RESEARCH COMMUNICATION

The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide

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The novel endogenous cannabinoid 2-arachidonoylglycerol (2-AG) was rapidly inactivated by intact rat basophilic leukaemia (RBL-2H3) and mouse neuroblastoma (N18TG2) cells through diffusion/hydrolysis/reacylation processes. The hydrolysis of 2-AG was inhibited by typical esterase inhibitors and by more specific blockers of 'fatty acid amide hydrolase' (FAAH), the enzyme catalysing the hydrolysis of the other 'endocannabinoid', anandamide (AEA). No evidence for a facilitated-diffusion process was found. A 2-AG-hydrolysing activity was detected in homogenates from both cell lines, with the highest levels in membrane fractions. It exhibited an optimal pH at 10, and recognized both 2- and 1(3)- isomers of monoarachidonoylglycerol with similar efficiencies. The apparent K_m and V_{max} values for [^{3}H]2-AG hydrolysis were 91 μM and 29 μM and 2.4 and 1.8 nmol·min^{-1}·mg of protein^{-1} respectively in N18TG2 and RBL-2H3 cells. [^{3}H]2-AG hydrolysis was inhibited by Cu^{2+}, Zn^{2+} and *p*-hydroxymercuribenzoate, and by 2- or 1(3)-monolinoleoyl- and -linolenoyl-glycerols, but not by the oleoyl, palmitoyl and myristoyl congeners. Purified fractions from solubilized membrane proteins catalysed, at pH 9.5, the hydrolysis of 2-AG as well as AEA. Accordingly, AEA as well as FAAH inhibitors, including arachidonoyltrifluoromethyl ketone (ATFMK), blocked [^{3}H]2-AG hydrolysis by N18TG2 and RBL-2H3 membranes, whereas 2-AG inhibited [^{14}C]AEA hydrolysis. FAAH blockade by ATFMK preserved from inactivation the 2-AG synthesized *de novo* by intact N18TG2 cells stimulated with ionomycin. These data suggest that FAAH may be one of the enzymes deputed to the physiological inactivation of 2-AG, and create intriguing possibilities for the cross-regulation of 2-AG and AEA levels.

INTRODUCTION

Anandamide [arachidonoylethanolamide; AEA) and 2-arachidonoylglycerol (2-AG) were isolated from both nervous and peripheral tissues [1–4] and were thought to act as endogenous ligands of the two CB_1 and CB_2 cannabinoid receptor subtypes (for a review, see [5]). Pathways for AEA biosynthesis and inactivation by intact cells were identified [6], and an enzyme catalysing AEA hydrolysis to arachidonate (AA) and ethanolamine was detected in several tissues [5,7–10], purified, cloned, sequenced and named 'fatty acid amide hydrolase' (FAAH) [10]. This enzyme is inhibited by classical serine- and cysteine-protease inhibitors, such as *N*-ethylmaleimide (NEM) and *p*-hydroxymercuribenzoate (*p*HMB) [7–10], as well as by AA derivatives such as arachidonoyltrifluoromethyl ketone (ATFMK) and arachidonoylmethyl fluorophosphonate (MAFP) [9,11]. When overexpressed in COS-7 cells, FAAH was shown to catalyse also the hydrolysis of the sleep-inducing factor *cis*-9-octadecenoamide (oleamide) [10] and of arachidonoyl methyl ester [12]. Only a few metabolic studies have been performed to date on 2-AG [13–15], some of which have shown potential connections between its formation and AEA production [13]. In mouse neuroblastoma N18TG2 cells, ionomycin triggers 2-AG biosynthesis and release from cells [14], and enzymic activities have been found for 2-AG release from diacylglycerols and (lyso)phosphoacylglycerols, and for 2-AG degradation to AA and glycerol [13,14]. These enzymes have not yet been studied, nor has the possibility that 2-AG be inactivated by living neuronal or peripheral cells. Here we report the results of a study aimed at assessing this possibility and characterizing the enzyme responsible for the physiological degradation of 2-AG. Together with N18TG2 cells, where seminal work on 2-AG/AEA biosynthesis and CB1-mediated actions was previously carried out [5,13,14,16,17], we used rat basophilic leukaemia RBL-2H3 cells, in which AEA biosynthesis, uptake and FAAH-mediated degradation have been previously demonstrated [8] and constitutive CB2 receptors are selectively expressed [18].

