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Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'

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Abstract The endogenous cannabimimetic substance, anandamide (*N*-arachidonoyl-ethanolamine) and the recently isolated sleep-inducing factor, oleoyl-amide (*cis*-9,10-octadecenoamide), belong to two neuroactive fatty acid amide classes whose action in mammals has been shown to be controlled by enzymatic amide bond hydrolysis. Here we report the partial characterisation and purification of 'anandamide amidohydrolase' from membrane fractions of N18 neuroblastoma cells, and provide evidence for a further and previously unsuspected role of this enzyme. An enzymatic activity catalysing the hydrolysis of [^{14}C]anandamide was found in both microsomal and 10,000 × *g* pellet fractions. The latter fractions, which displayed the highest V_{max} for anandamide, were used for further characterisation of the enzyme, and were found to catalyse the hydrolysis also of [^{14}C]oleoyl-amide, with an apparent K_m of 9.0 ± 2.2 μM. [^{14}C]anandamide- and [^{14}C]oleoyl-amide-hydrolysing activities: (i) exhibited identical pH- and temperature-dependency profiles; (ii) were inhibited by alkylating agents; (iii) were competitively inhibited by the phospholipase A_2 inhibitor arachidonyl-trifluoromethyl-ketone with the same IC_{50} (3 μM); (iv) were competitively inhibited by both anandamide (or other polyunsaturated fatty acid-ethanolamides) and oleoyl-amide. Proteins solubilised from 10,000 × *g* pellets were directly analysed by isoelectric focusing, yielding purified fractions capable of catalysing the hydrolysis of both [^{14}C]anandamide and [^{14}C]oleoyl-amide. These data suggest that 'anandamide amidohydrolase' enzymes, such as that characterised in this study, may be used by neuronal cells also to hydrolyse the novel sleep-inducing factor oleoyl-amide.

Key words: Anandamide; Arachidonoyl-ethanolamide; Fatty acid amide; Oleoyl-amide; Cannabinoid; Sleep-inducing factor

1. Introduction

Several recent findings have assigned to fatty acid amides an unprecedented role as possible neuroactive signalling molecules: (i) the discovery of arachidonoyl-ethanolamide (anandamide), proposed as the endogenous effector at the brain (CB-1) cannabinoid receptor [1]; (ii) the finding that anandamide is released from rat central neurons (challenged with depolarizing stimuli) together with other long chain acyl-ethanolamides [2], and that porcine brain contains two more cannabimimetic acyl-ethanolamides with polyunsaturated acyl chains [3]; (iii) the activation, in mast cells, of the peripheral (CB-2) cannabinoid receptor by a naturally-occurring anandamide congener, palmitoyl-ethanolamide [4]; (iv) the isolation, from the cerebrospinal fluid of sleep-deprived cats, rats, and humans, of a primary amide, *cis*-9,10-octadecenoamide (oleoyl-amide), with potent sleep-inducing activity [5]. Mechanisms for the inactivation of anandamide in central neurons have been proposed, showing that its degradation is secured primarily by amide bond hydrolysis, with subsequent formation of arachidonic acid and ethanolamine, which are then readily reincorporated into membrane phosphoglycerides [2]. The enzyme catalysing this hydrolysis, named 'anandamide amidohydrolase', has been partially characterised from rat brain [6] and porcine brain microsomes [7]. Anandamide amidohydrolase activities have been also found in non-microsomal membrane fractions from rat and porcine brain [6–9] as well as from mouse neuroblastoma and glioma cells [8], but they have not been fully characterised. The observation by Cravatt et al. [5] that oleoyl-amide is likewise hydrolysed to oleic acid upon incubation with rat brain membranes, prompted us to carry out the present study where we present evidence that 'anandamide amidohydrolase' from neuroblastoma cell membranes may efficiently catalyse also the breakdown of the novel sleep-inducing substance.

