tested, in cells prelabelled with \( ^{3}H \)AA, the effect on radiolabelled 2-AG, AA and AcAG levels of stimulation with exogenous \( C. \) perfringens PLC, \( S. \) chromofuscus PLD or pig pancreas PLA_{2}, we found a significant enhancement of \( ^{3}H \)2-AG levels only concomitantly with a corresponding increase in \( ^{3}H \)AcAG, i.e. when intact cells were treated with ionomycin or PLC, but not with PLD or PLA_{2} (Figure 2A). Moreover, incubation of cells with ionomycin in the presence of the Ca^{2+}-chelating agent EGTA inhibited the production not only of 2-AG but also of AcAGs (Figure 2A). These results seem to indicate a biosynthetic relationship between AcAGs and 2-AG in \( N_{18} \)TG_{2} cells. Accordingly, the incubation of whole-cell homogenates with synthetic \( sn-1 \)-stearyl-2-[\( ^{3}H \)]arachidonoylglycerol or \( sn-1 \)-arachidonoyl-2-[\( ^{3}H \)]arachidonoylglycerol led to the formation of \( ^{3}H \)2-AG (Figure 3), confirming that the
Cannabimimetic arachidonic acid derivative in neuroblastoma cells

Effect of various agents on the amount of \[^{3}H\]-2-AG, \[^{3}H\]-AcAG and \[^{3}H\]-AA produced by N18TG2 cells prelabelled with \[^{3}H\]-AA

The ionomycin concentration was 5 µM, and the concentration of the phospholipases was 10 units/ml in each case. \[^{3}H\]-2-AG was purified by reverse-phase HPLC of 2-AG-like fractions from the first TLC chromatographic step (Figure 1A) and quantified by liquid-scintillation counting of the corresponding HPLC fractions. \[^{3}H\]-AcAG and \[^{3}H\]-AA were purified by TLC (performed with diethyl ether/light petroleum (boiling range 40–60 °C)/NH\(_4\)OH (50:50:1, by vol.) of fractions with 0.9 < \(R_f\) < 1.0 from the first TLC chromatographic step (Figure 1A), and quantified by a one-dimensional TLC radioscanner, whose efficiency was estimated to be approx. 20% of that of liquid-scintillation counting. Bars marked with an ‘a’ have been divided by 10 so that they fit in the same scale as the others. The asterisks indicate statistically significant differences (\(P < 0.01, \text{unpaired Student’s } t\)-test) between ionomycin plus EGTA and ionomycin (A) and between ionomycin plus PLA\(_2\) and ionomycin (B). Values are means ± S.D. for three independent determinations. The values for ionomycin, PLC and PLA\(_2\) are repeated in (A) and (B). Abbreviations: iono, ionomycin; neomycin, neomycin sulphate.

Table 1 Effect of PMSF, AACOCF\(_3\) and anandamide on the rate of \[^{3}H\]-2-AG hydrolysis by N\(_{18}\)TG\(_2\) subcellular fractions

<table>
<thead>
<tr>
<th>Substance added</th>
<th>10,000 g pellet fraction</th>
<th>Microsomal fraction</th>
<th>Cytosolic fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PMSF (0.5 mM)</td>
<td>70.3 ± 5.0</td>
<td>52.8 ± 4.9*</td>
<td>70.2 ± 16.3</td>
</tr>
<tr>
<td>AACOCF(_3) (200 µM)</td>
<td>85.4 ± 7.1</td>
<td>77.0 ± 6.4</td>
<td>89.2 ± 0.9</td>
</tr>
<tr>
<td>Anandamide (100 µM)</td>
<td>78.3 ± 1.4</td>
<td>81.0 ± 7.8</td>
<td>92.4 ± 5.4</td>
</tr>
</tbody>
</table>

Rates of hydrolysis (% of control) for N18TG2 subcellular fractions

The rate of \[^{3}H\]-2-AG hydrolysis in the absence of inhibitors was taken as 100% and was respectively 51.3 ± 0.1, 70.0 ± 0.7 and 70.4 ± 5.8 pmol/min per mg of protein for cytosolic, microsomal and 10,000 g fractions respectively. Values are means ± S.E.M. for four independent determinations. *Statistically different from control (\(P < 0.05, \text{unpaired Student’s } t\)-test).

