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Potential Biosynthetic Connections between the Two Cannabimimetic Eicosanoids, Anandamide and 2-Arachidonoyl-Glycerol, in Mouse Neuroblastoma Cells

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Anandamide (arachidonoyl-ethanolamide, AnNH) and 2-arachidonoyl-glycerol (2-AG) have been suggested to act as endogenous agonists at the brain cannabinoid receptor, and their biosynthetic and degradative mechanisms in nervous tissues and cells have also been partially elucidated. Here we present evidence for the presence, in mouse $N_{18}TG_2$ neuroblastoma cells, of enzymatic activities potentially responsible for the biosynthesis of AnNH and 2-AG from a common phospholipid precursor. Cell homogenates were shown to catalyze: (a) the transfer of an arachidonoyl moiety from the *sn*-1 position of *sn*-1,2-di-arachidonoyl-phosphatidylcholine (AAPC) to phosphatidyl-ethanolamine (PE) to form *N*-arachidonoyl-PE (N-ArPE) and *sn*-1-lyso-2-arachidonoyl-PC (lyso-APC), (b) the hydrolysis of N-ArPE to AnNH, (c) the hydrolysis of lyso-APC to 2-AG, (d) the hydrolysis of AAPC to *sn*-1,2-di-arachidonoyl-glycerol (AAG), and (e) the hydrolysis of AAG to 2-AG. From these findings it is possible to suggest that AAPC may serve as precursor for both AnNH and 2-AG biosynthesis through three different pathways.

In much the same way that the discovery of morphine receptors had led to the characterization of endorphins in the 1970s, the recent finding and cloning of both central and peripheral cannabinoid receptors, christened CB1 and CB2 (1, 2), opened the way for the discovery of 'endocannabinoids', i.e. endogenous substances potentially capable of acting as physiological agonists at these receptors (3-6). Two of these compounds, anandamide (arachidonoyl-ethanolamide, AnNH) and 2-arachidonoyl-glycerol (2-AG), derive from the non-oxidative metabolism of arachidonic acid (AA) and belong to two well known lipid classes, the acyl-ethanolamides (7) and the monoglycerides. Apart from binding to both CB1 and, with a lower affinity, CB2 receptors, AnNH and 2-AG were found to share several of the cannabinoid receptor-mediated pharmacological actions of $(-)\Delta^9$-tetra-hydrocannabinol *in vitro* and *in vivo,* e.g. inhibition of forskolin-induced adenylate cyclase and of electrically-evoked mouse *vas deferens* contractions, induction of hypomotility, analgesia and hypothermy in rodents, and modulation of lymphocyte proliferation (3-6, and for a recent review 8).

Recent studies have addressed the question of how the inter- and extra-cellular levels of AnNH and 2-AG are regulated in the CNS, by investigating the molecular mechanisms of AnNH and 2-AG biosynthesis, release and inactivation by neuronal cells. In the case of AnNH, these mechanisms seem to be similar to those previously reported for other acyl-ethanolamides (7, 23), i.e. AnNH can be: a) degraded to arachidonic acid (AA) and ethanolamine through the action of an amidohydrolase (9-13), and b) produced from the hydrolysis, catalyzed by a phosphodiesterase of the D type, of a phospholipid precursor, *N*-arachidonoyl-phosphatidyl-ethanolamine (N-ArPE) (13-15, 19). It has also been suggested that depolarization of the