2. Experimental

N18TG2 cells were purchased from DSM (Germany) and grown in DMEM containing 10% fetal calf serum at 37°C and 5% CO_2. Confluent cells were harvested and homogenised in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, using a Dounce homogeniser. The homogenate was centrifuged sequentially at 800 × *g* (5 min), 10,000 × *g* (15 min) and 100,000 × *g* (30 min). The pellets as well as the supernatant from the last centrifugation were incubated, unless otherwise stated, in 0.5 ml of 50 mM Tris-HCl, pH 7.4 at 37 °C for 30 min in the presence of 10,000 cpm (2.4 μM) of either [^{14}C]anandamide (5.3 mCi/mmol, labelled on the ethanolamine moiety) or [^{14}C]oleoyl-amide (5.3 mCi/mmol, labelled on the oleic acid moiety). These were synthesized as described previously [5,10] using [^{14}C]ethanolamine (53 mCi/mmol, Amersham, UK) and [^{14}C]oleic acid (50 mCi/mmol, DuPont NEN, Germany), respectively. Other ^{14}C-labelled acyl-ethanolamides and [^{14}C]linoleoyl-amide were also prepared as described previously [5,10]. Incubations were terminated by adding 1 ml of chloroform/methanol (1 : 1, v/v), and lowering the temperature to 4°C. [^{14}C]Ethanolamine produced by the enzymatic hydrolysis of [^{14}C]acyl-ethanolamides was determined by open bed chromatography of the aqueous phase on Porapak mini-columns [2,6], followed by liquid scintillation counting. [^{14}C]oleic and -linoleic acids produced by the enzymatic hydrolysis of [^{14}C]oleoyl- and -linoleoyl-amides were determined by thin layer chromatography [5] of the organic phase, followed by direct β emission counting by a radioac-

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tivity scanner (Packard, USA). Experiments were carried out also with different concentrations of [^{14}C]acyl-ethanolamides or [^{14}C]oleoyl-amide and [^{14}C]linoleoyl-amide, for K_m and V_{max} determination, or in different buffers at different pH values, as described previously [6]. Thermal stability of the 10,000 × *g* pellet enzymatic activities was determined by keeping identical aliquots of the same 10,000 × *g* pellet suspension at various temperature for 5 min, and then conducting the incubation as usual [7]. The effect of various concentrations of arachidonoyl-trifluoromethyl-ketone (AACOCF$_3$) [8] was also determined by conducting the assay in identical aliquots of the same 10,000 × *g* pellet suspension and conducting the incubation as usual with and without the inhibitor. Incubation at different intervals of time were carried out by adding 300,000 cpm of the radiolabelled substrate to 10,000 × *g* pellets suspended in 2.25 ml of assay buffer; 250 μl aliquots were withdrawn at different times, and the products of the reaction analysed as described above. Incubations were also performed in the presence of either of the following substances: 1 mM dithiothreitol (DTT), 5mM EDTA, 0.1 mM phenyl-methyl-sulphonyl-fluoride (PMSF), 0.1 mM *p*-bromo-phenacyl-bromide, 0.1 mM *p*-hydroxy-mercuri-benzoate, 0.1 mM *o*-phenanthroline and 0.1 mM benzamidine, all purchased from Sigma, UK, and 0.1 mM oleoyl- and palmitoyl-ethanolamides, synthesised as described in [1], and oleoyl-amide.

Isoelectric focusing was performed using a 1% Triton X-100-, 12% glycerine-containing 5% T and 3% C matrix polyacrilamide gel (125 × 130 × 3 mm) with 3% ampholine in the pH range 5.6–8.13 (obtained by mixing ampholines in the pH range 3.5–10 plus ampholines in the pH range 7–9 in a 1:4 ratio). The anodic and cathodic solutions were 0.1% ampholine pH 7–9 and 1 M NaOH, respectively. The prefocusing run conditions were 30 min at 1000 V; 10,000 × *g* pellet proteins, solubilised by 12 h treatment with 1% Triton X-100 at 4°C and centrifuged for 30 min at 10,000 × *g*, were then loaded into one well (93 × 6 × 1.5 mm) and the run continued for 4 hours at 17 W (limiting voltage 2000 V), during which the gel was cooled at 10 °C. At the end of the run the pH gradient was measured with an Ingold combined microelectrode on a digital Corning pH-meter, and the gel was cut into 15 slices (5 mm). Proteins were then eluted by adding 3 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1mM DTT, to each slice, and then assayed as usual for [^{14}C]anandamide and [^{14}C]oleoyl-amide hydrolysis. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard [11].

