Biosynthesis of anandamide and related acylethanolamides in mouse J774 macrophages and N_{18} neuroblastoma cells

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Anandamide (arachidonoylethanolamide, AnNH) has been recently proposed as the endogenous ligand at the brain cannabinoid receptor CB1. Two alternative pathways have been suggested for the biosynthesis of this putative mediator in the central nervous system. Here we present data (1) substantiating further the mechanism by which AnNH is produced by phospholipase D (PLD)-catalysed hydrolysis of *N*-arachidonoylphosphatidylethanolamine in mouse neuroblastoma $N_{18}TG_2$ cells, and (2) suggesting for the first time that AnNH is biosynthesized via the same mechanism in a non-neuronal cell line, mouse J774 macrophages, together with other acylethanolamides and is possibly involved in the control of the immune/inflammatory response. Lipids from both neuroblastoma cells and J774 macrophages were shown to contain a family of *N*-acylphosphatidylethanolamines (N-aPEs), including the possible precursor of AnNH, *N*-arachidonoyl-PE. Treatment with exogenous PLD, but not with exogenous phospholipase A_2 and ethanolamine, resulted in the production of a series of acylethanolamides (AEs), including AnNH, from both cell types. The formation of AEs was accompanied by a decrease in the levels of the corresponding N–aPEs. Enzymically active homogenates from either neuroblastoma cells or J774 macrophages were shown to convert synthetic *N*-[^{3}H]arachidonoyl-PE into [^{3}H]AnNH, thus suggesting that in both cells an enzyme is present which is capable of catalysing the hydrolysis of N–aPE(s) to the corresponding AE(s). Finally, as previously shown in central neurons, on stimulation with ionomycin, J774 macrophages also produced a mixture of AEs including AnNH and palmitoylethanolamide, which has been proposed as the preferential endogenous ligand at the peripheral cannabinoid receptor CB2 and, consequently, as a possible down-modulator of mast cells. On the basis of this as well as previous findings it is now possible to hypothesize for AnNH and palmitoylethanolamide, co-synthesized by macrophages, a role as peripheral mediators with multiple actions on blood cell function.

INTRODUCTION

After the discovery of prostaglandins in the 1960s, leukotrienes in the late 1970s and lipoxins in the 1980s (for a review see ref. [1]), research on the large family of arachidonic acid metabolites, the eicosanoids, has recently experienced a new surge of enthusiasm with the finding that the long looked for endogenous agonist at the brain cannabinoid receptor (CB1) might be a lipid derived by non-oxidative metabolism of arachidonic acid. The ethanolamide of arachidonic acid [named anandamide (AnNH) from the sanskrit word for 'bliss'] was first isolated from porcine brain by Devane et al. in 1992 [2], and subsequently shown ([2,3] and, for a review, [4]) to mimick several of the pharmacological properties exerted by the major active principle of cannabis, Δ^9-tetrahydrocannabinol (THC), both *in vivo* and *in vitro*. More recent studies (reviewed in refs. [5,6]) have conclusively established the cannabimimetic properties of AnNH, and have led to: (1) the finding, in mammalian brain, of two other polyunsaturated fatty acid ethanolamides with THC-like properties, i.e. di-homo-γ-linolenoyl (20:3, $n-6$)- and adrenoyl (22:4, $n-6$)-ethanolamides [7]; (2) the characterization of the possible metabolic pathways responsible for AnNH biosynthesis and inactivation in the central nervous system [8,10]; and (3) the suggestion that another acylethanolamide (AE), i.e. palmitoylethanolamide, can act as a preferential agonist at the peripheral cannabinoid receptor (CB2) [11]. In particular, two alternative routes have been suggested to lead to AnNH synthesis in nervous cells: (a) the ATP-independent condensation of arachidonic acid with ethanolamine, which has been shown to occur in rabbit and rat brain membrane preparations, and to be catalysed by an 'AnNH synthase' requiring 27–130 mM concentrations of ethanolamine [8,9]; (b) the phosphodiesterase-catalysed hydrolysis of a membrane precursor, *N*-arachidonoylphosphatidylethanolamine (N-ArPE), which underlies the production and release of AnNH from intact rat central neurons stimulated with membrane-depolarizing agents [10] (for a critical discussion of these two mechanisms see also ref. [12]). The former mechanism, because of the high ethanolamine requirements of 'AnNH synthase' and the very low intracellular concentration of arachidonic acid, would need the prior as well as simultaneous activation of both phospholipases D (PLD) and A_2 (PLA_2) in order to occur. In order to lead to AnNH production, the second mechanism, in contrast, does not require PLA_2 activation (nor the presence of high amounts of arachidonic acid on the *sn*-2 position of phosphoglycerides), but requires instead the existence of an *N*-arachidonoyl acyl chain on phosphatidylethanolamine (PE) and a PLD-like enzyme capable of recognizing as a substrate this 'remodelled' form of PE. Indeed, an enzyme activity very similar to (if not identical with) this PLD was reported in the 1980s to catalyse, in dog brain, the hydrolysis of *N*-acylphosphatidylethanolamines (N-aPEs) to saturated and monounsaturated AEs (for a review see ref. [13]). In fact, the 'precursor' mechanism

Abbreviations used: AnNH, anandamide, N-ArPE, *N*-arachidonoylphosphatidylethanolamine; N-aPE, *N*-acylphosphatidylethanolamine; AE, acylethanolamide; PE, phosphatidylethanolamine; PLD, phospholipase D; PLA_2, phospholipase A_2; ATFMK, arachidonoyltrifluoromethylketone; THC, Δ^9-tetrahydrocannabinol; DMEM, Dulbecco's modified Eagle's medium.

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might be used, in principle, by central and peripheral cells also for the production, from the corresponding N-aPEs, of other bioactive AEs, e.g. the two other polyunsaturated cannabimimetic AEs found in porcine brain [7], the putative ligand of the peripheral CB2 receptor, palmitoylethanolamide [11] and oleoyl- and linolenoyl-ethanolamides, which have been shown to share with AnNH the capability of inhibiting astroglial-gap-junction-mediated Ca^{2+} fluxes, albeit at high concentrations [14]. Conversely, because of the high selectivity for arachidonic acid exhibited by 'AnNH synthase' [8,9], the 'condensation' mechanism cannot account for the production of these AEs, unless the existence of other 'fatty acid ethanolamide synthases' is assumed. Together with the above considerations, two other observations seem now to favour the 'phospholipid precursor' mechanism for AnNH biosynthesis in the central nervous system: (a) the lack of any evidence supporting the occurrence of the 'condensation mechanism' in intact cells, and (b) the recent finding [15] that the 'AnNH synthase' activity found in mammalian brain is likely to be the enzyme previously shown [10,16] to catalyse AnNH hydrolysis, i.e. 'AnNH amidohydrolase', working in reverse because of the high concentrations of ethanolamine used in the synthase assay.