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neuronal membrane with subsequent influx of Ca^{2+} may trigger AnNH biosynthesis (13), possibly by inducing the formation of N-ArPE through the transfer of arachidonate from the *sn*-1 position of another phospholipid, *sn*-1,2-di-arachidonoyl-phosphatidyl-choline (AAPC), to the *N*- position of phosphatidyl-ethanolamine (PE) (13-15). This process, which was known to underlie the biosynthesis of other *N*-acyl-PEs (7, 23), is catalyzed by a Ca^{2+}-dependent *trans*-acylase activity (14, 15), and leads also to the formation of *sn*-1-lyso-2-arachidonoyl-phosphatidyl-choline (lyso-APC). Also 2-AG has been shown to be biosynthesized and released by neuronal cells in a Ca^{2+}-dependent manner (16), and following the formation of *sn*-1-acyl-2-arachidonoyl-glycerols (16, 17). The latter metabolites may derive either from *de novo* synthesis, *via* phosphatidic acid, or through the stimulus-induced action of phospholipase C (PLC) enzymes on inositol- and choline-phosphoglycerides. An alternative pathway for 2-AG biosynthesis may be the hydrolysis of *sn*-1-lyso-2-arachidonoyl-phospholipids. Indeed, a PLC selective for *sn*-1-lyso-phosphatidylinositols, but capable of catalyzing, at a lower rate, also the hydrolysis of *sn*-1-lyso-phosphatidyl-choline and -serine, has been recently characterized in rat brain (18). Therefore, by simply looking at the biochemical pathways proposed so far for the two 'endocannabinoids', it is tempting to speculate that AAPC may serve as a common ultimate precursor for AnNH and 2-AG, and that a by-product of AnNH biosynthesis, i.e. lyso-APC, may also be utilized as a direct precursor for 2-AG biosynthesis. In order to test this hypothesis and, therefore, to throw the bases for future studies on the existence in neurons of common regulative mechanisms for the two 'endocannabinoids', we have undertaken the present study. We describe the presence, in homogenates of mouse $N_{18}TG_2$ neuroblastoma cells (a neuronal cell line extensively employed for studies of 'endocannabinoid' action, biosynthesis and degradation [12, 19, and references cited therein]), of enzymatic activities potentially responsible for the formation of both AnNH and 2-AG from AAPC.

MATERIALS AND METHODS

$N_{18}TG_2$ cells were purchased from DSM (Germany) and grown in DMEM containing 10% fetal calf serum at 37°C and 5% CO_2. Synthetic 2-AG was kindly donated by Prof. R. Mechoulam, The Hebrew University of Jerusalem. Synthetic [^{3}H]-2-AG was prepared from [^{3}H]-AA (230 mCi/mmol, NEN Dupont), glycerol and benzaldheyde following the procedure previously described ([5] and references cited therein). Synthetic [^{3}H]-N-ArPE (5 mCi/mmol) was prepared as described previously (13, 19), starting from [^{3}H]-AA and PE (Sigma). Synthetic *sn*-1-stearoyl-2-[^{3}H]-arachidonoyl-PC (35 Ci/mmol) was purchased from NEN Dupont. Synthetic AAPC radiolabelled on arachidonate on the *sn*-2 (5 mCi/mmol) or on both *sn*-1 and *sn*-2 positions (10 mCi/mmol) was prepared as described previously (15). Synthetic *sn*-1-arachidonoyl-2-[^{3}H]-arachidonoyl-glycerol (AAG, 5 mCi/mmol) was prepared by digestion of the corresponding PC-derivative with *C. perfringens* phospholipase C (PLC, Sigma, 10 units/ml) in 50 mM Tris-HCl, pH 7.4 + 1mM $CaCl_2$ for 1 hour at 37°C. Synthetic *sn*-1-lyso-2-[^{3}H]-arachidonoyl-PC was prepared by digesting *sn*-di-[^{3}H]-arachidonoyl-PC with 10 mg *Rhizopus delemar* lipase (Seikagaku Kogyo, Tokyo) for 1 h at 25°C in 1 ml of 50 mM acetate buffer pH 5.6 containing 4.5 mg bovine serum albumin, 0.1 M NaCl and 10 mM $CaCl_2$. In both cases, enzymatic digestion was followed by lipid extraction and purification by thin layer chromatography (TLC).

$N_{18}TG_2$ cell homogenates were prepared by homogenizing confluent cells in 20 mM Hepes buffer pH 7.4 containing 2 mM $CaCl_2$ and 2 mM dithiothreitol. The homogenates were centrifuged at 800 × *g* for 15 min at 4°C, and the supernatants (1 ml, 0.5-2.4 mg total proteins) were used for incubations, carried out at 37°C as described in the corresponding Legends to Figures. In each case, the incubations were carried out also with or without 2 mM EGTA or with homogenates boiled for 5 min, and were stopped by lowering the temperature to 4°C and by adding 2 ml of chloroform/methanol 2:1 (v/v). After sonication for 2 min at 4°C, the organic phase was dried down under reduced pressure, redissolved in chloroform and analyzed by TLC using silica gel-coated polyethylene TLC plates (Merck) developed with one of the following solvent systems: chloroform/methanol/CH_3COOH 85/15/1 (v/v) (solvent system A), which allows to separate N-ArPE (Rf=0.40) from AnNH (Rf=0.8) and AA+diacylglycerols+2-AG (Rf=0.95); the organic phase of a mixture of *iso*-octane/ethyl acetate/water/acetic acid 50:110:100:20 (v/v) (solvent system B), which allows to separate AnNH (Rf=0.6) from 2-AG (Rf=0.75) and AA+diacylglycerols (Rf=0.95); diethyl ether/petroleum ether/NH_4OH 50:50:1 (v/v) (solvent system C), which allows to separate AA (Rf=0), 2-AG (Rf=0.15), diacylglycerols (Rf=0.5) and triglycerides (Rf=0.9). Radioactivity incorporated into metabolites was measured first by a one-dimensional TLC radioscanner (Packard), and then, in order to achieve a more efficient measurement, by cutting TLC plates at 0.5 cm intervals and measuring β-emission in scintillation vials containing 1 ml methanol and 10 ml scintillation liquid (Ultima Gold, Packard). When produced in sufficient amounts, 2-AG-like fractions were