3. Results and discussion

Intact mouse N18TG2 neuroblastoma cells uptake and degrade both [^{14}C]anandamide [8] and [^{14}C]oleoyl-amide (Di Marzo et al., unpublished observations). Moreover, this cell line has been used in a recent study on possible inhibitors of anandamide hydrolysis [12]. Therefore we used N18TG2 cells as source for the enzymatic activity(ies) responsible for the hydrolysis of the two neuroactive fatty acid amides. Anandamide amidohydrolase activity was found in the 800 × *g* (cellular debris, 6.6%), 10,000 × *g* (77.8%) and 100,000 × *g* (microsomes, 14.0%) pellets from cell homogenates, while negligible activity was found in the supernatant from the 100,000 × *g* centrifugation (cytosol, 1.5%). Anandamide amidohydrolase activities from 10,000 × *g* pellets and microsomes were compared on the basis of their substrate specificity, dependency from pH and incubation time, and sensitivity to either irreversible or competitive inhibitors, and displayed similar profiles (Table 1 and Fig. 1a,b,c). Among the acyl-ethanolamides tested, both activities showed the highest affinity for anandamide, as it must be expected from an anandamide amidohydrolase-like enzyme [6,7,9]. The microsomal fraction, however, exhibited: (i) respectively, a slightly lower and a significantly higher apparent K_m for anandamide and for its monounsaturated analogue, *cis*-13-docosenoyl-(erucoyl)-ethanolamide (Table 1); (ii) a higher sensitivity to inhibition by the competitive phospholipase A$_2$ inhibitor arachidonoyl-trifluoromethyl-

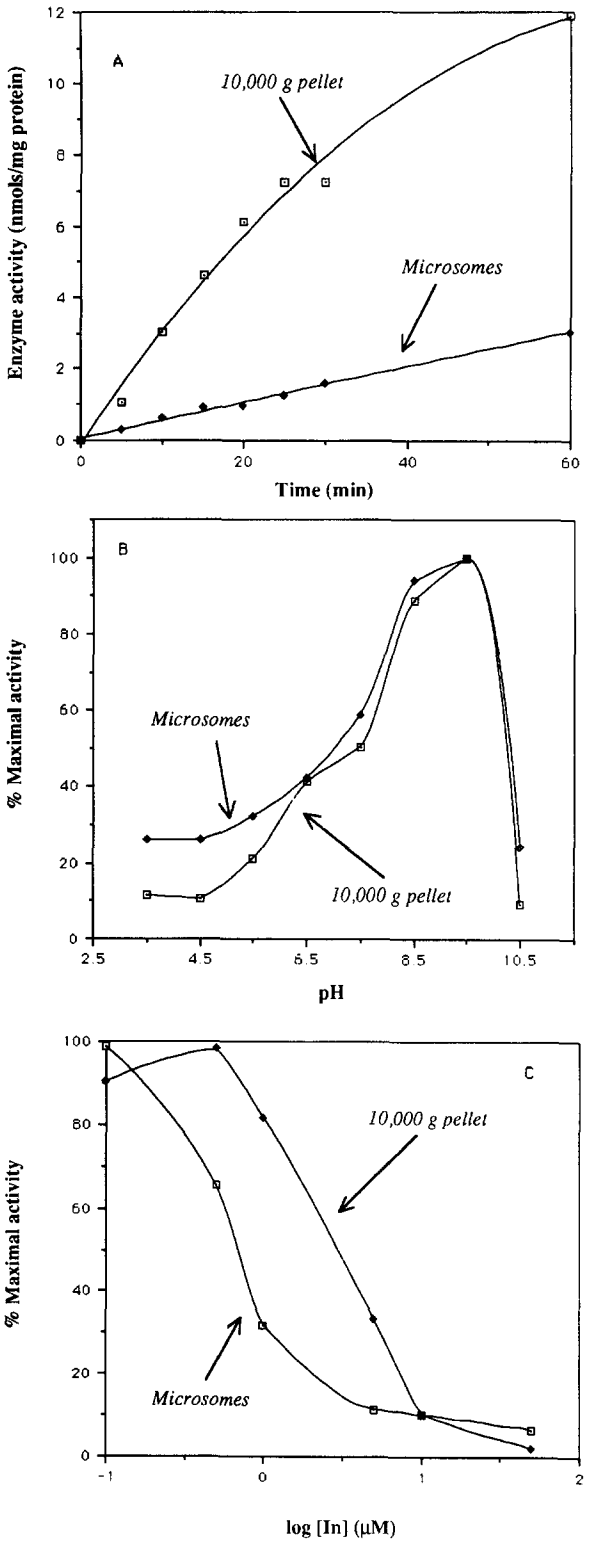

Fig. 1. Effect of (A) incubation time, (B) pH, and (C) arachidonoyl-trifluoromethyl-ketone (AACOCF$_3$) on the rate of [^{14}C]anandamide hydrolysis by N$_{18}$ neuroblastoma microsomal and 10,000 × *g* pellet fractions. Incubations, in duplicates, were conducted as described in section 2, by using, in (B) and (C), 30 μg proteins and 10,000 cpm of [^{14}C]anandamide (2.4 μM). In (A), [^{14}C]anandamide concentration was 16 μM and protein concentration was 200 μg/ml. Data are representative of at least three separate experiments.