In the present study we have addressed two open questions concerning AnNH biosynthesis in mammalian cells. First, we have investigated the mechanism by which AnNH is biosynthesized in mouse neuroblastoma $N_{18}TG_2$ cells, a cell line that, because of the presence of constitutive CB1 receptors, has been extensively used in studies on AnNH action (for a review see ref. [4]), and degradation [17–19]. These cells have been shown to produce AnNH on stimulation with THC, and this effect was accompanied by arachidonate release and was counteracted by wortmannin at concentrations that inhibit both PLD and phospholipase C [20]. Therefore, AnNH biosynthesis in $N_{18}TG_2$ cells might be due to either of the two alternative biosynthetic routes described above. Secondly, and, to our judgement, more importantly, we have applied the same experimental strategy used here and previously to investigate neuronal AnNH release [10,21], to mouse J774 macrophages. We present evidence suggesting, for the first time in an immunocompetent blood cell, that J774 cells, like neurons, on stimulation with either ionomycin or exogenous PLD, release AnNH, palmitoylethanolamide and other AEs from a family of membrane phospholipid precursors.

MATERIALS AND METHODS

AnNH and other AEs were synthesized by using the diisopropylcarbodi-imide method described previously [21], starting from ethanolamine and the non-esterified fatty acids. N-ArPE and N-[^{3}H]ArPE (0.6 Ci/mmol) were also prepared by this method, but using 1,2-*sn*-dipalmitoylphosphatidylethanolamine (Sigma) and either unlabelled or [^{3}H]-labelled arachidonic acid [10].

$N_{18}TG_2$ and J774A.1 cells were purchased from DSM and cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10 % foetal bovine serum (Sigma) plus 1 % penicillin/streptomycin (Sigma) in 5 % CO_2 at 37 °C. $N_{18}TG_2$ cell culture medium also contained 60 μM 6-thioguanine (Sigma).

Cells were prelabelled overnight with either [^{14}C]ethanolamine (Amersham; 53 mCi/mmol; 0.2 μCi/ml of culture medium) or [^{3}H]arachidonic acid (NEN–DuPont de Nemours; 60 Ci/mmol; 0.1 μCi/ml of culture medium) and then rinsed twice with serum-free DMEM before stimulation. Confluent cells from at least five 100 mm Petri dishes (1.5×10^7 cells/dish) were stimulated for 20 min with either PLD from *Streptomyces chromofuscus* (Sigma; 10 units/ml in 3 ml of serum-free DMEM) or ionomycin (Sigma; 2–5 μM in 3 ml of serum-free DMEM). Cell viability after stimulation was checked with Trypan Blue and found to be higher than 95 %. Control incubations were conducted with the same number of confluent dishes treated with 3 ml of DMEM only. Arachidonoyltrifluoromethylketone (ATFMK; 5 μM; Cascade), an inhibitor of both 'AnNH amidohydrolase' and PLA_2 [18] was also introduced, in both control and stimulated cells, 10 min before stimulation in order to minimize AnNH degradation and to rule out the participation of endogenous PLA_2 in AnNH release. Incubations of $N_{18}TG_2$ cells were carried out also with 10 mM ethanolamine plus porcine pancreatic PLA_2 (Sigma; 10 units/ml in 3 ml of serum-free DMEM). After the incubation, 3 ml of methanol was added to each dish and cells were transferred to Falcon tubes before the addition of 6 ml of chloroform. The mixtures were then sonicated for 2 min and the two phases allowed to separate by centrifugation at 800 ***g***.

The organic phases were then dried down under reduced pressure, redissolved in chloroform/methanol (9:1, v/v) (0.5 ml/dish) and loaded on to Pasteur pipettes containing 1 ml of silica gel (Merck) in chloroform/methanol (9:1, v/v). One silica mini-column was used for the prepurification of lipids from each dish. The silica was eluted with 2 ml of chloroform/methanol (9:1, v/v) first and then with 4 ml of chloroform/methanol (6:4, v/v), in order to elute AnNH-like material and N-aPE-like material respectively [21]. The two eluates were then dried down under a flow of nitrogen and purified separately by TLC on analytical (0.25 mm × 20 cm × 20 cm) silica-coated plastic plates (Merck). The 6:4 fraction was purified by using developing system A [chloroform/methanol/NH_3 (85:15:1 by vol)]. The band with R_F 0.35–0.42 corresponding to that of synthetic N-ArPE was scraped off the plate and eluted with chloroform/methanol (6:4, v/v). An aliquot of this component was analysed by TLC developed with solvent system B [chloroform/methanol/acetic acid (85:15:1, by vol.)], and the remainder was first analysed by ^{1}H-NMR spectroscopy, carried out in [^{2}H]chloroform/[^{2}H]methanol (95:5, v/v) on a 500 MHz Brucker apparatus, and subsequently digested with PLD from *S. chromofuscus* (10 units/ml) for 1 h at 37 °C in a two-phase mixture of ethyl ether and 50 mM Tris/HCl, pH 7.4. After the digestion, the ethereal phase was dried down and analysed by TLC first (developing system C, see below) and then by reverse-phase HPLC using a Spherisorb ODS-2 C_{18} column (5 μm; 4.5 mm × 25 cm) eluted with a methanol/water gradient as described previously [21]. The radioactivity present in each 1 ml HPLC fraction was measured by adding 4 ml of scintillation liquid (Ultima Gold; Packard) and counting β-emission by a liquid-scintillation counter (Packard). The 9:1 fraction from Silica-gel chromatography was purified by using developing system C [chloroform/methanol (95:5, v/v)]. The band with R_F 0.35 corresponding to synthetic AnNH was scraped off the plate, eluted with chloroform/methanol (8:2, v/v) and then analysed by reverse-phase HPLC as described above. In some cases, the HPLC peak at 24 min, corresponding to the retention time of both synthetic AnNH and linoleoylethanolamide, was analysed further by TLC using the organic phase of a mixture of isooctane/ethyl acetate/water/acetic acid (50:110:100:20, by vol.) (developing system D), which allows a better resolution between AnNH and some of the other AEs. In some experiments, samples from J774 cells containing AnNH-like material labelled with [^{3}H]arachidonic acid were also analysed by two-dimensional TLC. In this case, AnNH synthetic standard (10 μg) was spotted together with the sample, and the developing systems used were, for the first migration, system A, which ensures good separation between AnNH and arachidonic acid [21], and, for the second