analyzed also by reversed phase high pressure liquid chromatography (HPLC), carried out as described previously (19), and by TLC on borate-treated silica gel-coated plates developed with chloroform/acetone 96:4 (v/v), in order to separate 1-AG (Rf=0.2) from 2-AG (Rf=0.35). Borate-impregnated plates were prepared by a quick immersion of silica gel-coated glass plates (Merck) in a 0.4 M boric acid solution, followed by heating at 110°C for two hours.

RESULTS AND DISCUSSION

Biosynthesis of [^3H]*-N-ArPE,* [^3H]*-AAG and* [^3H]*-2-AG from* [^3H]*-AAPC.* When incubated with PE and synthetic AAPC radiolabelled with [^{3}H]-AA on both *sn*-1 and *sn*-2 positions, $N_{18}TG_2$ cell homogenates were found to produce a radioactive lipid co-migrating with synthetic standards of N-ArPE in TLC analyses (Rf=0.40, developing solvent system A, Fig. 1A). This metabolite was not produced in measurable amounts from incubations carried out with boiled homogenates or in the presence of 2 mM EGTA (Fig. 1B), nor when AAPC radiolabelled only on the arachidonate on the *sn*-2 position or *sn*-1-stearoyl-2-[^{3}H]-arachidonoyl-PC were used as precursors (Fig. 1B). The chemical structure of the radioactive compound as that of N-ArPE was confirmed by digestion with *S. chromofuscus* phospholipase D, carried out as described previously (19), which yielded a radioactive peak co-migrating with synthetic standards of AnNH in TLC analyses (developing solvent system B, not shown). These data are in agreement with the presence in neurons of a Ca^{2+}-dependent *trans*-acylase activity catalyzing the transfer of arachidonate from the *sn*-1 position of AAPC to the *N*-position of PE, and reported in previous studies conducted in rat cortical neurons and brain (14, 15). However, we also observed, in the lipid extracts of incubates, the presence of another radioactive TLC peak (Fig. 1A) which was negligible when using boiled homogenates. When scraped off the TLC plates, and re-analyzed by TLC carried out using either solvent system B or C, this second component could be separated into three radioactive peaks respectively co-migrating with synthetic standards of AA, 1-stearoyl-2-arachidonoyl-glycerol and 2-AG (Fig. 1A, insets). These findings indicate the presence, in $N_{18}TG_2$ cell homogenates, of enzymatic activities capable of converting AAPC into AAG (which, under the chromatographic conditions used has the same Rf as 1-stearoyl-2-arachidonoyl-glycerol) and mono-arachidonoyl-glycerol. Since similar results were obtained when the homogenates were incubated with AAPC radiolabelled only on the *sn*-2 arachidonate (data not shown and Fig. 1C), it can be suggested that the radiolabelled monoarachidonoyl-glycerol produced during the incubation is the 2-isomer. Interestingly, the rate of conversion of either radiolabelled AAPC species into [^{3}H]-AAG was significantly inhibited by co-incubation with 2 mM EGTA (Fig. 1C).

Conversion of [^3H]*-N-ArPE into* [^3H]*-AnNH.* In agreement with previous data (19), incubation of $N_{18}TG_2$ cell homogenates with synthetic [^{3}H]-N-ArPE led to the formation of a radioactive metabolite co-eluting with synthetic standards of AnNH in TLC analyses carried out with two different developing systems (solvent systems A and B, data not shown). The conversion of [^{3}H]-N-ArPE into [^{3}H]-AnNH was not measurable in incubations carried out with boiled homogenates, and was not significantly affected by the presence of 2 mM EGTA in the incubation medium (Fig. 1D). The amount of [^{3}H]-AnNH formed during the incubation may have been underestimated due to the presence in the homogenates of a potent AnNH-hydrolyzing activity (12).