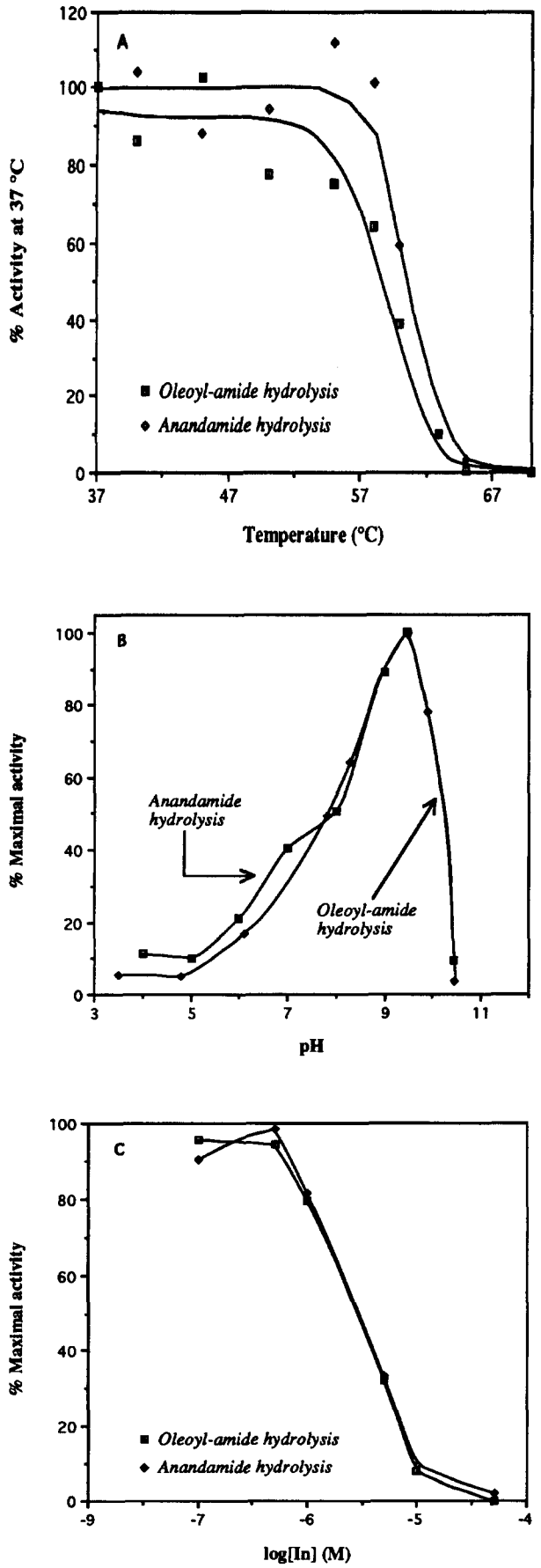

Fig. 2. Effect of (A) temperature, (B) pH, and (C) arachidonoyl-trifluoromethyl-ketone ($AACOCF_3$) on the rate of [^{14}C]anandamide or [^{14}C]oleoyl-amide hydrolysis by N_{18} neuroblastoma 10,000 × *g* pellet fractions. Incubations, in duplicates, were conducted as described in section 2. In (A), (B), (C) 30 μg of 10,000 × *g* pellet proteins and 10,000 cpm of each substrate (2.4 μM) were used. In (B) and (C), data relative to [^{14}C]anandamide hydrolysis are the same as in Fig. 1B and C. Data are representative of three separate experiments.

ketone ($AACOCF_3$, IC_{50} = 0.7 μM vs. 3 μM in 10,000 × *g* pellet fractions, Fig. 1c), and (iii) a slower rate of anandamide hydrolysis (V_{max} = 0.948 ± 0.363 nmol·min^{-1}·mg protein^{-1} vs. 2.259 ±