Cannabimimetic acylglycerol might derive from AcAGs and suggesting the presence of an sn-1 DAG lipase activity in N\(_{18}\)TG\(_2\) cells. The rate of conversion was not significantly affected by co-incubation with EGTA (results not shown) and was higher after incubations of 15 min than of 30 min (200.0 ± 10.6 compared with 116.0 ± 3.4 pmol/min per mg of protein, and 12.6 ± 0.6 compared with 7.4 ± 0.6 fmol/min per mg of protein respectively for sn-1-arachidonoyl- and sn-1-stearoyl-2-[\(^{3}H\)]arachidonoyl-glycerol; means ± S.D. for three experiments), possibly because of the presence of the 2-AG-hydrolysing activity described in this study.

Figure 2 Effect of various agents on the amount of \[^{3}H\]-2-AG, \[^{3}H\]-AcAG and \[^{3}H\]-AA produced by N\(_{18}\)TG\(_2\) cells prelabelled with \[^{3}H\]-AA

Whole homogenates (1 ml, approx. 0.5 mg of total proteins) from N\(_{18}\)TG\(_2\) cells were incubated with 50,000 c.p.m. of either sn-1-arachidonoyl-2-[\(^{3}H\)]arachidonoylglycerol (9 nmol) (A) or sn-1-stearoyl-2-[\(^{3}H\)]arachidonoylglycerol (1.3 pmol) (B) at 37 °C for either 15 min (middle traces) or 30 min (upper traces). Incubations were also performed with boiled homogenates (lower traces). After the incubation, lipids were extracted and analysed by TLC with solvent system A. The \(R_f\) values of synthetic 2-AG and AcAGs are shown. Traces are not aligned, to avoid overlapping of peaks. The chemical nature of the 2-AG-like peaks was confirmed by HPLC and TLC on borate-impregnated plates. Results are from one experiment representative of three separate experiments.

Figure 3 Conversion of \[^{3}H\]-AcAGs into \[^{3}H\]-2-AG by N\(_{18}\)TG\(_2\) cell homogenates

Table 1 Effect of PMSF, AACOCF\(_3\) and anandamide on the rate of \[^{3}H\]-2-AG hydrolysis by N\(_{18}\)TG\(_2\) subcellular fractions
The results described above on the effect of ionomycin on AA and AcAG formation did not allow us to clarify whether this substance acts on N₆TG₂ cells by activating PLA₄, PLC or both. Ionomycin, like other Ca²⁺ ionophores, is known to provoke Ca²⁺-evoked events, such as PLA₄ stimulation, independently of PLC activation ([30], and references therein). However, to make a preliminary investigation of the participation of PLC in ionomycin-induced AcAG and 2-AG formation, we addressed the question of whether this effect could be influenced by the PLC inhibitor neomycin sulphate (1 mM). Interestingly, the inhibitor did not counteract the ionomycin-induced increase of either 2-AG or AcAG levels but, probably because of its weak ionophore properties, slightly potentiated it (Figure 2B), suggesting that the effect of ionomycin might not be due to PLC activation. Accordingly, pretreatment of cells with PLA₄ did not inhibit but, on the contrary, caused a 2.5-3-fold potentiation of the ionomycin double stimulatory action on AcAG and 2-AG levels (Figure 2B). In fact, if 2-AG produced by the stimulation of neurons with ionomycin were derived from AcAGs originating from the PLC-catalysed hydrolysis of phospholipid precursors, pretreatment of cells with PLA₄, which decreases significantly the amount of AA on the sn-2 position of phosphoacylglycerols (Figure 2A), should have led to an inhibition of ionomycin-induced AcAG and 2-AG formation. In fact, treatment of neuroblastoma cells with exogenous PLA₄ (as well as with 1 mM neomycin sulphate) inhibited the formation of AcAG and 2-AG induced by exogenous PLC (results not shown). In contrast, in accord with AcAG participation in ionomycin-induced 2-AG biosynthesis, the ionophore did not cause a further enhancement of the effect of exogenous PLC, which was likely to have caused a saturation of 2-AG and AcAG levels that could not be increased further by ionomycin.