Biosynthesis of [^3H]*-2-AG from* [^3H]*-AAG.* Incubation of $N_{18}TG_2$ cell homogenates with synthetic [^{3}H]-AAG led to the formation of a radioactive component which was absent from incubates with boiled homogenates and exhibited the same Rf as synthetic 2-AG in TLC (developing solvent system B, Fig. 2A) and in reversed phase HPLC (not shown) analyses. The isomeric composition of this mono-arachidonoyl-glycerol was determined by TLC analyses carried out on borate-impregnated silica TLC plates, which revealed the presence of 29.1 % and 70.9 % of the 1- and 2-isomers, respectively. The presence of the former isomer is probably due, to some extent, to the occurrence of spontaneous acyl migration from the *sn*-2 to the less sterically hindered *sn*-1 position

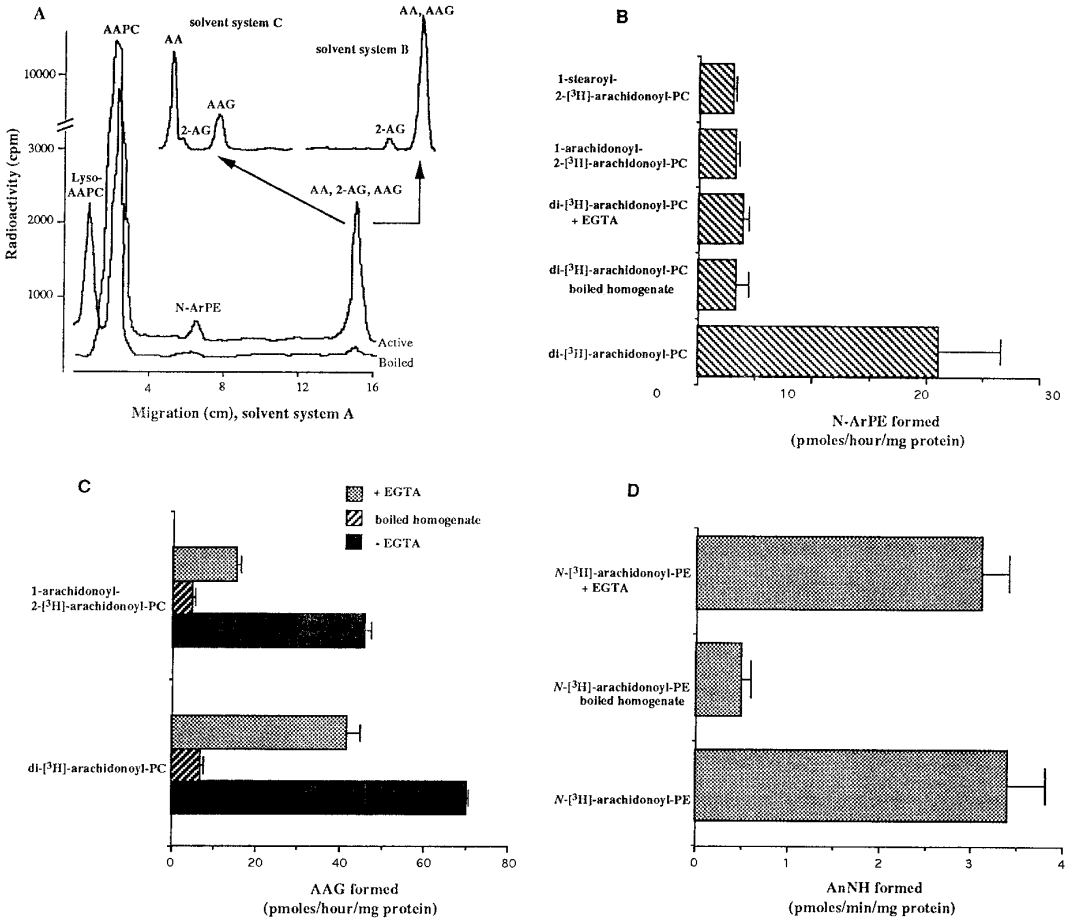

FIG. 1. (A) Conversion of di-[^{3}H]-arachidonoyl-phosphatidyl-choline (AAPC) into *N*-[^{3}H]-arachidonoyl-phosphatidyl-ethanolamine (N-ArPE), di-[^{3}H]-arachidonoyl-glycerol (AAG) and 2-[^{3}H]-arachidonoyl-glycerol (2-AG) in $N_{18}TG_2$ cell homogenates. Incubations were carried out with 200,000 cpm (18.2 nmol) [^{3}H]-AAPC and 250 nmol PE in 0.36 ml homogenate (0.8 mg proteins) in Hepes buffer for 2 hours at 37°C. Lipids extracted from the incubates were analyzed by TLC (solvent system A). The peak with Rf=0.95 was scraped off the plate and re-analyzed either with solvent system B or C (insets). The Rf values of synthetic AAPC, N-ArPE, arachidonic acid (AA), 2-AG, 1-stearoyl-2-arachidonoyl-glycerol (which under these conditions has the same chromatographic behavior as