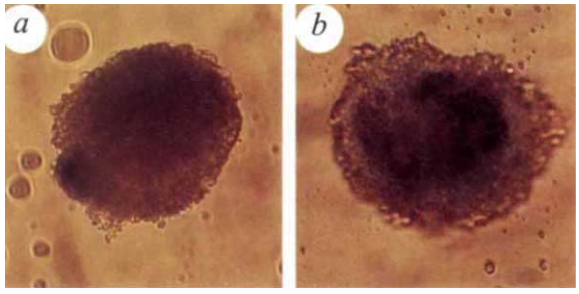
sulphate staining[3], in explants of isolated rhombomeres cultured in control medium or after treatment with BMP4 (Fig. 4). Although there are a few scattered dead cells in all cases when rhombomere 3 is treated with BMP4, a focus of intensely staining cells is evident (Fig. 4). We also used transmission electron microscopy to examine the morphology of the dying cells. We find that in explants of rhombomere 3 treated with BMP4 there is a localized region of dead or dying cells which have morphological features typical of apoptosis (Fig. 4*B*).

We have previously demonstrated that the apoptotic elimination of neural crest cells in rhombomeres 3 and 5 is effected by an interaction with the neighbouring even-numbered rhombomeres. The results presented here are consistent with that effect being elicited through the induction of high-level *Bmp4* expression in the neural crest primordium of rhombomeres 3 and 5. Thus *Bmp4* can be seen to be pivotal in this process, acting to stimulate *msx2* expression and the depletion of neural crest cells from rhombomeres 3 and 5 through the induction of apoptosis. These observations also strengthen the association between *msx2* expression and morphogenetic cell death in the rhombencephalon at these early stages of development. Thus, it is not the direct action of even-numbered rhombomeres upon rhombomeres 3 and 5 that effects the cell death programme, rather this is carried out by the induction of *Bmp4* in rhombomeres 3 and 5 themselves. We have also demonstrated that *Bmp4* will cause the depletion of neural crest from isolated r3 and r5 but not from r4. This suggests that there are intrinsic differences between rhombomeres 3 and 5 and rhombomere 4, possibly in the form of the deployment of as-yet undefined vertebrate BMP4 receptors. The control of cell death by BMP4 in this system also appears to involve an autocrine loop in which *Bmp4* expression depends upon BMP4 (ref. 7). This raises the possibility that cells expressing *Bmp4* are then eliminated by it in a manner analogous to autocrine survival function of brain-derived neurotrophic factor in dorsal root ganglion cells[10]. This work provides further evidence to support the idea that the sculpting of neural crest into discrete streams which will populate and pattern the branchial arches is achieved by a mechanism intrinsic to the neuroepithelium. □



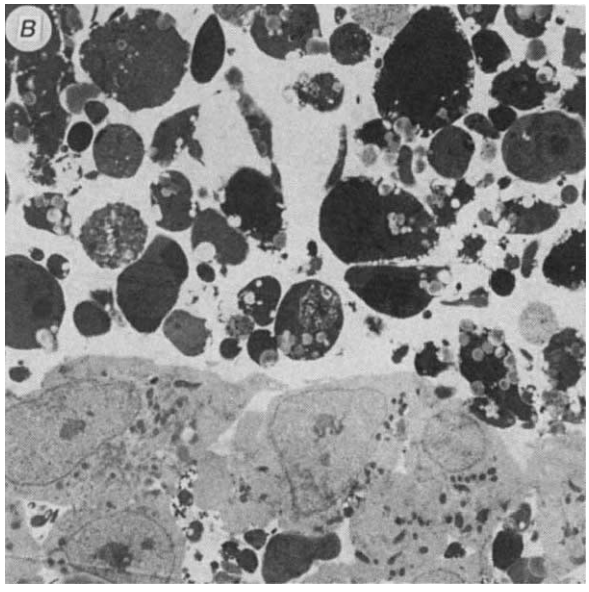


FIG. 4 BMP4 effects depletion of neural crest cells from r3 and r5 through stimulation of apoptosis. *A*, Assessment of cell death in explants using Nile blue sulphate staining. *a*, Rhombomere 3 cultured in F-12 stains weakly; and *b*, treatment of r3 with 10 ng ml^{-1} BMP4 induces programmed cell death, as indicated by strong localized Nile blue sulphate staining. Little staining is evident in r4 cultured in either F-12 or in BMP4 at 10 ng ml^{-1} (data not shown). *B*, Transmission electron micrograph of the foci of dead cells present in r3 after BMP4 treatment at 10 ng ml^{-1}, showing cells displaying features typical of apoptosis such as pyknotic nuclei with condensed chromatin.
METHODS. Rhombomeres were dissected out[3] and incubated for 6 h in either F-12 or F-12 with recombinant BMP4 at 10 ng ml^{-1}. Explants were incubated in 4-well dishes (Nunc) which were first coated with 0.33% agarose to prevent explants adhering. Explants were stained with Nile blue sulphate (1:133,000 dilution[3]) and incubated for 10 min at 37 °C, washed and photographed.