migration, solvent system D. Spots and bands on TLC plates were visualized by brief exposure to iodine vapour. Radioactivity profiles of both one-dimensional and two-dimensional TLC analyses were determined either by using a one-dimensional radioactivity scanner (Packard), or by cutting the plates at 1 cm intervals and measuring the radioactivity bound to silica in plastic vials containing 1 ml of methanol and 10 ml of scintillation liquid.

The yields of AnNH and N-ArPE after the extraction and TLC purification procedures described above were calculated by using a known amount of the radiolabelled compounds and were respectively 62 and 45 % (see also ref. [21]). PLD digestion of N-[^{3}H]ArPE under the conditions described above had a 95 % yield. Finally, the yield of [^{14}C]AnNH after the reverse-phase HPLC step was 75 %. Therefore total yield of AnNH was 46.7 %, and total yield of N-ArPE, when quantified as AnNH produced by PLD digestion, was 32.1 %.

Experiments on the conversion of N-[^{3}H]ArPE (0.6 Ci/mmol) into [^{3}H]AnNH by cell homogenates were carried out as described previously [10]. Homogenates (about 1 mg of total protein/ml) were prepared in 50 mM Tris/HCl, pH 7.4, from 1×10^8–2×10^8 cells by using a Dounce homogenizer, and incubated at 37 °C for 10 min with 50000 c.p.m./ml N-[^{3}H]ArPE. No protease or amidohydrolase inhibitor was introduced into the incubation mixtures. After the incubation, 2 vol. of chloroform/methanol (2:1, v/v) was added and the lipids were extracted. The organic phase was dried down under reduced pressure and analysed by TLC using developing system D.

RESULTS

$N_{18}TG_2$ and J774 cells contain a family of N–aPEs

Prepurified lipid fractions from both $N_{18}TG_2$ and J774 cells prelabelled with [^{14}C]ethanolamine contain a radioactive component with the same chromatographic behaviour as synthetic standards of N-ArPE and N-aPEs in TLC analyses carried out using developing system A (Figure 1). In both cases, the levels of this component were significantly decreased by incubation of intact cells with exogenous PLD from *S. chromofuscus*, suggesting that the lipid can be a substrate for this phosphodiesterase (Figure 1). When the N-ArPE-like component was scraped off the TLC plate and analysed by TLC using developing system B, a radioactive peak co-chromatographing with synthetic N-ArPE at R_F 0.45 was again found (not shown). The metabolite was then subjected to digestion with *S. chromofuscus* PLD, thus yielding a mixture of lipids which all co-chromatographed with synthetic AnNH in TLC analyses, and were co-eluted with synthetic standards of AnNH and linolenoyl-, linoleoyl-, palmitoyl-, oleoyl- and stearoyl-ethanolamides in reverse-phase HPLC analyses (Figure 2). These data strongly suggest that both $N_{18}TG_2$ and J774 cells contain a family of phospholipids with chromatographic properties identical with those of N-aPEs and capable of releasing a corresponding family of AEs on treatment with PLD. This suggestion was confirmed by ^{1}H-NMR analysis [500 MHz; [^{2}H]chloroform/[^{2}H]methanol (95:5, v/v)] of the N-aPE-like component from neuroblastoma cells which revealed the presence of signals typical of N-aPEs, in particular those at $\delta = 5.05$ (1H, multiplet) assigned to the proton on the C *sn*-2 of the glycerol moiety, $\delta = 4.20$ and 3.97 (2H, double doublets) assigned to the protons on the C *sn*-1 of the glycerol moiety, $\delta = 3.72$ (2H, multiplet) assigned to the protons on the C *sn*-3 of the glycerol moiety, $\delta = 3.70$ and 3.25 (2H × 2, multiplets) assigned to the four methylene protons of the ethanolamine moiety, and $\delta = 2.02$ (2H, triplet) assigned to the methylene protons α to the amide bond.

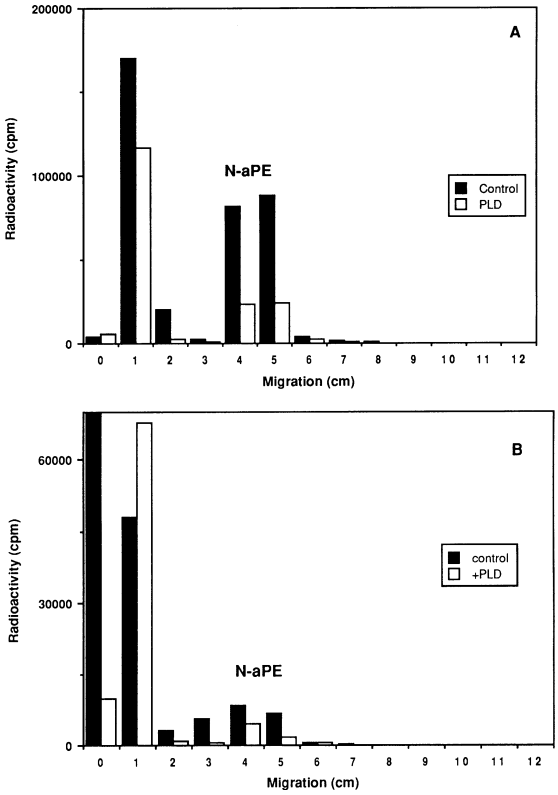

Figure 1 TLC radioactivity profiles of prepurified lipid extracts from $N_{18}TG_2$ mouse neuroblastoma cells (ten Petri dishes) (A) and J774 mouse macrophages (three Petri dishes) (B) prelabelled with [^{14}C]ethanolamine

Fractions eluted with chloroform/methanol (6:4, v/v) from silica gel mini-columns were analysed on analytical TLC plates developed with solvent system A. Under these conditions, synthetic standards of *N*-arachidonoyl-and *N*-acyl-phosphatidylethanolamines (N-aPE) migrated as a large spot with $0.35 < R_F < 0.42$. Extracts from either unstimulated cells (■) or cells stimulated for 20 min with exogenous PLD from *S. chromofuscus* (□) were analysed. These profiles are representative of three different experiments.