AAG) and lyso-arachidonoyl-PC (lyso-APC) are shown. The peak of lyso-APC is likely to come from the hydrolysis of AAPC and, to a smaller extent, from the transfer of arachidonate from AAPC to PE. (B) Rates of N-ArPE formation in $N_{18}TG_2$ cell homogenates under the conditions described in (A), and using also either different radiolabelled precursors (1-arachidonoyl-2-[^{3}H]-arachidonoyl-phosphatidylcholine [100,000 cpm, 18.2 nmol], 1-stearoyl-2-[^{3}H]-arachidonoyl-phosphatidyl-choline [1,000,000 cpm, 13 pmols]), boiled homogenates or homogenates containing 2 mM EGTA. (C) Rates of the conversion of synthetic [^{3}H]-AAPC into [^{3}H]-AAG in $N_{18}TG_2$ cell homogenates incubated and analyzed as described in (A). Experiments were conducted by using [^{3}H]-AAPC radiolabelled either on the *sn*-2 position only or on both *sn*-1 and *sn*-2 positions (see also [B]). EGTA (2 mM)-containing or boiled homogenates were also used. (D) Conversion of synthetic [^{3}H]-N-ArPE into [^{3}H]-anandamide (AnNH) in $N_{18}TG_2$ cell homogenates. Incubations were carried out with 100,000 cpm (18.2 nmol) [^{3}H]-N-ArPE in 1 ml homogenate (2.4 mg proteins) in Hepes buffer for 15 min at 37°C, and also with boiled homogenates or in the presence of 2mM EGTA. All data are mens ± S.E.M. of at least three separate experiments.

during the enzymatic reaction or the purification procedure. The rate of 2-AG formation from the hydrolysis of [^{3}H]-AAG was not significantly affected by the presence of 2 mM EGTA in the incubation medium (Fig. 2C), and may have been underestimated due to the presence in the