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Formation and inactivation of endogenous cannabinoid anandamide in central neurons

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ANANDAMIDE (*N*-arachidonoyl-ethanolamine) was recently identified as a brain arachidonate derivative that binds to and activates cannabinoid receptors[1–4], yet the mechanisms underlying formation, release and inactivation of this putative messenger molecule are still unclear. Here we report that anandamide is produced in and released from cultured brain neurons in a calcium ion-dependent manner when the neurons are stimulated with membrane-depolarizing agents. Anandamide formation occurs through phosphodiesterase-mediated cleavage of a novel phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine. A similar mechanism

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also governs the formation of a family of anandamide congeners, whose possible roles in neuronal signalling remain unknown. Our results and those of others[5,6] indicate therefore that multiple biochemical pathways may participate in anandamide formation in brain tissue. The life span of extracellular anandamide is limited by a rapid and selective process of cellular uptake, which is accompanied by hydrolytic degradation to ethanolamine and arachidonate. Our results thus strongly support the proposed role of anandamide as an endogenous neuronal messenger.

We studied anandamide formation in primary cultures of rat brain striatal or cortical neurons, labelled with [^{3}H]ethanolamine and stimulated with the Ca^{2+} ionophore, ionomycin, or with various membrane-depolarizing agents. Ionomycin stimulates formation of a radioactive component coeluting with anandamide on high-performance liquid chromatography (HPLC) (Fig. 1*a–c*). A similar stimulation is produced by high K^+, kainate (a glutamate-receptor agonist) and 4-aminopyridine (a K^+ channel blocker) (Fig. 1*c*). Chelating extracellular Ca^{2+} with EGTA prevents the effect of high K^+ suggesting that anandamide formation is Ca^{2+} dependent (Fig. 1*c*).

By analogy with neurotransmitters and various lipid mediators[7–9], anandamide is expected to have to exit neurons to activate cannabinoid receptors on neighbouring cells. We find, however, that only ~20% of the radioactive material in anandamide fractions is recovered in the medium after stimulation with ionomycin, whereas ~80% remains associated with cells (Fig. 1*d*). Because neurons and glia rapidly take up extracellular anandamide (see below), to demonstrate anandamide release we stimulated neurons with ionomycin in the presence of exogenous anandamide (which saturates the uptake system, see below) or bovine serum albumin (which traps hydrophobic compounds). Both treatments increase anandamide-associated radioactivity in the medium and decrease it within cells, suggesting that anandamide is released and subsequently recaptured by cells (Fig. 1*d*). The radioactive material coeluting with anandamide is further resolved by HPLC into several components, which coelute with the following *N*-acyl-ethanolamines (NAE): (1) *N*-γ-linolenoyl-; (2) *N*-arachidonoyl- (=anandamide) plus *N*-linoleoyl-; (3) *N*-palmitoyl-; (4) *N*-oleoyl-; and (5) *N*-stearoyl-ethanolamine (Fig. 1*e*). Additional HPLC analysis allowed us to resolve component 2 into two products coeluting with anandamide (~80%) and *N*-linoleoyl-ethanolamine (~20%). This identification was confirmed by gas chromatography/mass spectrometry (GC/MS). From the mass spectrum of synthetic anandamide (Fig. 1*f*), we chose characteristic ions for mass fragmentography. Analysis of stimulated neurons reveals that a product is eluted from the GC at the retention time of anandamide (~0.5 pmol per dish with ionomycin and ~2 pmol per dish with kainate). Fragmentation of this product yields ions characteristic of anandamide, at *m/z* 329, 244, 145 and 85 (Fig. 1*f*, $g_{(1)}$ and data not shown). GC elution and MS fragmentation confirm that stimulated neurons produce *N*-linoleoyl-, *N*-oleoyl-, *N*-stearoyl- and *N*-palmitoyl-ethanolamine (Fig. 1$g_{(2)-(5)}$). Ions characteristic of *N*-γ-linolenoyl-ethanolamine were not detectable and identification of this product must therefore be tentative.

To determine whether striatal or cortical astroglia contribute to anandamide formation, we stimulated neuron-free cultures of astrocytes[10] with ionomycin after [^{3}H]ethanolamine labelling. We found no radioactivity coeluting with anandamide, indicating that formation occurs in neurons (control, 82 ± 34 d.p.m. per dish; ionomycin, 89 ± 26 d.p.m. per dish, background, 43 ± 3 d.p.m. per dish, $n = 3$).

Previous studies on NAE suggest two possible mechanisms of anandamide formation: (1) energy-independent condensation of free arachidonate with ethanolamine[5,6,11–13], or (2) release by phospholipase D-mediated cleavage of the phospholipid precursor, *N*-arachidonoyl phosphatidylethanolamine (PE) (Fig. 2). This phospholipid has not yet been isolated from mammalian tissues, but the occurrence of related *N*-acyl PEs has been demonstrated[14–16].

Because levels of free ethanolamine and arachidonate in resting neurons are low[7,16], for condensation to occur multiple enzyme activities should be stimulated concomitantly: phospholipase A_2 (PLA_2) or phospholipase C (PLC) (to generate arachidonate) and phospholipase D (PLD) (to generate ethanolamine)[5,6]. We find that two selective PLA_2 inhibitors, dimethyleicosadienoic acid and octadecylbenzoacrylic acid, and the PLC inhibitor, neomycin, have no effect on ionomycin-stimulated anandamide formation. Moreover, incubation of intact neurons with exogenous PLD (*Streptomyces chromofuscus*, 40 units ml^{-1}, 10 min), but not PLC or PLA_2, is sufficient to produce anandamide and other NAE. Despite the large quantities of ethanolamine released by exogenous PLD, addition of arachidonate (60 μM) does not increase anandamide formation, indicating that condensation does not occur in the intact neurons (data not shown).