Since N-ArPE had not been found previously in non-nerve cells, we performed a series of experiments in order to gain further evidence for the presence of this compound in J774 cells. The latter were prelabelled with [^{3}H]arachidonic acid leading to 13.3 % incorporation of the labelled fatty acid into cells (mostly in the *sn*-2 position of phosphoglycerides as determined by digestion of purified phospholipids with porcine pancreatic PLA_2). Prepurified lipids were fractionated by TLC carried out using developing system A. Again, a radioactive component with the same R_F as synthetic N-ArPE was found (not shown). This metabolite was scraped off the TLC plate and digested with *S. chromofuscus* PLD. The lipids produced by the enzymic reaction were then analysed by two-dimensional TLC, thus using a technique which had previously proven suitable for identifying AnNH biosynthesis in $N_{18}TG_2$ cells [20]. As shown in Figure 6(A), along with a radioactive peak co-eluted with AnNH, a more hydrophobic and more abundant peak was also found. This component, the chemical structure of which is currently under study, may be another ethanolamide of a fatty acid biogenetically related to arachidonic acid [e.g. adrenic (C22:4) acid or eicosapentaenoic (C20:5) acid]. This result was confirmed by reverse-phase HPLC analysis of the sample from PLD digestion of the N-ArPE-like material, which again showed the

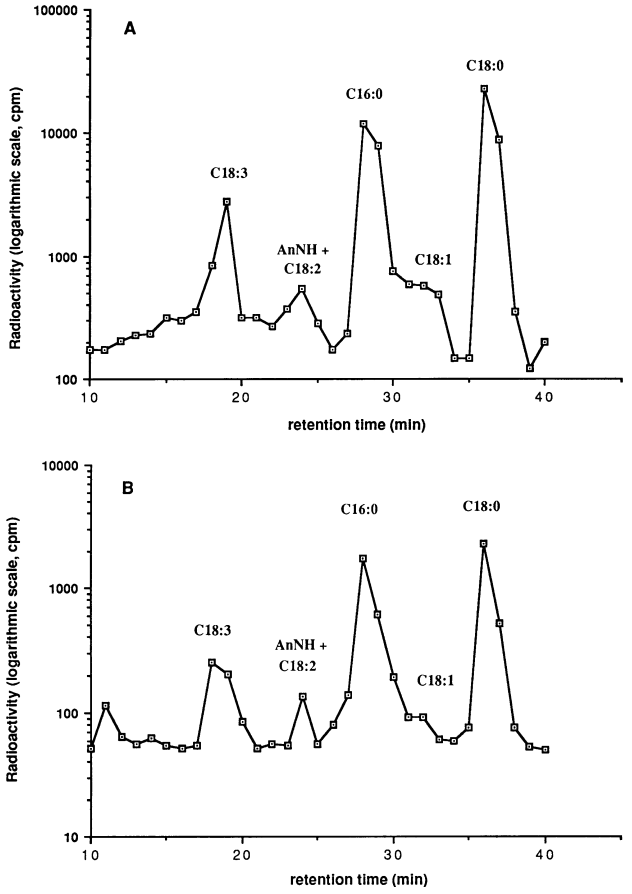

Figure 2 Reverse-phase HPLC radioactivity profiles of lipids obtained after PLD digestion of N-aPE-like material

The TLC peaks shown in Figure 1 corresponding to N-aPEs were scraped off the plates, eluted with chloroform/methanol (6:4, v/v), dried down and digested with PLD from *S. chromofuscus*. The digests were purified by TLC carried out with solvent system C, and radioactive peaks corresponding to AnNH standards scraped off the silica and analysed by HPLC. Samples were co-injected with synthetic standards of AnNH and γ-linolenoyl- (C18:3), linoleoyl- (C18:2), palmitoyl- (C16:0), oleoyl- (C18:1) and stearoyl- (C18:0) ethanolamides, the retention time of which are shown. These profiles are representative of three different experiments. (**A**) $N_{18}TG_2$ cells; (**B**) J774 cells.

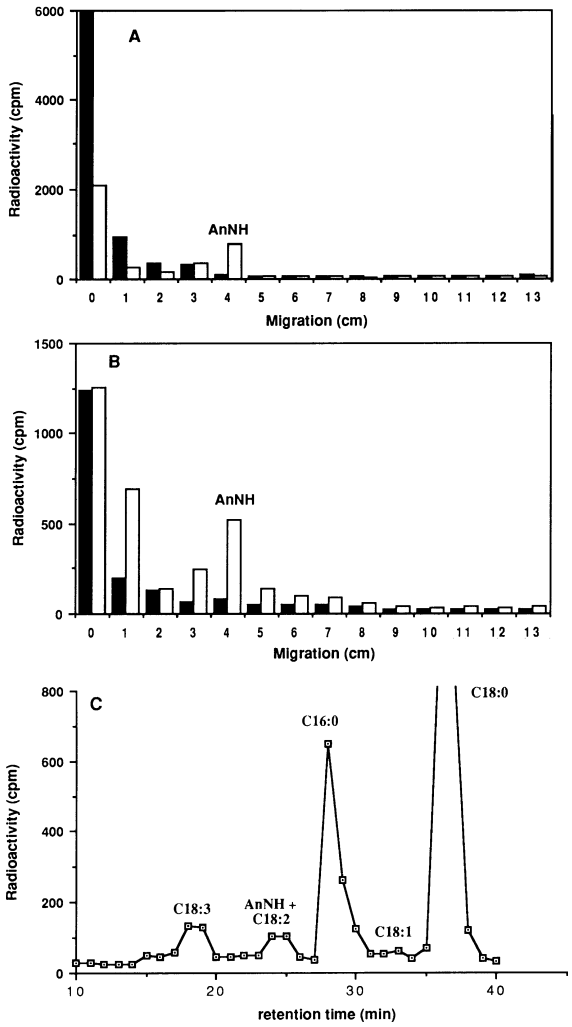

Figure 3 TLC radioactivity profiles of prepurified lipid extracts from $N_{18}TG_2$ mouse neuroblastoma cells prelabelled with [^{14}C]ethanolamine and stimulated with PLD from *S. chromofuscus* (A) or with either ethanolamine plus porcine pancreatic PLA_2 or *S. chromofuscus* PLD (B)