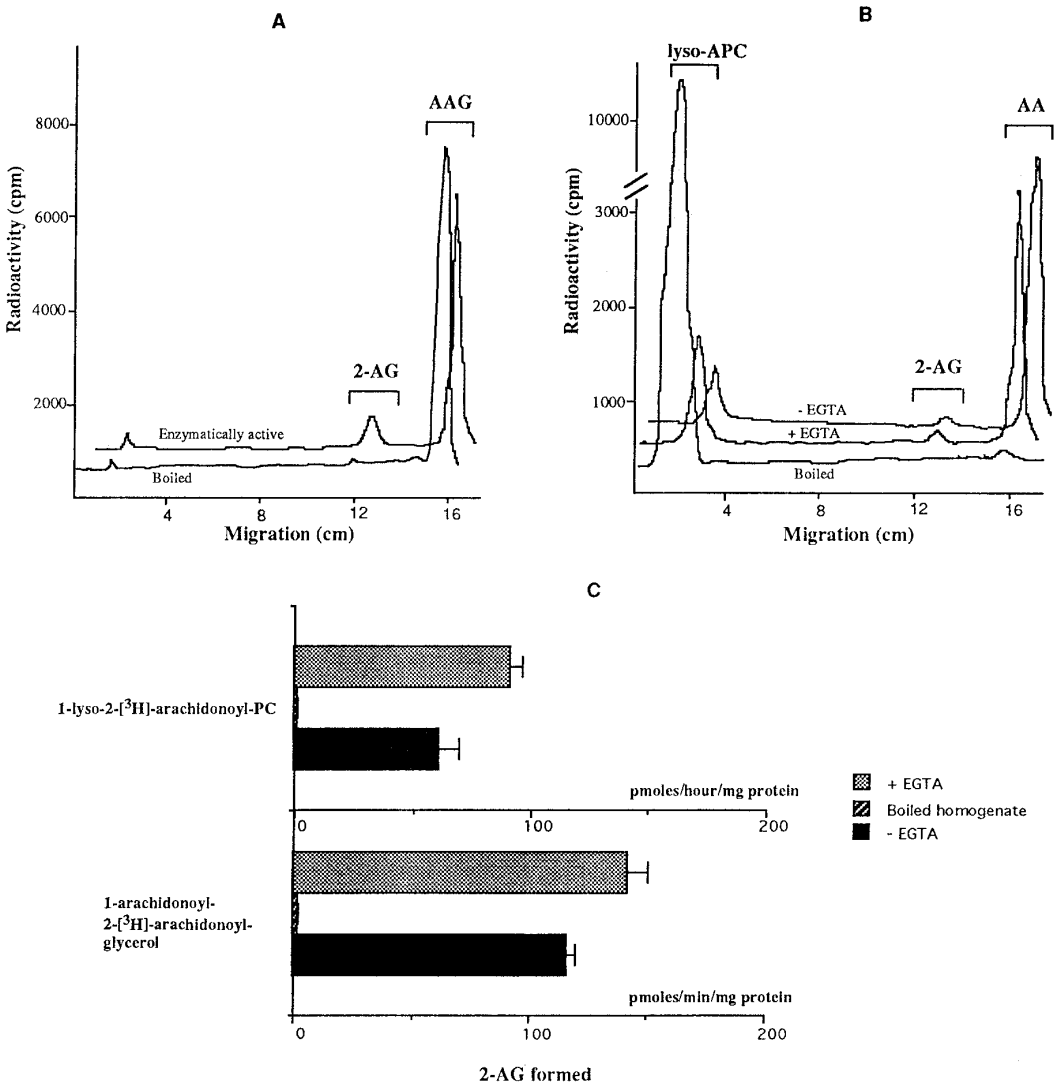

FIG. 2. (A) Conversion of di-[^{3}H]-arachidonoyl-glycerol (AAG) into 2-[^{3}H]-arachidonoyl-glycerol (2-AG) in $N_{18}TG_2$ cell homogenates. Homogenates (1 ml, 0.5 mg proteins) were incubated with 50,000 cpm (9.1 nmol) synthetic [^{3}H]-AAG for 30 min at 37°C. Incubations were also carried out with boiled homogenates (lower trace), or in the presence of 2 mM EGTA (not shown). Lipids extracted from the incubates were analyzed by TLC (solvent system B). The Rf values of synthetic 2-AG and 1-stearoyl-2-arachidonoyl-glycerol (AAG) are shown. (B) Conversion of 1-lyso-2-[^{3}H]-arachidonoyl-PC (lyso-APC) into 2-[^{3}H]-AG in $N_{18}TG_2$ cell homogenates. Homogenates (0.5 ml, 2 mg proteins) were incubated with 100,000 cpm (18.2 nmol) synthetic [^{3}H]-lyso-APC for 30 min at 37°C. Incubations were also carried out with boiled homogenates (lower trace), or in the presence of 2 mM EGTA (middle trace). Lipids extracted from the incubates were analyzed by TLC (solvent system B). The Rf values of synthetic 2-AG, lyso-APC and arachidonic acid (AA) are shown. (C) Rates of 2-[^{3}H]-AG formation from either synthetic [^{3}H]-lyso-APC (upper bars) or [^{3}H]-AAG (lower bars) in incubations with $N_{18}TG_2$ cell homogenates carried out as described in (B) and (A). Data are means ± S.E.M. of three separate experiments. In (A) and (B) chromatograms are not alligned for the sake of clarity and are representative of three separate experiments.

homogenates of 2-AG-hydrolyzing activity(ies) (16). No significant conversion of 2-AG into AAG was observed when cell homogenates were incubated with synthetic [^{3}H]-2-AG under conditions similar to those described here (data not shown).