MATERIALS AND METHODS

N18TG2 and RBL-2H3 cells were cultured as described in [8,17]. [^{3}H]2-AG, [^{3}H]1(3)-AG and [^{14}C]AEA (5 mCi/mmol) were synthesized from [^{3}H]AA plus 1,3-benzylideneglycerol or DL-1,2-isopropylideneglycerol and [^{14}C]ethanolamine plus AA as described in [3,6]. [^{3}H]2-AG and [^{3}H]1(3)-AG were purified by TLC on borate-impregnated silica [14]. AEA, 1(3)-AG, oleamide and palmitoylethanolamide were synthesized as described in [8,14,17]. 2-AG and its non-hydrolysable analogue AGE (the exact structure of which is presently the subject of a patent application) were

Abbreviations used: 2-AG, 2-arachidonoylglycerol; FAAH, 'fatty acid amide hydrolase'; AEA, anandamide; ATFMK, arachidonoyltrifluoromethyl ketone; AA, arachidonate; NEM, *N*-ethylmaleimide; *p*HMB, *p*-hydroxymercuribenzoate; MAFP, arachidonoyl methylfluorophosphonate; oleamide, *cis*-9-octadecenoamide; AEG, 1-arachidonyl ethylene glycol; MArG, monoarachidonoylglycerol.

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kindly donated by Dr. R. Mechoulam (Faculty of Medicine, The Hebrew University, Jerusalem, Israel) and 1-arachidonoyl-ethylene glycol (AEG) by Dr. V. Bezuglov (Shemyakin–Ovchinnikov Institute, Moscow, Russia). Monoacylglycerols and esterase inhibitors were purchased from Sigma. Experiments with intact cells were carried out as described previously [8] in six-well dishes (about 0.5×10^6 cells/well). Cells were incubated at 37 °C with 0.5 ml of serum-free media containing 10000 c.p.m. of either [^{3}H]2-AG or [^{14}C]AEA for different periods of time, or for 30 min with or without the substances shown in Figure 1(B) (below). After the incubation, [^{3}H]2-AG, [^{3}H]AA and [^{3}H]phospholipids, or [^{14}C]AEA and [^{14}C]ethanolamine, present in the incubation media or cells, were purified and quantified as described in [8,13,14]. Before extraction, cells were washed three times with 0.2%-BSA-containing medium. [^{3}H]2-AG and [^{14}C]AEA hydrolysis by cell-free fractions was measured by using either 10000 or 100000 ***g*** pellets or the 100000 ***g*** supernatant, prepared as described in [8,13,14]. In most experiments the 10000 and 100000 ***g*** pellets were pooled ('membrane' fraction), and aliquots (0.05–0.1 mg of protein) were incubated in 0.5 ml of 50 mM Tris/HCl, pH 9.5 (buffer A) with 20000 c.p.m. (8 μM) of either [^{3}H]2-AG or [^{14}C]AEA for 30 min at 37 °C, with or without one of the substances shown in Figures 2(A) and 2(B) (below). Incubations were also carried out in buffers at different pH values prepared as described in [8,17] or in buffer A with increasing amounts of either proteins or [^{3}H]2-AG and [^{3}H]1(3)-AG. Apparent V_{max} and K_m values were calculated from Lineweaver–Burk profiles using least-squares fitting (CricketGraph) and are expressed as means ± S.E.M. ($n = 3$). Membranes were solubilized in buffer A containing 1% reduced Triton X-100, and purified by ion-exchange chromatography, carried out on a Q anion-exchange column (7 mm × 52 mm; Bio-Rad) as described in [11], followed by salt removal on PD-10 minicolumns (Pharmacia) and hydroxyapatite chromatography. This was carried out with a CHT-2 column (7 mm × 52 mm; Bio-Rad) eluted with 30 ml of 10 mM Na_3PO_4 buffer, pH 7.6, containing 0.05% reduced Triton X-100, followed by a 30 ml gradient to a 420 mM Na_3PO_4/Triton X-100 buffer, pH 7.6 (flow rate 1 ml/min). Aliquots of each fraction were adjusted to pH 9.5 with 50 mM Tris/HCl, pH 11 (final volume 0.5 ml) and tested for their ability to hydrolyse either [^{3}H]2-AG or [^{14}C]AEA. The effect of the FAAH inhibitor ATFMK (10 μM; Biomol, Plymouth Meeting, PA, U.S.A.) on the levels of [^{3}H]2-AG, produced by ionomycin (5 μM) stimulation of N18TG2 cells (five confluent 100 mm-diameter Petri dishes) prelabelled overnight with [^{3}H]AA (NEN–DuPont; 230 Ci/mmol), and purified as described previously [14], was studied.