Fractions eluted with chloroform/methanol (95:5, v/v) from silica gel mini-columns were analysed on analytical TLC plates developed with solvent system C. Under these conditions synthetic AnNH migrated with R_F 0.35. In separate experiments, the effects of 20 min incubations with (**A**) either PLD (□) or no stimulant (■) and (**B**) either PLD (□) or 10 mM ethanolamine plus porcine pancreatic PLA_2 (■) were examined. The bands corresponding to AnNH were scraped off the plates, dried down and analysed by reverse-phase HPLC together with a mixture of synthetic AnNH and γ-linolenoyl-(C18:2), palmitoyl- (C16:0), oleoyl- (C18:1) and stearoyl- (C18:0) ethanolamides. A typical radioactivity profile of this analysis is shown in (**C**).

presence of a radioactive peak co-eluted with AnNH at 24 min and a more hydrophobic larger peak at 30 min (not shown).

Treatment of $N_{18}TG_2$ cells with exogenous PLD, but not PLA_2 plus ethanolamine, leads to formation of AnNH and AE

When $N_{18}TG_2$ cells prelabelled with [^{14}C]ethanolamine were treated with exogenous *S. chromofuscus* PLD, a significant amount of a radioactive component having the same R_F as synthetic AnNH and AEs on TLC was found in prepurified lipid fractions (Figure 3A). Treatment with ionomycin, at doses (2–5 μM) previously reported to induce AnNH release from rat central neurons [10], caused the formation of much smaller amounts ($130 \pm 9\%$ with respect to unstimulated cells, $n = 3$) of this radioactive peak (not shown). When cells were treated under the same conditions with exogenous porcine pancreatic PLA_2 plus 10 mM ethanolamine, this radioactive peak was no higher than that present in control cells (Figure 3B). Notably, PLA_2 treatment of $N_{18}TG_2$ cells prelabelled with [^{3}H]arachidonic acid resulted in the release of high levels ($320 \pm 15\%$ with respect to unstimulated cells, $n = 3$) of free [^{3}H]arachidonate (arachidonic acid is one of the most abundant fatty acids on the *sn*-2 position of N_{18} cell phosphoglycerides [22]), whereas PLD stimulation of these cells did not induce arachidonate release (not shown). When the radioactive AnNH-like component obtained by PLD stimulation was scraped off the TLC plate and analysed by reverse-phase HPLC, a series of radioactive peaks were co-eluted with synthetic standards of AnNH and of linolenoyl-, palmitoyl-, oleoyl- and stearoyl-ethanolamides (Figure 3C), thus demonstrating that in neuroblastoma $N_{18}TG_2$, as in rat central neurons, it is possible to detect, along with the previously reported biosynthesis of AnNH [20], the formation of other AEs.

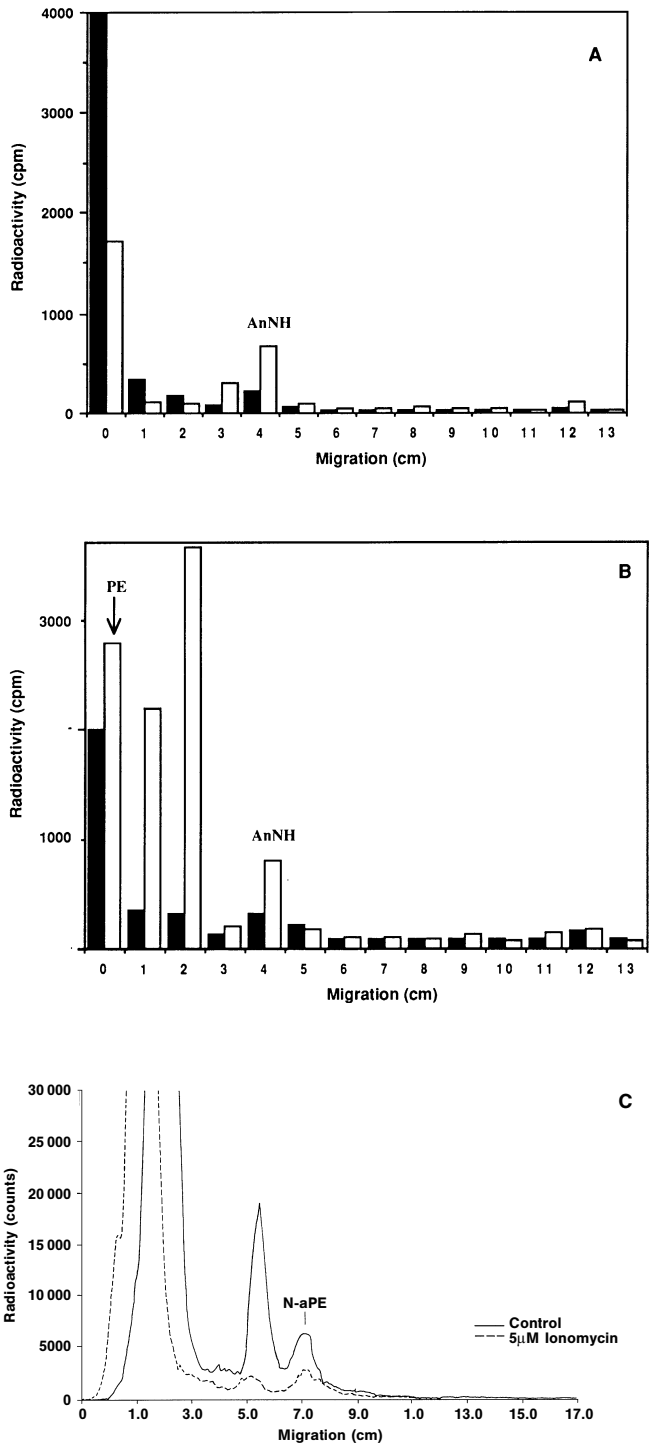

Figure 4 TLC radioactivity profiles of prepurified lipid extracts from J774 mouse macrophages prelabelled with [^{14}C]ethanolamine and stimulated with PLD from *S. chromofuscus* (A) or ionomycin (B)

Fractions eluted with chloroform/methanol (95:5, v/v) from silica-gel mini-columns were analysed on analytical TLC plates developed with solvent system C. Under these conditions synthetic AnNH migrated with R_F 0.35. In separate experiments, the effects of 20 min incubations with (**A**) either PLD (□) or no stimulant (■) and (**B**) either 5 μM ionomycin (□) or no stimulant (■) were examined. In experiments conducted with ionomycin, the fraction eluted with chloroform/methanol (6:4, v/v) from silica gel mini-columns was also analysed by TLC, carried out using solvent system A. The radioactivity profiles, obtained using a TLC radioactivity scanner, of a typical example of these latter analyses are shown in (**C**), where the migration of synthetic N-ArPE (or N-aPE) is also indicated.