Biosynthesis of [^{3}H]-2-AG from [^{3}H]-lysoAPC. Incubation of $N_{18}TG_2$ cell homogenates with synthetic [^{3}H]-lysoAPC led to the formation of two radioactive components which were absent from incubates with boiled homogenates and exhibited the same Rf as synthetic 2-AG and AA in TLC analyses (developing solvent system B, Fig. 2B). Interestingly, the rate of 2-AG formation from the hydrolysis of [^{3}H]-lysoAPC was significantly augmented by the presence of 2 mM EGTA in the incubation medium (Fig. 2C, 148.0±8.0 % of the rate without EGTA, n=3). This was very probably due to inhibition of a Ca^{2+}-dependent (lyso)-phospholipase A-like activity, whose presence in $N_{18}TG_2$ homogenates would explain the formation of high levels of [^{3}H]-AA (Fig. 2A), and would minimize the formation of [^{3}H]-2-AG from [^{3}H]-lysoAPC by subtracting AA from the *sn*-2 position of the lyso-phosphoglyceride. Accordingly, co-incubation with EGTA reduced the amount of [^{3}H]-AA produced during the incubation (Fig. 2B, 57.5±6.6 % of the amounts without EGTA, n=3). Moreover, also in this case the levels of [^{3}H]-2-AG may have been underestimated due to the presence in the homogenates of 2-AG-hydrolyzing activit(ies) (16). No significant conversion of 2-AG into lysoAPC was observed when cell homogenates were incubated with synthetic [^{3}H]-2-AG under conditions similar to those described here (data not shown).

CONCLUSIONS

In the present paper evidence has been described for the presence, in mouse $N_{18}TG_2$ neuroblastoma cells (a neuronal model widely employed for studies on the endogenous 'cannabinergic system') of enzymatic activities potentially responsible for AnNH and 2-AG biosynthesis from a common precursor, AAPC, a phospholipid species known to be a component of rat brain (15) and testis (21) membranes. The formation of AnNH has been suggested here to occur through the Ca^{2+}-dependent transfer of a *sn*-1 arachidonate from AAPC to PE, followed by the phospholipase D-mediated hydrolysis of the N-ArPE thereby produced, thus extending to mouse nerve cells a finding obtained previously in rat cortical neurons (13, 14), brain (14, 15) and testis (21). This pathway is currently regarded as the most likely candidate route to the physiological formation of AnNH, since: a) in mouse as well as rat brain, N-ArPE cannot be obtained from direct condensation of PE with AA-CoA (personal communication by Prof. K. Chapman, University of North Texas, Denton, and ref. 15); b) direct condensation of arachidonic acid and ethanolamine to AnNH in $N_{18}TG_2$ cell and brain homogenates only occurs with non-physiological concentrations of the two compounds (15, 19, for review [8]). In agreement with what observed in rat brain (15) and testis (21), the rate of N-ArPE formation in $N_{18}TG_2$ cells appeared to be much lower than that of N-ArPE conversion into AnNH.

2-AG has been recently suggested to be produced by ionomycin-stimulated $N_{18}TG_2$ cells mostly through a diacylglycerol-mediated, as well as PLC-independent, pathway (16). From the findings described here, however, it is possible to hypothesize for 2-AG biosynthesis two additional potential pathways starting with AAPC (Fig. 3). The first pathway would occur concomitantly to AnNH biosynthesis by exploiting the *trans*-acylase-catalyzed formation of lyso-APC (and N-ArPE) and the PLC-catalyzed hydrolysis of lyso-APC. The second AAPC-dependent pathway for 2-AG biosynthesis would start with the activation of an EGTA-sensitive PLC, and might compete with AnNH biosynthesis by subtracting AAPC from the *trans*-acylase-catalyzed reaction described above, unless the existence of distinct subcellular AAPC pools for each of the two pathways is assumed. It must be pointed out, however, that, until further data on the substrate specificities of the enzymatic activities described here are available, both these pathways may, in principle, yield 2-AG also independently from AnNH formation, and that the amounts of AAPC may not be so crucial to 2-AG as to AnNH biosynthesis. The lyso-APC hydrolyzing-activity described here may utilize also *sn*-2-arachidonate-containing 1-lysophosphoglycerides derived from the activation of phospholipase A_1 enzymes (18). Also the AAPC-hydrolyzing activity reported here may recognize phospholipid species different