The inhibitory effect of the substances shown in Figures 1 and 2 (below) on 2-AG hydrolysis was calculated as the percentage of the [^{3}H]AA produced with only vehicle added (control) after 30 min incubations, and expressed as means ± S.E.M. for at least three independent experiments. Statistically significant differences between means ± S.D. of the raw data (i.e. as c.p.m. and not as percentage of control) were evaluated by means of the Student's unpaired t test, using a P value of < 0.05 as the threshold for significance.

RESULTS AND DISCUSSION

[^{3}H]2-AG was found to disappear rapidly from the incubation medium of living cells (Figure 1A). As a result of this process, which was half-maximal after 17 and 27 min respectively in RBL-2H3 and N18TG2 cells, the radioactivity was found to be partitioned among: (a) residual 2-AG in medium, (b) 2-AG diffused into cells, (c) AA and (d) phospholipids. Although no evidence was found for a facilitated-diffusion process, 2-AG hydrolysis was inhibited by 2-linoleoylglycerol (but not by the palmitoyl analogue) and AEG, by alkylating agents such as NEM and *p*HMB (100 μM), and by AEA or substances previously found to specifically inhibit FAAH-catalysed AEA hydrolysis, i.e. MAFP (50 nM) and ATFMK (10 μM) (Figure 1B; results not shown). Interestingly, ATFMK and AEG decreased the formation of [^{3}H]phospholipids to a lower extent than [^{3}H]2-AG hydrolysis (respectively 88.8 and 59.6% of control). This may suggest that [^{3}H]phospholipids may derive not only from [^{3}H]AA produced from [^{3}H]2-AG hydrolysis, as previously shown for AEA hydrolysis by rat intact neurons [6], but also from the reacylation of [^{3}H]2-AG [4], which is probably not inhibited by ATFMK and AEG. These findings, taken together, indicate that 2-AG is not only released from [14,15], but also inactivated and recycled by, living cells through diffusion/hydrolysis/reacylation mechanisms.