These results argue against a role for energy-independent condensation in anandamide formation and suggest that a phospholipid precursor of anandamide and other NAE exists in neurons. Because *N*-arachidonoyl PE and other *N*-acyl PE meet the structural criteria for such a precursor (Fig. 2), we purified *N*-acyl PE from cultured neurons, and identified it by thin-layer chromatography, HPLC, enzymatic digestion and proton nuclear magnetic resonance spectroscopy. When purified *N*-acyl PE is digested with *S. chromofuscus* PLD, the spectrum of NAE formed (determined by HPLC and GC/MS) is qualitatively similar to that produced by ionomycin and comprises anandamide (H.C., S. Gaillet, V.D.M. and D.P., manuscript in preparation). These results indicate that *N*-acyl PE is present in neurons and is composed of multiple molecular species which include *N*-arachidonoyl PE.

To obtain direct evidence that *N*-acyl PE serves as a precursor for anandamide and other NAE, we determined whether there is in neurons a phosphodiesterase activity that hydrolyses this phospholipid. Neuron homogenates convert purified [^{3}H]*N*-acyl PE or synthetic [^{3}H]*N*-arachidonoyl PE into anandamide and other NAE, and this reaction is inhibited by boiling (Fig. 3*a*). Although we have not characterized the phosphodiesterase activity involved in *N*-arachidonoyl PE hydrolysis, a PLD activity that hydrolyses *N*-acyl PE forming NAE has been described[17,18].

If *N*-acyl PE served as precursor for anandamide and other NAE, stimuli that enhance formation of these metabolites should also increase *N*-acyl PE turnover. To test this, we incubated neurons with [^{3}H]ethanolamine for 20 min, and measured [^{3}H]*N*-acyl PE. Ionomycin produces a 2.6-fold enhancement of [^{3}H]ethanolamine incorporation into *N*-acyl PE, without affecting [^{3}H]PE, indicating that *N*-acyl PE turnover is selectively enhanced (Fig. 3*b*, *c*).

Next, we determined whether mechanisms exist in neural cells for the inactivation of anandamide. Exogenous [^{3}H]anandamide is rapidly cleared from the media of neuronal cultures (Fig. 4*a*). This clearance is accompanied by association of [^{3}H]anandamide with cells, a process that fulfills several criteria of a carrier-mediated uptake. [^{3}H]Anandamide association with cells is: (1) rapid ($t_{1/2} = 2.5$ min) (Fig. 4*b*); (2) temperature-dependent (Fig. 4*b*); (3) saturable at 37 °C (Fig. 4*c*), and consequently reduced in a concentration-dependent manner by the addition of nonradioactive anandamide (Fig. 4*d*); (4) selective. [^{3}H] N-palmitoyl-ethanolamine is not recaptured (Fig. 4*a*) and other NAE do not compete for [^{3}H]anandamide uptake (Fig. 4*d*). Uptake of [^{3}H]ethanolamine-labelled anandamide is accompanied by formation of free [^{3}H]ethanolamine and, to a minor extent, [^{3}H]PE (Fig. 4*e*, *f*). Moreover, when [^{3}H]arachidonate-labelled anandamide is used, radioactivity is recovered in all phospholipid classes, but not in free arachidonate (Fig. 4*e*, *f*). These results suggest that an endogenous amidohydrolase activity[11,17] catalyses the cleavage of anandamide to ethanolamine and arachidonate, which are then incorporated into phospholipids (Fig. 2). Astrocytes in culture take up and degrade [^{3}H]anandamide to a degree similar to neurons,