A possible explanation for the potentiation of ionomycin effect by exogenous PLA₄ (which alone did not cause 2-AG formation; Figure 2A) is that the extremely high levels of AA released by this phospholipase (Figures 2A and 2B) might induce the formation of AcAGs and/or 2-AG, provided that a simultaneous influx of calcium into cells is evoked. Accordingly, an analogous pre-incubation of cells with exogenous phospholipase B from Vibrio spp. (Sigma; 10 units/ml), which is not selective for the sn-2 position of phosphoacylglycerols, and leads to the formation of both 1-lyso- and 2-lyso-phosphoacylglycerols, did not result in a significant alteration of ionomycin-induced [³H]2-AG, [³H]AcAG or [³H]AA formation (respectively 110.7±7.1%, 126.7±15.1% and 102.6±3.0% of the levels obtained without preincubation, means±S.D., n = 2), suggesting that the PLA₄-induced release of AA and not of lysophospholipids might be the cause of the potentiation of ionomycin effect. As outlined above, AA, derived from the possible ionomycin-induced activation of endogenous PLA₄, might indeed lead to 2-AG biosynthesis by generating AcAGs via AA-CoA:monoaicylglycerol-3-phosphate acyltransferase followed by phosphatidic acid phosphatase. Biosynthesis experiments in vitro conducted by incubating AA-CoA and [¹⁴C]glycerol with N₆TG₂ cell whole homogenates in an attempt to produce [¹⁴C]-2-AG were not successful (results not shown). However, [¹⁴C]-2-AG accumulation in these experiments might have been prevented by the action of the potent 2-AG-hydrolysing activity or activities described in this study, for which no inhibitors have been found. Therefore the existence in N₆TG₂ cells of a biosynthetic mechanism involving AA in 2-AG formation could not be ruled out nor proved in this study.

In summary, our results are strongly suggestive of a role for AcAGs as immediate precursors for 2-AG biosynthesis, possibly via the catalysis of sn-1-specific DAG lipase, as previously proposed for other tissues [22–24]. Moreover, a clue that PLA₄-derived AA participates in ionomycin-induced 2-AG biosynthesis has been provided. However, the molecular mechanisms by which, respectively, ionomycin stimulation leads to AcAG formation or AA facilitates 2-AG formation were not planned to be investigated in detail in this study, and their full understanding will require further experiments. In a separate study [31], N₆TG₂ cell homogenates were found to contain distinct PLC-like enzymic activities capable of slowly but significantly converting diarachidonylphosphatidylethanolamine and 1-lyso-2-arachidonylphosphatidylethanolamine (respectively the precursor and the by-product of the biosynthetic reaction leading to the formation of N-arachidonylphosphatidylethanolamine [9]) into 2-AG. Therefore, although not supported by results presented here for ionomycin-treated cells, it is possible that, as in Swiss 3T3 cells [22], PLC-mediated formation of 2-AG might occur when neuronal cells are stimulated with physiological agonists capable of up-regulating the activity of specific PLC-like enzymes that recognize sn-2-arachidonate-containing (lyso)phospholipids.

In conclusion, the present study, by describing evidence for the presence in neuronal cells of mechanisms for 2-AG inactivation and its Ca²⁺-dependent biosynthesis and release, has provided biochemical grounds for the previous hypotheses that this monoacylglycerol might act either as one of the physiological agonists at the central CB1 cannabinoid receptor [20] or as a second messenger modulating Na⁺/K⁻-ATPase activity [32]. Future studies will have to be aimed at a deeper understanding of the molecular mechanisms underlying both 2-AG formation and its regulation, as well as at clarifying the physiological function of 2-AG in both the central nervous system and peripheral tissues, also in relation to its possible co-existence [31] with other proposed ‘endocannabinoids’ such as palmitoylethanolamide [6] and AnNH.
Cannabimimetic arachidonic acid derivative in neuroblastoma cells