A [^{3}H]2-AG-hydrolysing activity was found in N18TG2 and RBL-2H3 cell homogenates, with the highest levels in membrane fractions (73.2 and and 78.2% of total activity respectively). This activity displayed an optimal pH at 10.0 and another apparent active-site pK_a value at ≈ pH 6.0 (Figure 1C), and it varied linearly with protein amounts up to 40 μg/ml, being maximal between 200 and 300 μg/ml (Figure 1D). Both [^{3}H]2-AG and [^{3}H]1(3)-AG were hydrolysed with apparent K_m values of 91 ± 7 and 85 ± 9 μM in N18TG2 cells and 29 ± 4 and 53 ± 6 μM in RBL-2H3 cells. Apparent V_{max} values were, respectively, 2.4 ± 0.7 and 4.0 ± 0.9 $min^{-1}\cdot$mg of protein^{-1} in N18TG2 cells and 1.8 ± 0.2 and 2.1 ± 0.3 nmol·$min^{-1}\cdot$mg of protein^{-1} in RBL-2H3 cells. Thus RBL-2H3 membranes catalysed [^{3}H]2-AG hydrolysis more efficiently than N18TG2 membranes ($V_{max}/K_m = 0.062$ and 0.026 respectively), in agreement with data obtained in intact cells (Figures 1A and 1B). In both cell lines [^{3}H]2-AG hydrolysis was inhibited by 1(3)-AG and [^{3}H]1(3)-AG hydrolysis by 2-AG, whereas the hydrolysis of either substrate was inhibited by the same agents (Figures 2A and 2B; results not shown), suggesting that the same enzyme(s) catalyse(s) the hydrolysis of both mono-arachidonoylglycerol (MArG) regioisomers. This is particularly important in view of the observation that both isomers have similar biological activities and that 1(3)-AG is derived from 2-AG following acyl migration [14,15]. [^{3}H]2-AG hydrolysis by membranes was inhibited by 1(3)- and/or 2-linoleoylglycerol [1(3)- and 2-18:2] and 1(3)-linolenoylglycerol [1(3)-18:3], but much less by 1(3)- and/or 2-myristoyl- (14:0), -palmitoyl- (16:0) and -oleoyl- (18:1) glycerols (Figures 2A and 2B; results not shown), suggesting an increasing affinity of the enzyme for monoacylglycerols with increasing degree of unsaturation, as previously described for FAAH towards fatty acid amides [8,10,17]. Moreover, the AEG and, to a lesser extent, the AGE analogues also inhibited [^{3}H]2-AG hydrolysis. However, 1(3)- and 2-AG, as well as other di- and poly-unsaturated analogues and homologues, inhibited [^{3}H]2-AG hydrolysis less with N18TG2 than with RBL-2H3 cell membranes, suggesting that in the former cell type some less selective enzyme(s) may also contribute to MArG hydrolysis.

Like some previously described lipases [19–22], the 'MArG lipase' was inhibited by Zn^{2+}, Cu^{2+} and *p*HMB, and unaffected by Mg^{2+} and EDTA (Figures 2A and 2B). More interestingly, the enzyme was significantly inhibited by AEA, the preferential substrate of FAAH [10], and ATFMK or MAFP, two inhibitors of this enzyme [9,11]. FAAH was previously shown to be expressed in both cell types under study, to display a pH-dependency profile identical with that shown in Figure 1(C) for [^{3}H]2-AG hydrolysis [8,17] and to catalyse the hydrolysis of arachidonoyl methyl ester [12]. Oleamide and palmitoylethanol-