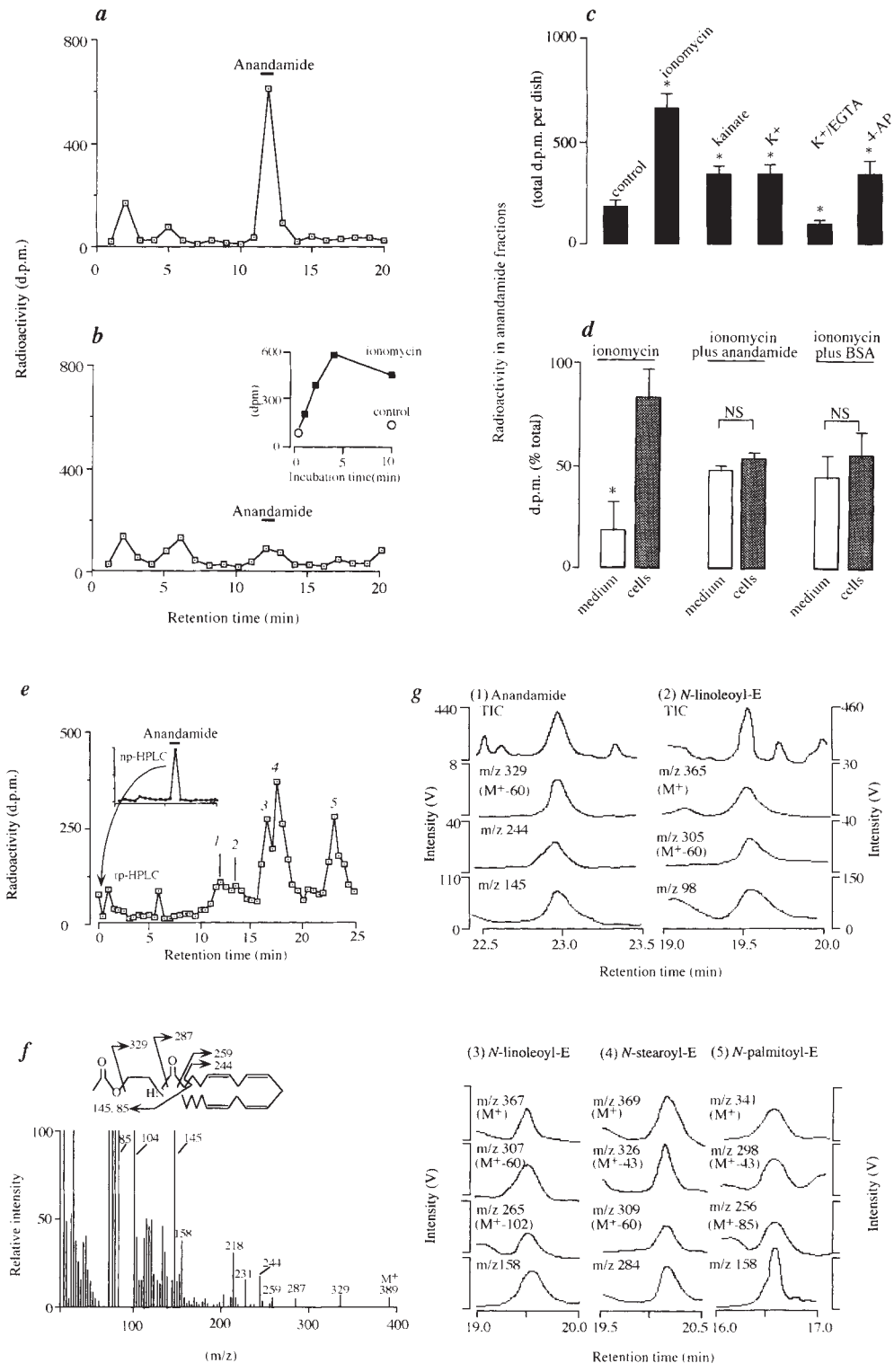


FIG. 1 Formation and release of anandamide from neurons in primary culture. *a–c*, In rat striatal neurons labelled by incubation with [^{3}H]ethanolamine, ionomycin and depolarizing stimuli evoke formation of radioactive material eluting at the retention time of anandamide. Representative normal-phase (np)HPLC fractionation of samples from the 10-min incubation of neurons (one dish) *a*, with ionomycin (1 μM) and *b*, without ionomycin. Bars indicate the retention time of synthetic anandamide. The inset in *b* depicts the time-course of the ionomycin effect. *c*, Effects of ionomycin, kainate (200 μM), KCl (40 mM) and 4-aminopyridine (4-AP, 3 mM) on the formation of radioactive material in anandamide np-HPLC fractions. Results represent the mean ±s.e.m. of 3–5 experiments, each done on one dish of neuronal cultures. *, $P < 0.05$ (ANOVA). *d*, Release from striatal neurons of radioactive material in anandamide fractions. Incubations were in culture medium containing either exogenous anandamide (100 μM) or fatty acid-free bovine serum albumin (BSA, 0.5%). Results represent the mean ±s.e.m. of 3–5 experiments. * Statistically different, or not significant (NS), $P < 0.05$ (Student's *t*-test). *e*, HPLC identification of anandamide and related *N*-acyl-ethanolamines formed in ionomycin-stimulated cortical neurons. Representative np- and rp-HPLC fractionations of samples from the 10-min incubation (four dishes) with ionomycin of neurons labelled with [^{3}H]ethanolamine. Numbers indicate the retention times of the following synthetic *N*-acyl-ethanolamines: (1) *N*-γ-linolenoyl-ethanolamine; (2) *N*-linoleoyl-ethanolamine plus anandamide; (3) *N*-palmitoyl-ethanolamine; (4) *N*-oleoyl-ethanolamine; (5) *N*-stearoyl-ethanolamine. *f*, *g*, GC/MS identification of anandamide and related *N*-acyl-ethanolamines. *f*, Electron-impact mass spectrum (70 eV) of synthetic anandamide (*O*-acetyl derivative). *g*, Mass fragmentographic analyses of anandamide and related *N*-acyl-ethanolamines (*N*-acyl-E) produced by cortical neurons stimulated with ionomycin. (1) Anandamide; (2) *N*-linoleoyl-E; (3) *N*-oleoyl-E; (4) *N*-stearoyl-E; (5) *N*-palmitoyl-E. Intensities are omitted from (3)–(5) for clarity, but the relative abundances of the diagnostic ions correspond to those obtained with synthetic standards (A.F., V.D.M., H.C. and D.P., manuscript in preparation). TIC, total ion current.