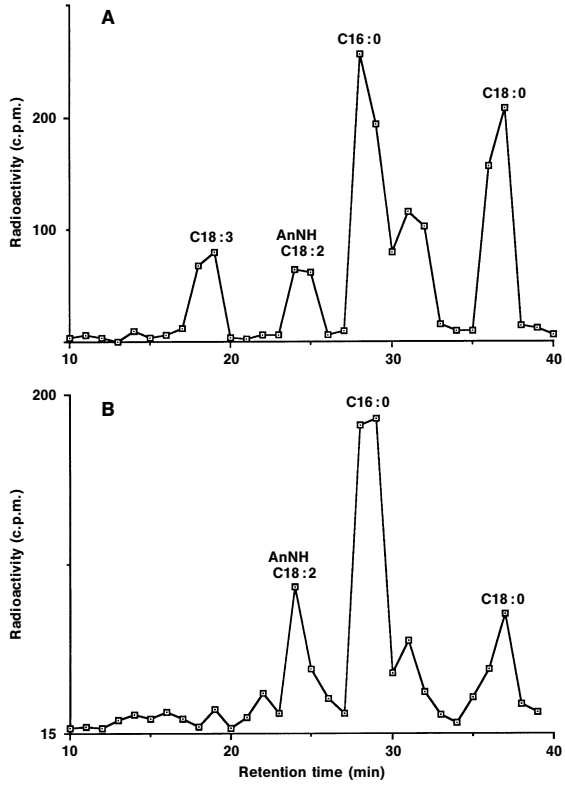

Figure 5 Reverse-phase HPLC radioactivity profiles of radioactive lipids with R_F value identical with that of synthetic AnNH from J774 macrophages stimulated for 20 min with either *S. chromofuscus* PLD (A) or ionomycin (B)

The TLC peaks shown in Figures 4(A) and 4(B) were scraped off the TLC plates, eluted with chloroform/methanol (8:2, v.v), dried down and co-injected on to the HPLC column together with synthetic standards of AnNH and of γ-linolenoyl- (C18:3), linoleoyl- (C18:2), palmitoyl- (C16:0), oleoyl- (C18:1) and stearoyl- (C18:0) ethanolamides, the retention times of which are shown. These profiles are representative of three different experiments. The contributions of radioactive AnNH and linoleoylethanolamide to the peak with a retention time of 24 min were determined by TLC analysis of this peak (carried out with solvent system D; not shown) and found to be respectively 54.3 and 45.7% in (**A**) and 62.1 and 37.9% in (**B**).

Treatment of J774 macrophages with either exogenous PLD or ionomycin leads to formation of AnNH and AEs

When J774 macrophages prelabelled with [^{14}C]ethanolamine were treated with exogenous PLD from *S. chromofuscus*, a significant amount of a radioactive component having the same R_F as synthetic AnNH on TLC was found in lipid extracts (Figure 4A). Treatment with 5 μM ionomycin of radiolabelled macrophages also resulted in the production of this radioactive peak (Figure 4B) as well as in the decrease in the levels of N-aPE-like metabolite(s) in the same lipid extract (Figure 4C). When the metabolite produced by either type of stimulation was scraped off the TLC plates and analysed by reverse-phase HPLC, a family of radioactive peaks was co-eluted with synthetic AEs in both cases (Figure 5). The chemical composition of these AE mixtures, however, depended on the type of stimulus used. AnNH and linolenoyl-, linoleoyl-, palmitoyl-, oleoyl- and stearoyl-ethanolamides were all produced by exogenous PLD whereas only AnNH and linoleoyl-, palmitoyl- and stearoyl-ethanolamides were found as the major AEs when ionomycin was used. Since the reverse-phase HPLC conditions used in this study do not allow separation of AnNH and linoleoylethanolamide [21], the relative contribution of these two metabolites to

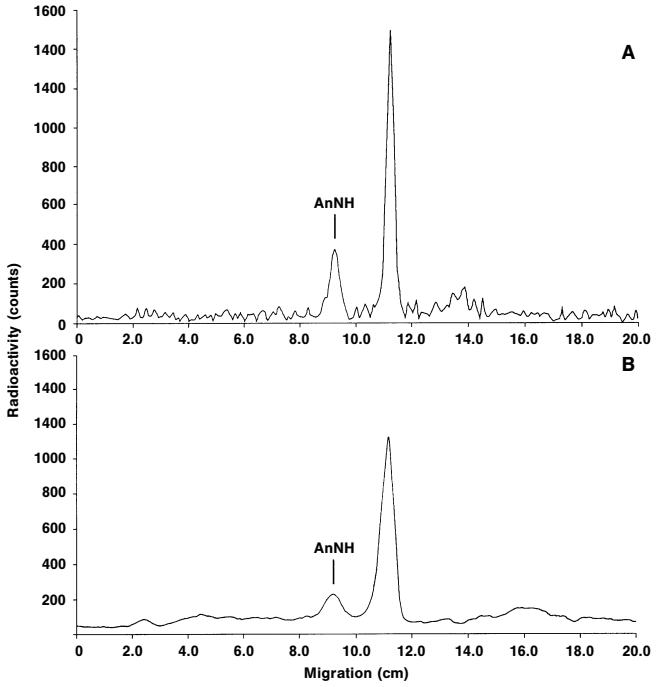

Figure 6 One-dimensional scan of two-dimensional TLC analyses of AnNH-like material from either (A) a PLD digest of the N-aPE fraction or (B) purified lipids, obtained from J774 macrophages prelabelled with [^{3}H]arachidonic acid

Prelabelled J774 macrophages were stimulated for 20 min with PLD from *S. chromofuscus* and subsequently extracted. AnNH obtained from either the chloroform/methanol (95:5, v/v) silica mini-column fraction (B) or PLD digestion of the prepurified N-aPE fraction (A) was analysed by two-dimensional TLC, using solvent system A for the first migration and solvent system D for the second migration, which was conducted at 90° with respect to the first migration. The radioactivity profile of the second migration of the lipids displaying the same R_F as AnNH in the first migration (i.e. R_F 0.8) was obtained with a one-dimensional radioactivity scanner, and is shown here.