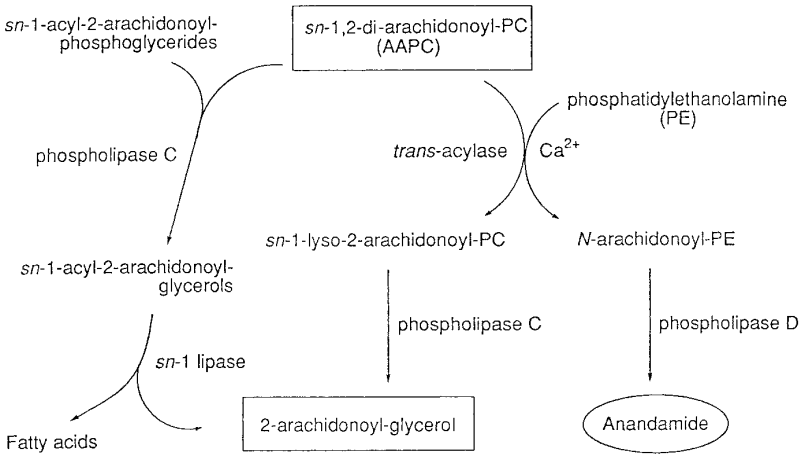

FIG. 3. Possible biosynthetic links between 2-arachidonoyl-glycerol and anandamide. Based on results presented here and previously (13-15).

from AAPC and containing a *sn*-2 arachidonate, thus leading to the formation of *sn*-1-acyl-2-arachidonoyl-glycerol species which may be potential substrates for the *sn*-1 diacylglycerol lipase-like activity described here. In both cases, non AAPC-derived precursors for 2-AG would be available. On the other hand, lyso-APC may also originate from the *trans*-acylase-catalyzed transfer of fatty acid residues other than arachidonate from the *sn*-1 position of 1-acyl-2-arachidonoyl-PC to the *N*-position of PE. This process leads to the formation of *N*-acyl-PEs, precursors of bioactive acyl-ethanolamides (7, 23), one of which, e.g. palmitoyl-ethanolamide, has been recently proposed to act as a physiological ligand of peripheral CB2 receptors and to play a protective role against glutamate-induced excitotoxicity in neurons (22 and references cited therein). Therefore, 2-AG may be also produced concomitantly to cannabimimetic acyl-ethanolamides different from AnNH.

In summary, the data reported here allow to hypothesize that stimuli which elicit in neurons the *de novo* biosynthesis of N-ArPE (or other N-acyl-PEs) and AnNH (or other acyl-ethanolamides) (see ref. [14]) may, through the action of the lyso-APC-hydrolyzing activity reported here, also evoke 2-AG biosynthesis. Conversely, the formation of 2-AG may occur completely independently from (16) or, partly, simultaneously to or in competition with (Fig. 3) AnNH biosynthesis, depending on whether neurons are stimulated with agents coupled only to Ca^{2+} influx (16) or also to the activation of one or more PLC enzymes (17) with more or less stringent substrate selectivities. Experiments in intact cells under physiological conditions will have to be performed in order to determine to what extent each of the enzymatic reactions described here contributes to basal or physiologically-stimulated levels of 2-AG, and to assess how the biosynthesis of this cannabimimetic glyceride is regulated in relation to AnNH formation. Moreover, a further characterization of the novel enzymatic activities described here (catalyzing, respectively, the conversion of AAPC into AAG, of lyso-APC into 2-AG, and of AAG into 2-AG) will have to be carried out, particularly in order to obtain their accurate classification and, more importantly, to clarify their substrate specificities, cofactor requirements and regulation. However, the finding of a potential common precursor, as well as of a possible common biosynthetic pathway, for AnNH and 2-AG opens now the way to studies aimed at investigating the possibility of joint regulatory mechanisms for the two putative 'endocannabinoids'. Interestingly, the rate, *in vitro*, of the enzymatic reactions leading to the formation of the AAPC-derived putative precursors of AnNH and 2-AG, i.e. N-ArPE, lyso-

APC and AAG, appeared to be significantly lower than the rate of their hydrolysis to the corresponding 'endocannabinoids' (Fig. 1 and 2), possibly suggesting that the former reactions may be targets for such regulatory mechanisms. Also the levels of AAPC, present as a minor membrane component of both unstimulated rat brain (15) and testis (20, 21) (respectively, 0.3% and 3.4% of total phosphatidylcholine [15, 21]), may be subject to up-regulation, for example through a putative Ca^{2+}- and *trans*-acylase-dependent *O*-arachidonoylation of lyso-APC (23).

In conclusion, the present paper has provided the first report of potential biosynthetic connections between AnNH and 2-AG, two putative physiological ligands of central cannabinoid receptors. Future investigations in this new direction may lead to the suggestion that what previously found for endorphins, which often share polypeptidic precursors and regulatory mechanisms, may in some way also apply to 'endocannabinoids'.

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