METHODS. Neuronal cultures were prepared from 16-day-old rat embryos, and maintained in serum-supplemented culture medium as described[22]. Striatal neurons were used after 10 days *in vitro*, and cortical neurons after 5–6 days *in vitro*. The neurons (90-mm dishes, plated at a density of 2.5×10^7 cells per dish), labelled by incubation (16–20 h) with [^{3}H]ethanolamine (1 μCi ml^{-1}, 34 Ci mmol^{-1}, Amersham), were washed with Dulbecco's modified Eagle's medium (DMEM) and incubated for various times in DMEM (5 ml) containing drugs at the indicated concentrations. Incubations were stopped by adding methanol, immediately followed by chloroform extraction. Fractionation and analyses of anandamide and related *N*-acyl-ethanolamines will be described in detail elsewhere (A.F., V.D.M., H.C. and D.P., manuscript in preparation). Chemical syntheses of *N*-acyl-ethanolamines used as standards were as described[1]. Identities of the synthetic products were verified by proton nuclear magnetic resonance spectroscopy (NMR) and by GC/MS, after purification of the products by HPLC.

indicating that glia contribute to [^{3}H]anandamide disposition (data not shown).

Our results show that brain neurons produce, release and inactivate anandamide, strongly supporting a role for this arachidonate derivative as an endogenous cannabinoid substance. They also indicate that anandamide, unlike neurotransmitters, neuropeptides and eicosanoids, may be produced through a single-step, phosphodiesterase-mediated cleavage of a novel phospholipid, *N*-arachidonoyl PE (Fig. 2), and that a family of chemically related NAE may be coreleased through a similar mechanism. The biological roles of the NAE remain unknown, but their pharmacological effects on excitable tissues suggest that

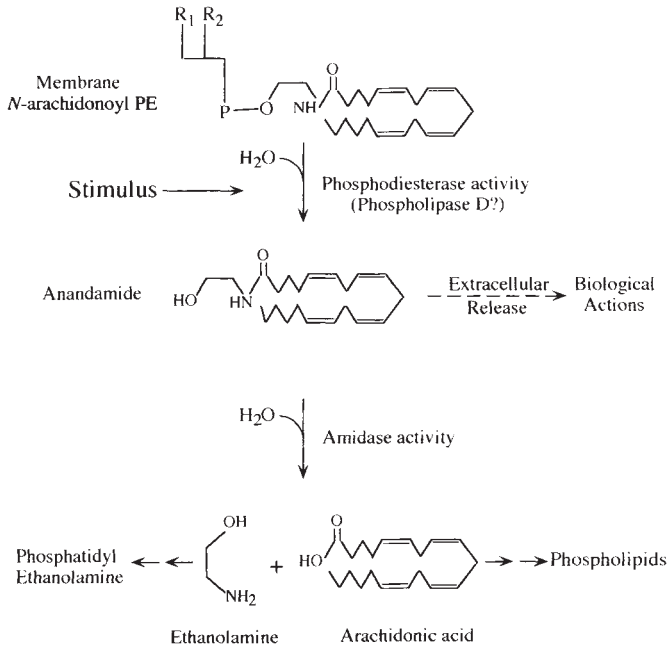
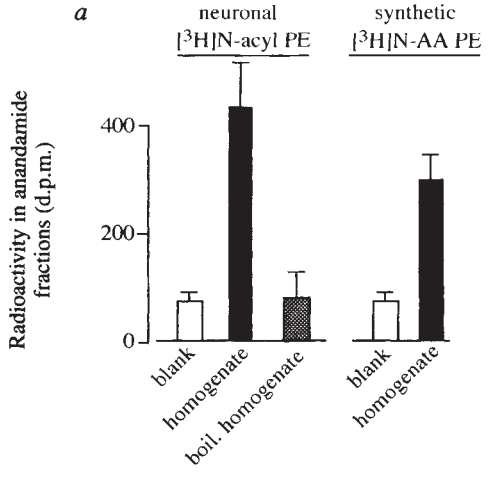


FIG. 2 Proposed pathway for the formation and inactivation of anandamide in brain neurons. According to this model, stimuli (such as increased intracellular Ca^{2+}) evoke the hydrolytic cleavage of a phospholipid precursor in neuronal membranes, *N*-arachidonoyl phosphatidylethanolamine (*N*-arachidonoyl PE). Hydrolysis of *N*-arachidonoyl PE may be catalysed by a D-type phosphodiesterase activity (phospholipase D), producing anandamide in a single-step reaction, or by C-type phosphodiesterase activity (phospholipase C), producing a phosphorylated form of anandamide which would be converted to anandamide by phosphatase activity. The family of *N*-acyl-ethanolamines described in this study are probably generated from *N*-acyl PE through a similar mechanism. An additional mechanism of anandamide formation, the enzymatic condensation of arachidonate with ethanolamine, was also proposed[5,6]. Newly generated anandamide may exit neurons to activate receptors on target cells. The biological actions of anandamide may be terminated by two concurrent processes, cellular uptake (not shown) and hydrolytic degradation to arachidonate and ethanolamine, which are both incorporated into cellular phospholipids.