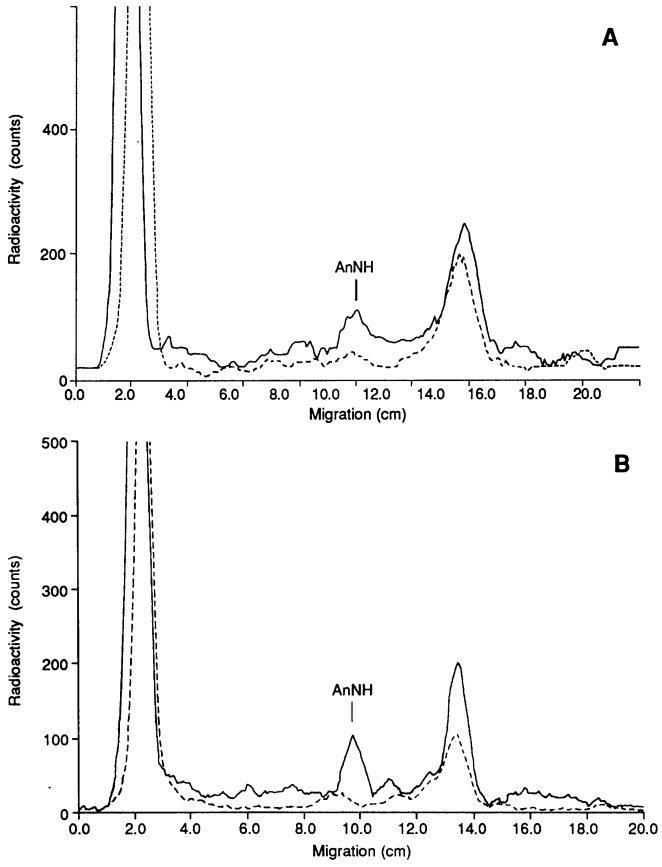

Figure 7 TLC radioactivity profiles of lipids from 10 min incubations of synthetic N-[^{3}H]ArPE with whole homogenates from either $N_{18}TG_2$ mouse neuroblastoma cells (A) or J774 mouse macrophages (B)

The homogenates were either enzymically active (——) or inactivated by heating at 100 °C for 5 min (– – – –). Lipids from the chloroform/methanol (2:1, v/v) extracts of the incubation mixtures were analysed by TLC, carried out using solvent system D. Under these conditions AnNH migrates with R_F 0.45–0.50. Radioactivity was measured with a one-dimensional radioactivity scanner. These profiles are representative of three different experiments.

the HPLC peak eluted after 24 min and shown in Figures 5(A) and 5(B) was determined by analysing this peak by TLC using developing system D, which allows good separation of AnNH and linoleoylethanolamide (R_F 0.45 and 0.40 respectively).

Since AnNH had never been found previously in macrophages, we wanted to gain further evidence for the chemical nature of the AnNH-like metabolite produced by J774 macrophages, by prelabelling the cells with [^{3}H]arachidonic acid (see above for details on the radioactivity incorporated into cells) and then analysing the lipids produced by stimulation with *S. chromofuscus* PLD by two-dimensional TLC (see above). As shown in Figure 6(B), along with a radioactive peak co-eluted with AnNH, a more hydrophobic and more abundant peak (the possible nature of which has been discussed above) was again found. This result was confirmed by reverse-phase HPLC analysis of an aliquot of the prepurified lipids from PLD stimulation (not shown).

Enzymically active homogenates from both $N_{18}TG_2$ and J774 cells convert N-[^{3}H]ArPE into [^{3}H]AnNH

When whole homogenates prepared from either $N_{18}TG_2$ or J774 cells were incubated for 10 min with synthetic N-[^{3}H]ArPE, the formation of a radioactive peak co-eluted with AnNH in TLC analyses carried out with developing system D was observed (Figure 7). This peak was absent from lipids extracted from incubates of N-[^{3}H]ArPE with homogenates that had been previously inactivated by heating at 100 °C for 5 min.

DISCUSSION

In this study we have presented evidence for the 'preformed phospholipid precursor' mechanism for AnNH biosynthesis in both $N_{18}TG_2$ mouse neuroblastoma cells and J774 mouse macrophages. First we have shown that both cell types contain a family of N-aPEs which might serve as substrates for a phosphodiesterase of the D type, thus being hydrolysed directly to the corresponding AEs. Both N-aPEs and palmitoyl- as well as stearoyl-ethanolamide had been previously found in N_{18} neuroblastoma cells in amounts that could be sensibly decreased by 5′-bromodeoxyuridine-induced differentiation [22]. However, neither N-ArPE nor AnNH nor any of the other AEs described in the present investigation were detected by Gulaya and co-workers [22]. Next, we have presented data showing that treatment of both cell types with an exogenous PLD leads to the formation of a family of AEs including AnNH. It is worth noting that the chemical composition of AEs produced on PLD stimulation of cells (Figures 3C and 5A) reflects the *N*-acyl composition of the N-aPE families of the two cell types (Figure 2A and 2B