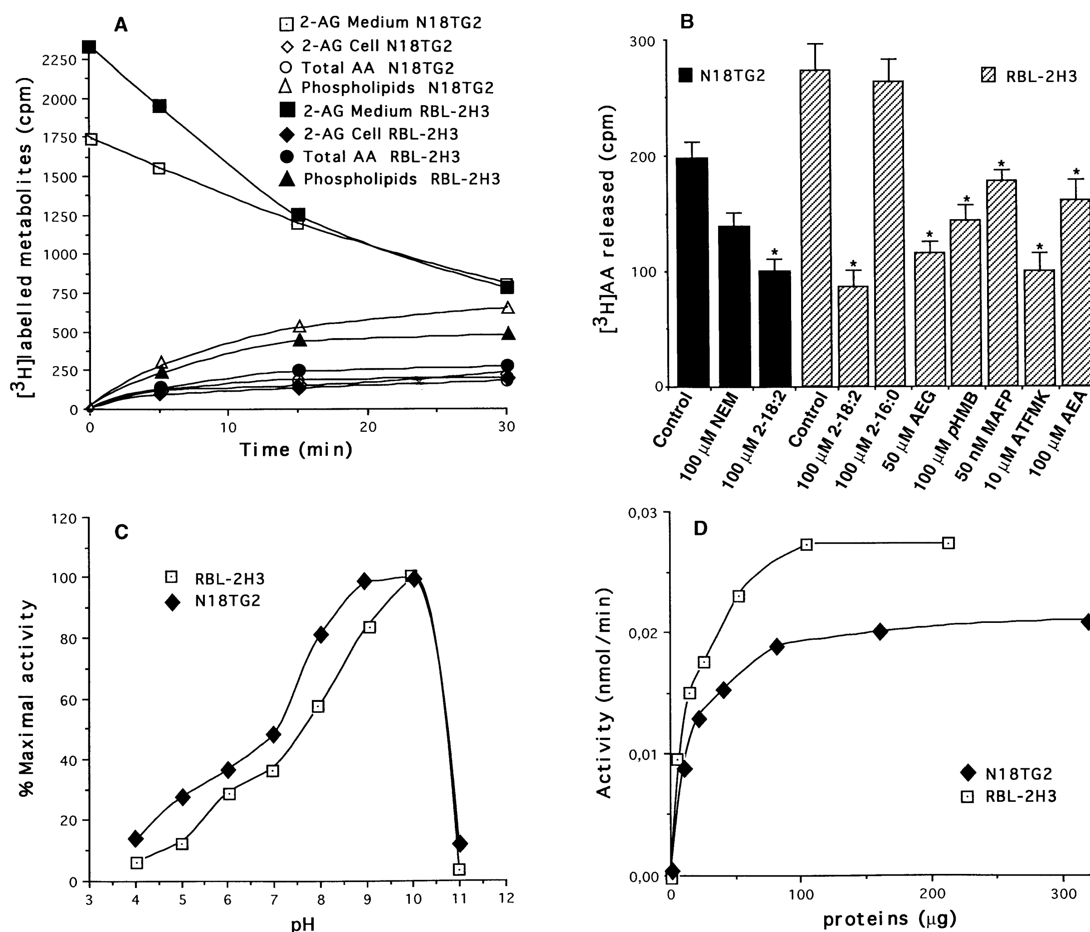

Figure 1 Inactivation of 2-AG by RBL-2H3 and N18TG2 cells

Clearance of [^{3}H]2-AG and corresponding incorporation of radioactivity into AA and phospholipids by intact cells at different intervals of time (**A**) and after 30 min in the presence of various substances selected from those tested (**B**). The radioactivity found in BSA washes was added to 2-AG 'Medium'. (**C**) pH- and (**D**) protein-dependency profile for [^{3}H]2-AG hydrolysis by RBL-2H3 and N18TG2 cell membranes. Activity was measured as the amount of [^{3}H]AA (in c.p.m.) produced from [^{3}H]2-AG hydrolysis. Results are means ± S.E.M., $n = 3$. Monoacylglycerols are indicated by the position, length and degree of unsaturation of their fatty acid chains, i.e.: 2-18:2, 2-linoleoylglycerol; 2-16:0, 2-palmitoylglycerol. $^*P < 0.05$ versus control.