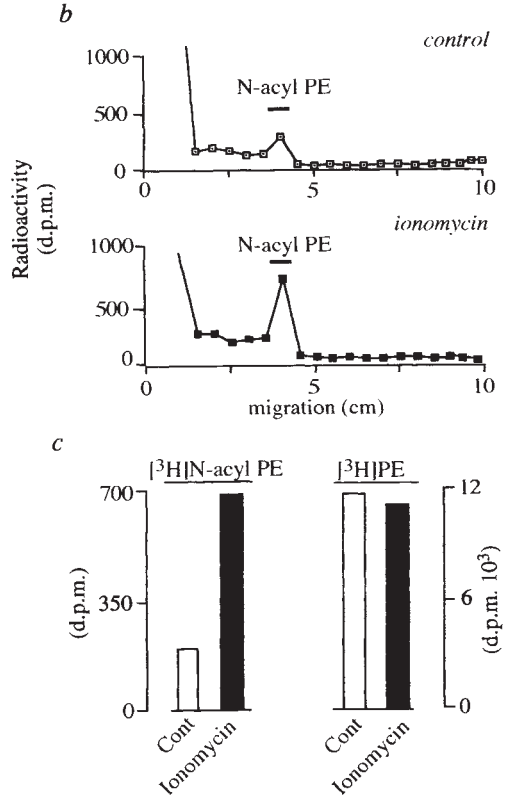


FIG. 3 Enzymatic hydrolysis and ionomycin-evoked turnover of *N*-acyl PE. *a*, Hydrolysis of purified [^{3}H]*N*-acyl PE and synthetic [^{3}H]*N*-arachidonoyl PE by neuron homogenates. Results present the mean ±s.e.m. of 3–7 experiments. *b*, Effect of ionomycin on the labelling of *N*-acyl PE. Representative TLC fractionation of samples from a 20-min incubation with [^{3}H]ethanolamine, alone (top), or together with ionomycin (1 μM, bottom). *c*, Effects of ionomycin on the labelling of *N*-acyl PE with [^{3}H]ethanolamine. Results are from the experiment shown in *b*. Similar results were obtained in two additional experiments.

METHODS. Neuronal [^{3}H]*N*-acyl PE was fractionated by silica gel G column chromatography followed by TLC using a solvent system of chloroform, methanol, ammonium hydroxide (80/20/1, vol/vol/vol). Neuron homogenates were incubated for 10 min at 37 °C in Tris buffer (25 mM, pH 7, 0.4 ml) containing purified [^{3}H]*N*-acyl PE or synthetic [^{3}H]arachidonoyl PE (10,000 d.p.m.). *N*-arachidonoyl PE was synthesized by reacting arachidonic acid with L-α-dipalmitoyl-phosphatidylethanolamine in the presence of diisopropylcarbodiimide. Identity of synthetic *N*-arachidonoyl PE was verified, after TLC purification, by proton nuclear magnetic resonance spectroscopy. An additional sample of *N*-arachidonoyl PE was provided by Avanti Polar Lipids Inc. (Alabaster, AL, USA). [^{3}H](Arachidonoyl)*N*-arachidonoyl PE, synthesized as described above using [^{3}H] arachidonic acid (20 μCi), was purified by TLC.