respectively), with the arachidonoyl- and linoleoyl- chains as the less abundant and palmitoyl- and stearoyl-chains as the most abundant fatty acid chains in both AEs and N-aPEs (although it is possible that the observed levels of AnNH, either as the 'free' amide or released by PLD digestion of N-ArPE, may have been underestimated because of losses incurred during the purification procedure). This observation suggests that AnNH and AE production in the two cell types under study is dependent on the *N*-fatty acid composition of PE rather than on the *sn*-2-fatty acid composition of phosphoglycerides {where arachidonic acid is one of the most abundant esterified fatty acids (this study; [22])}, again in agreement with the 'precursor hypothesis'. Further support for this hypothesis comes from the finding that, in $N_{18}TG_2$ cells, treatment with PLA_2 plus ethanolamine does not elicit the formation of AnNH or any of the other AEs, thus suggesting that the biosynthesis of AnNH by these cells does not occur through the condensation of ethanolamine and arachidonic acid, a mechanism that would require the previous activation of PLA_2 and the presence of high amounts of ethanolamine. In other words, when cells were treated with millimolar concentrations of ethanolamine under conditions that produce the liberation of high amounts of free arachidonate, thus satisfying the requirements of 'AnNH synthase', no formation of AnNH was observed. Accordingly, when $N_{18}TG_2$ cell homogenates were incubated with ethanolamine and [^{3}H]arachidonic acid under conditions reported previously to lead to AnNH synthesis [8,9], no [^{3}H]AnNH was detected (not shown). Interestingly, $N_{18}TG_2$ neuroblastoma cells responded hardly at all to ionomycin stimulation, thus behaving differently from rat central neurons in primary cultures. This behaviour, which may be due to the fact that neuroblastoma cells are differentiating neurons, was previously also observed in neuroblastoma × glioma NG108 × 15 cells (V. Di Marzo and D. Piomelli, unpublished work).

On the basis of these findings, it is possible to provide a likely explanation for THC-induced formation of AnNH in $N_{18}TG_2$ neuroblastoma cells, previously reported by Burstein and Hunter [20], as the consequence of THC-induced activation of one or more PLD-like enzymes including the one responsible for N-ArPE and, more generally, N-aPE hydrolysis. It is also possible to interpret the inhibition of THC-induced AnNH formation by 1 μM wortmannin as an inhibition of this PLD. THC has been reported to induce PLD activation in rat peritoneal cells [23], and this activation was indeed counteracted by 1 μM wortmannin. Moreover, a Ca^{2+}-sensitive microsomal PLD, capable of catalysing the hydrolysis of N-aPEs with saturated and monounsaturated *N*-acyl chains, has been previously characterized from dog brain [24]. The presence of a similar enzyme also in mouse neuronal cells might account for our data on the conversion by $N_{18}TG_2$ cell homogenates of N-ArPE into AnNH (Figure 7A).

Once produced by stimuli causing PLD activation, AEs other than AnNH may also play a role as neuromodulators, by acting, if not at the CB1 cannabinoid receptor (which does not bind saturated or monounsaturated long-chain AEs), at other membrane or intracellular targets [25]. Palmitoyl- and stearoyl-ethanolamides have been shown to inhibit both basal and veratridine-induced Rb^+ efflux from N_{18} neuroblastoma cells by blocking fast Na^+ channels at concentrations ranging between 0.5 and 5 μM [26]. Linolenoyl- and oleoyl-ethanolamides have been recently reported to inhibit astroglial-gap-junction-mediated Ca^{2+} fluxes, although at higher concentrations and to a lower extent than AnNH [14]. Therefore a mechanism by which AnNH and long-chain AEs with multiple neuromodulatory roles are simultaneously produced by the same neuron on stimulation may turn out to be, if confirmed by further investigations, of extreme physiological importance.

Finally, we have shown, for the first time in a non-neuronal cell, that pseudo-physiological conditions leading to Ca^{2+} influx into the cell, such as those occurring during stimulation with ionomycin, evoke the formation of AnNH, palmitoyl-ethanolamide, linoleoyl-ethanolamide and stearoyl-ethanolamide in J774 macrophages, while causing a significant decrease in the levels of the corresponding N-aPEs. The involvement of cytosolic PLA_2 in this mechanism seems unlikely, since incubations of cells with ionomycin were conducted in the presence of ATFMK, a selective inhibitor of this enzyme. The chemical composition of AEs released on ionomycin stimulation was different from that obtained with exogenous PLD, which instead reflected the fatty acid composition of the *N*-acyl chains of the N-aPEs found in J774 cell lipid extracts. This finding is in agreement with analogous data reported previously for rat central neurons [10] and can be explained by assuming that Ca^{2+} influx into J774 macrophages stimulates a PLD that has a different substrate specificity from that of PLD from *S. chromofuscus*. Indeed, the Ca^{2+}-sensitive N-aPE-specific PLD activities, previously characterized from both nervous and peripheral tissues, were shown to have a low affinity for non-*N*-acylated phosphoglycerides (for a review see ref. [13]). It is also possible that different cells express different types of N-aPE-specific PLD enzymes with different specificities towards N-aPEs with different *N*-acyl chains, and this might explain why the percentage composition of ionomycin-evoked AEs in J774 macrophages differs also from that reported previously for ionomycin-stimulated rat central neurons [10,21]. Further studies aimed at characterizing and purifying N-aPE-specific PLDs from both peripheral tissues and central nervous system are now needed. Our finding of an enzyme that converts N-ArPE into AnNH in J774 cell homogenates (Figure 7B), apart from supporting the hypothesis of a preformed precursor for AnNH formation in J774 macrophages, might prompt the use of these cells as a possible source of N-aPE-specific PLDs.

The finding of a mechanism by which macrophage-like blood cells such as J774 cells, on Ca^{2+} influx, can release at once both AnNH and palmitoylethanolamide is of particular importance considering the several pharmacological actions that have been recently ascribed to these two lipids in immunocompetent/inflammatory blood cells (reviewed in ref. [6]). AnNH has been reported to inhibit lymphocyte proliferation and to induce lymphocyte apoptosis [27]. Palmitoylethanolamide has been shown to bind with higher affinity than AnNH to cannabinoid CB2 receptors in rat peritoneal mast cells and RBL-2H3 cells, thereby inhibiting 5-hydroxytryptamine release, whereas AnNH has been found to antagonize this effect [11]. Finally, AnNH, but not palmitoylethanolamide, can release arachidonic acid from J774 macrophages in a fashion that is sensitive to pertussis toxin and cAMP [28]. By presenting data that suggest, for the first time, that a blood cell can indeed synthesize AEs, we have provided physiological grounds for these pharmacological effects. AnNH and palmitoylethanolamide produced by Ca^{2+}-activated hydrolysis of membrane phospholipid precursors may either exit the cell and act on neighbouring lymphocytes and mast cells or act on the same cell, thus behaving as autocrine inflammatory mediators. Macrophages, which are already known to play a major role in the chemical communication between leucocytes, would thus exhibit yet another means of co-ordinating blood cell activity and functionality during inflammatory and immune responses.

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