amide also inhibited [^{3}H]2-AG hydrolysis (Figures 2A and 2B) to an extent very similar to that previously observed for their inhibition of FAAH respectively in N18TG2 and RBL-2H3 cells [8,17]. On the other hand, 2-AG (100 μM) and AEG (50 μM) significantly inhibited [^{14}C]AEA hydrolysis (respectively 41.2 % and 34.6 % of control in N18TG2, and 24.6 % and 19.4 % of control in RBL-2H3 cell membranes). Accordingly, when partially purified fractions from solubilized N18TG2 membrane proteins were assayed for their ability to catalyse [^{3}H]2-AG and [^{14}C]AEA hydrolysis, the FAAH activity always co-eluted with the 'MArG lipase' activity (Figures 2C and 2D). These data, taken together, suggest that FAAH, or a very similar enzyme, may be the major source of the 'MArG lipase' activity described here. Previous experiments, showing that 2-AG hydrolysis is not catalysed by partially purified FAAH fractions, were carried out at pH 7.4 [14], which is not optimal for the 'MArG lipase' (Figure 1C). Indeed, even at pH 9.5, the rate of [^{3}H]2-AG hydrolysis by purified fractions was lower than that of [^{14}C]AEA hydrolysis, in agreement with the catalytic efficiencies observed here and previously [17] for N18TG2 cell membranes when using [^{3}H]2-AG and [^{14}C]AEA as substrates ($V_{max}/K_m = 0.026$ and 0.15 respectively). A recent study revealed that membranes from cells overexpressing FAAH can efficiently catalyse 2-AG hydrolysis to AA, whereas untransfected control cells cannot (Professor S. Yamamoto, personal communication). This finding indicates that recombinant FAAH has an intrinsic 'MArG lipase' activity, and strongly suggests that this enzyme may catalyse 2-AG hydrolysis also in N18TG2 and RBL-2H3 membranes. ATFMK, at the same concentration (10 μM) shown here (Figure 1B) and previously [9] to inhibit 2-AG hydrolysis and FAAH in intact cells, significantly enhanced the levels of 2-AG biosynthesized *de novo* in ionomycin-stimulated N18TG2 cells (372 ± 48 and 640 ± 65 c.p.m. respectively in control and ATFMK-treated cells, means ± S.D., $n = 3$, $P < 0.05$; basal [^{3}H]2-AG release was not significantly affected by ATFMK). This suggests that FAAH may contribute to 2-AG inactivation also in living cells. However, two small peaks of 'MArG lipase' activity (peaks C and D in Figure 2D) were consistently found not to coincide with FAAH activity, suggesting that other lipases may contribute to 2-AG hydrolysis. Among the several monoacylglycerol lipases identified so far, those from platelet microsomes and pancreatic islet membranes resemble the enzyme described here inasmuch as they are selective for 1(3)-AG or 2-AG and optimally active in alkaline buffers [19,20]. These