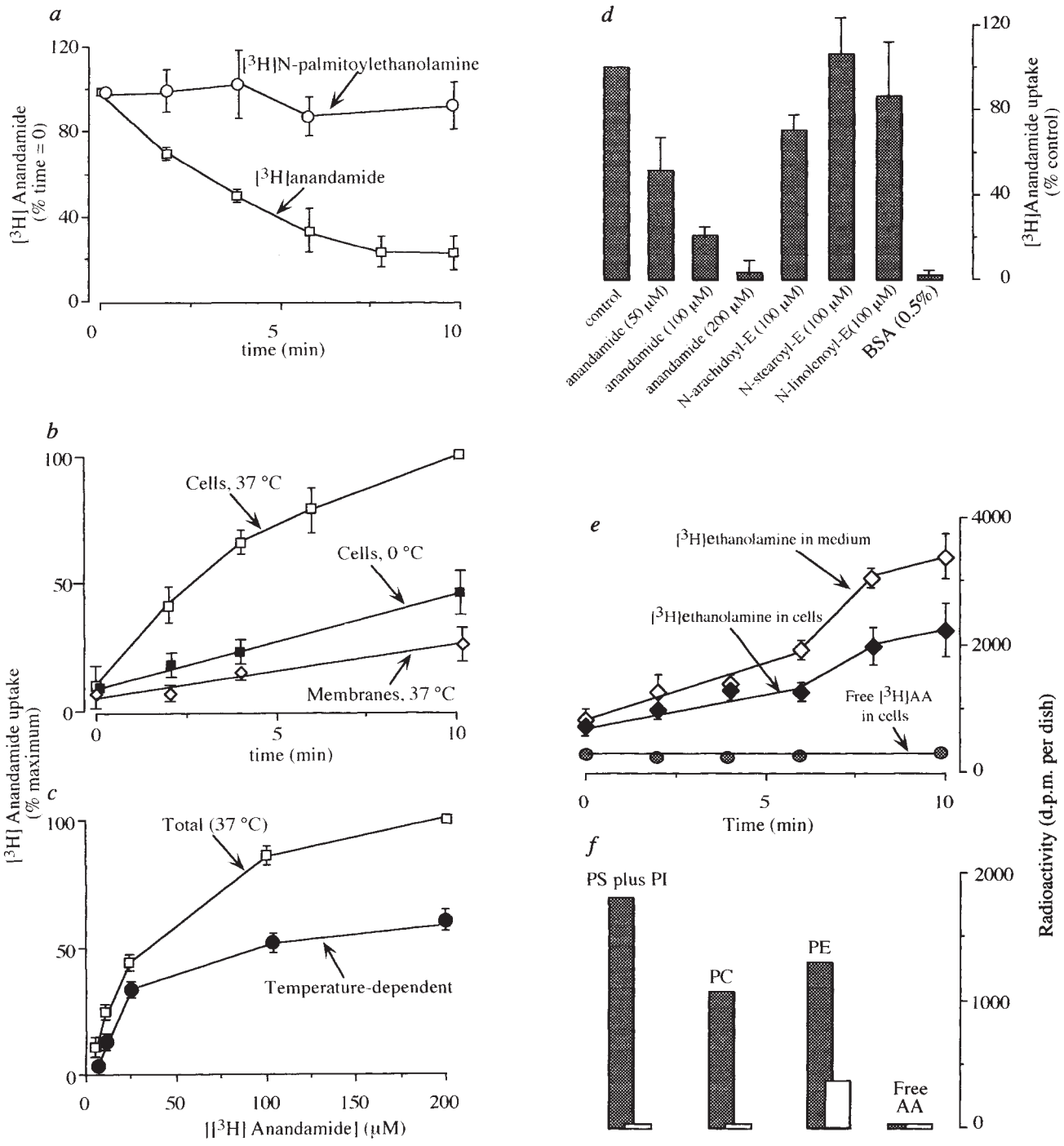


FIG. 4 Uptake and degradation of exogenous [^{3}H]anandamide. *a*, Time-dependent clearance of [^{3}H]anandamide from the incubation medium of cultures of cortical neurons (□). [^{3}H]*N*-palmitoyl-ethanolamine is not cleared from medium (○). *b*, Time-dependent association of [^{3}H]anandamide with intact neuronal cultures at 37 °C (□), intact neuronal cultures at 0–4 °C (■), or neuronal particulate fraction at 37 °C (◇). *c*, Kinetics of [^{3}H]anandamide association (2 min) with neuronal cultures. Temperature-dependent association (●), calculated by subtracting association at 0–4 °C (not shown) from total association at 37 °C (□). *d*, Effects of anandamide, *N*-acyl-ethanolamines (*N*-acyl-E) and BSA on the temperature-dependent association of [^{3}H]anandamide with neuronal cultures. Bars indicate the mean ±s.e.m. from 3 experiments. *e*, *f*, Hydrolytic cleavage of [^{3}H]anandamide to [^{3}H]ethanolamine and [^{3}H]arachidonate. *e*, Accumulation of [^{3}H]ethanolamine inside (◆) and outside (◇) cultures incubated with [^{3}H]anandamide, labelled on either the ethanolamine or the arachidonate moiety. Free [^{3}H]arachidonate (AA) does not accumulate under these conditions (shaded circle). *f*, Accumulation of [^{3}H]arachidonate (filled bars) or [^{3}H]ethanolamine (empty bars) in phospholipids of neuronal cultures incubated with [^{3}H]anandamide. AA, Arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

METHODS. Neuronal cultures were incubated at 37 °C or 0–4 °C in DMEM containing [^{3}H]anandamide (6.25–200 μM, 0.3 mCi mmol^{-1}) or [^{3}H]*N*-palmitoyl-ethanolamine (10 μM, 3.5 mCi mmol^{-1}). Samples of media were taken at the indicated times, and extracted immediately with methanol/chloroform. Cells were washed with repeated applications of DMEM containing BSA (0.5%) before extraction. [^{3}H]Anandamide and [^{3}H]*N*-palmitoyl-ethanolamine were measured in the lipid extracts by silica gel G column chromatography. In preliminary experiments, [^{3}H] anandamide was measured by HPLC, with identical results. [^{3}H]Ethanolamine was determined as detailed elsewhere (F. Desarnaud, H.C. and D.P., manuscript in preparation).

they may participate in cellular signalling[19,20]. Anandamide may be also synthesized by condensation of arachidonate with ethanolamine, a reaction catalysed by NAE amidohydrolase acting in reverse[11] or by anandamide synthetase[5,6]. Although our results may not be accounted for by such mechanism, they do not rule out its participation in anandamide formation under different conditions. It is possible for instance that, by analogy with platelet-activating factor[21], distinct physiological stimuli may cause anandamide formation by activating distinct biochemical pathways. □

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Recognition of a lipid antigen by CD1-restricted αβ⁺ T cells