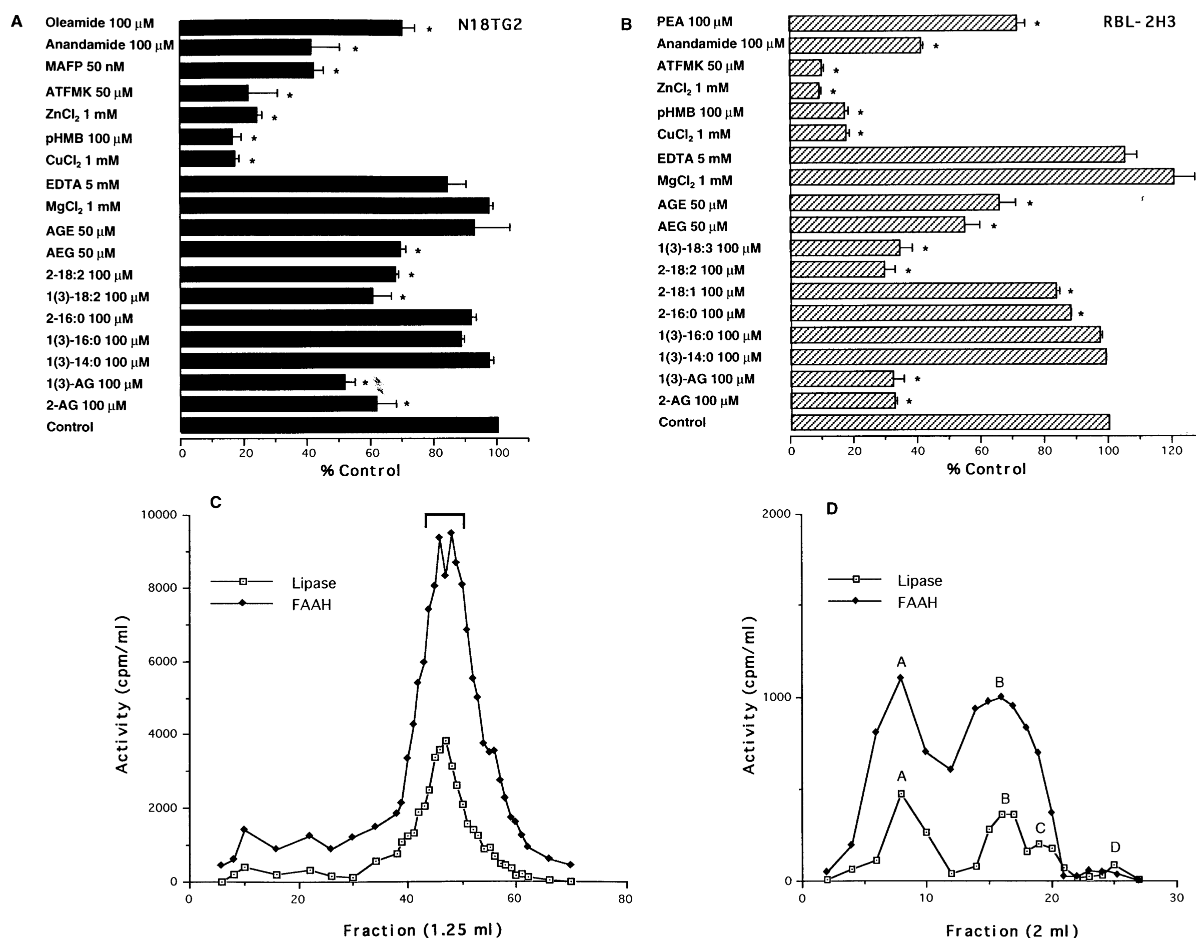

Figure 2 Effects of various substances on RBL-2H3 and N18TG2 cell 'MArG lipase' and its partial purification

In (**A**) and (**B**) the effects of selected inhibitors and 2-AG analogues, expressed as percentage of [^{3}H]2-AG hydrolysis with no substance added, are shown for N18TG2 (**A**) and RBL-2H3 (**B**) cell membranes. Anion-exchange (**C**) and hydroxyapatite (**D**) chromatography of solubilized N18TG2 membrane proteins. The fractions shown by a bar were passed through PD-10 mini-columns prior to the hydroxyapatite step. □, [^{3}H]AA released when incubating [^{3}H]2-AG (c.p.m./ml of fraction); ◆, [^{14}C]ethanolamine released when incubating [^{14}C]AEA (c.p.m./ml of fraction). In (**D**) the first peak of activity corresponds to the void volume and may be due to overloading. Data in (**A**) and (**B**) are means ± S.E.M., $n = 3$. Further abbreviations: PEA, palmitoylethanolamide; 1(3)-18:3, 1(3)-linolenoylglycerol. $^{*}P < 0.05$ versus control.

enzymes were suggested to contribute to phospholipase A_2-independent AA release, but may be also involved in the inactivation of cannabimimetic 2-AG. The microsomal enzyme from rat liver (the tissue with the highest FAAH levels [10]) also exhibits an optimal alkaline pH, and displays an approximate molecular mass of 62 kDa, very similar to that reported for FAAH [21]. The intriguing possibility that one of these enzymes is identical with FAAH may be a subject for future studies. The adipocyte monoacylglycerol lipase has been cloned and sequenced [22], but this enzyme is not selective for 2-AG and has a molecular mass different from that of FAAH.

In conclusion, the present study, apart from approaching the subject of the inactivation of 2-AG for the first time since the finding of its cannabimimetic properties, opens the way to possible cross-regulatory mechanisms between this metabolite and AEA. 2-AG is present in tissues in levels 170–500 times higher than those of AEA [1–5,15] and, by counteracting AEA hydrolysis (see above), may prolong AEA action. Indeed, when intact RBL-2H3 cells were co-incubated with 50 μM 2-AG and 4 μM [^{14}C]AEA, the hydrolysis, but not the uptake, of the latter compound was significantly inhibited (60.0 and 94.2 % respectively of control). This may suggest for 2-AG, which is less potent than AEA in cannabinoid-receptor-binding assays [3,4], an additional mechanism for the reinforcement of AEA cannabimimetic actions.

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