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MAJOR histocompatibility complex (MHC) class I and class II molecules bind immunogenic peptides and present them to lymphocytes bearing the αβ T-cell antigen receptor (TCR)[1–4]. An analogous antigen-presenting function also has been proposed for the non-MHC-encoded CD1 molecules[5], a family of non-polymorphic, β_2-microglobulin-associated glycoproteins[5–8] expressed on most professional antigen-presenting cells[9–11]. In support of this hypothesis, CD1 molecules are recognized by selected CD4⁻CD8⁻ αβ or γδTCR⁺ T-cell clones[12–14], and we have recently shown that CD1 molecules restrict the recognition of foreign microbial antigens by αβTCR⁺ T cells[10]. But the substantial structural divergence of CD1 from MHC class I and class II molecules[7], raises the possibility that the antigens presented by the CD1 system may differ fundamentally from those presented by MHC-encoded molecules. Here we report that a purified CD1b-restricted antigen of *Mycobacterium tuberculosis* presented to αβTCR⁺ T cells is mycolic acid, a family of α-branched, β-hydroxy, long-chain fatty acids found in mycobacteria[15,16]. This example of non-protein microbial antigen recognition suggests that αβTCR⁺ T cells recognize a broader range of antigens than previously appreciated and that at least one member of the CD1 family has evolved the ability to present lipid antigens.

We have previously described the CD1b-restricted recognition of an exogenous antigen contained in sonicated extracts of *M. tuberculosis* by the CD4⁻CD8⁻ αβTCR⁺ T-cell line DN1. Additional studies demonstrated a requirement for a chloroquine-sensitive antigen-processing step which was similar, but not identical to that required by MHC class II[10]. However, in contrast to MHC class II-restricted protein antigens, an initial assessment of the chemical nature of the CD1b-restricted mycobacterial antigen revealed that the antigen was resistant to protease treatment (Fig. 1).

Further studies using detergents and cell fractionation methods suggested that the CD1b-restricted antigen was a hydrophobic molecule associated with the bacterial cell wall. Given that mycobacteria contain a large variety of unique lipids as integral components of their cell walls[15,16], we consid-

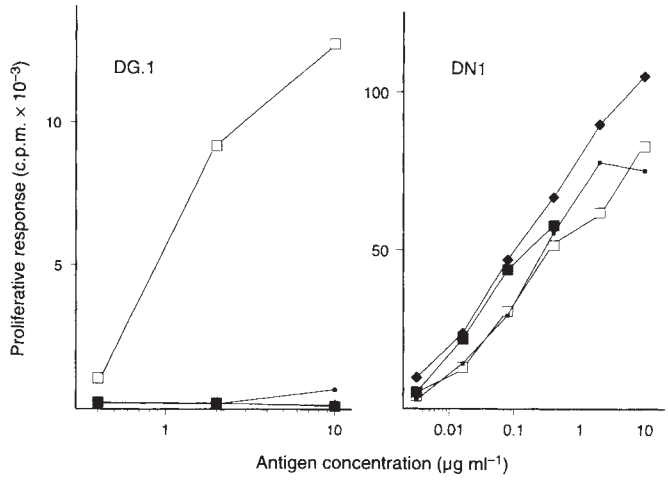

FIG. 1 Protease resistance of CD1b-restricted antigen recognition. The *M. tuberculosis* antigen preparation was digested with proteases having either limited amino-acid specificity (trypsin type XIII (Sigma) (Lys, Arg)(◆), V8 protease type XIII-B (Sigma) (acidic) (■)), or broad amino-acid recognition (proteinase K (Boehringer Mannheim) (■)). Identical aliquots were used to stimulate the proliferation of either the CD1b-restricted DN1 cell line or a *M. tuberculosis*-specific HLA-DR7-restricted CD4⁺ T cell line DG.1 (ref. 10). Left, Proliferative responses (c.p.m. of ^{3}H-thymidine incorporated) of the CD4⁺ T-cell line DG.1 to mock (□) or protease digestions of *M. tuberculosis*. Antigen concentration expressed as amount of *M. tuberculosis* protein (μg ml^{-1}) is shown on the x-axis. Right, Proliferative responses of the CD1b-restricted T-cell line DN1 to the identical samples.
METHODS. Proliferation assays were as previously described[10]. The antigen preparation used was a 50% ammonium sulphate precipitate of an aqueous sonicate of *M. tuberculosis* (strain H37Ra, Difco) prepared as described previously[10] and dialysed against 0.05 M NH_4HCO_3, pH 8.1 using a 12–14K molecular weight cutoff membrane to remove any contaminating peptides. A 100 μg (protein content) sample of the *M. tuberculosis* preparation was mixed with 10 μg of appropriate enzyme in total volume of 200 μl and incubated at 37 °C. To ensure complete digestion, incubations were done for 2 h, heat-inactivated (65 °C, 5 min) and a second aliquot of enzyme added for 15 h. The proteinase K digest contained 1 mM $CaCl_2$ in addition to the above buffer. Antigen-presenting cells were GM-CSF plus IL-4 treated (to induce CD1 expression[10]) monocytes (DN1), or EBV-transformed B cells derived from an HLA-DR7⁺ individual (DG.1). In experiments not shown, digestions with other broadly reactive proteases (pronase, subtilisin) or sequential digestions with V-8 protease, pronase and subtilisin, also had no effect on the CD1b-restricted mycobacterial antigen.