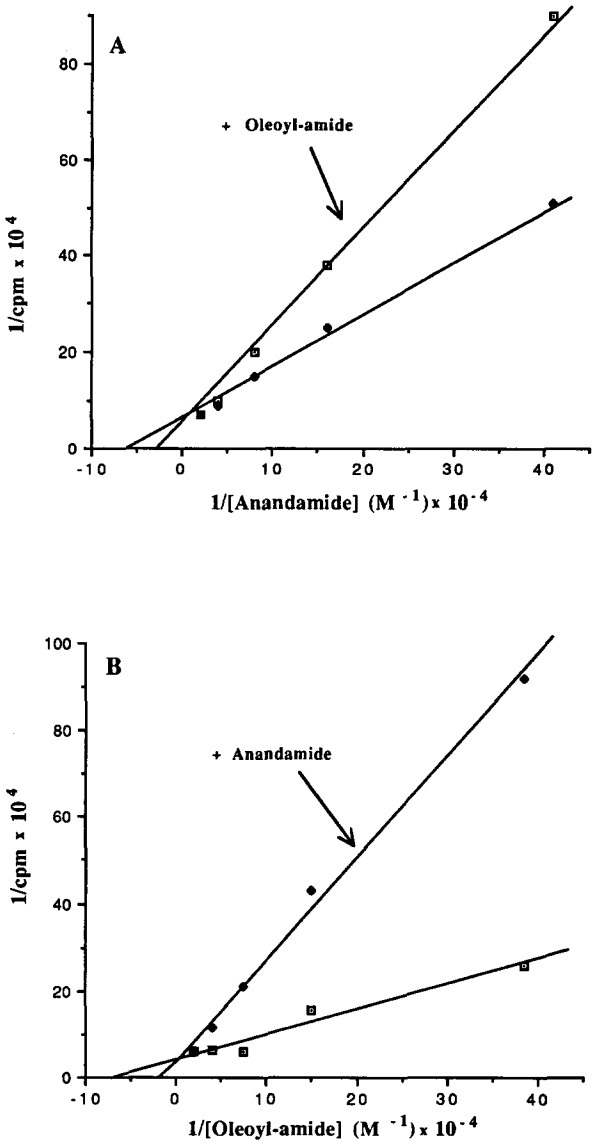

Fig. 3. Lineweaver–Burk profiles for [^{14}C]anandamide (A) or [^{14}C]oleoyl-amide (B) hydrolysis by N_{18} neuroblastoma 10,000 × *g* pellet fractions in the presence or absence of oleoyl-amide (20 μM) and anandamide (20 μM), respectively. Incubations were conducted as described in the legend to Table 1. Data points at the lowest substrate concentration (1.2 μM) were omitted for the sake of clarity. Reciprocals of background subtracted radioactivity associated to [^{14}C]ethanolamine or [^{14}C]oleic acid, formed from the hydrolysis of the two substrates, which is directly proportional to enzyme activity, are reported on the Y axis. Reciprocals of substrate molar concentration are reported on the X axis. Data are representative of two separate experiments.

Table 1
Substrate specificity of N_{18} neuroblastoma 10,000 × g pellet and microsomal anandamide amidohydrolase activity

Substrate	K_m (μM)		V_{max} (nmol·min^{-1}·mg protein^{-1})	
	10,000 × g pellet	Microsomes	10,000 × g pellet	Microsomes
[^{14}C]Arachidonoyl-ethanolamide	15.0	6.9	2.259	0.948
[^{14}C]Linoleoyl-ethanolamide	20.0	28.0	1.406	0.143
[^{14}C]*cis*-Eicosadienoyl-ethanolamide	33.0	–	2.093	–
[^{14}C]Erucoyl-ethanolamide	50.0	>100	0.549	<0.100
[^{14}C]Palmitoyl-ethanolamide	80.0	67.0	1.317	0.274
[^{14}C]Oleoyl-amide	9.0	14.4	0.941	0.340
[^{14}C]Linoleoyl-amide	6.8	–	1.448	–

Proteins (30 μg) from either 10,000 × g pellet or microsomal fractions, prepared as described in section 2, were incubated with 1.2, 2.4, 6.1, 12.2, 24 and 48 μM of either [^{14}C]anandamide (arachidonoyl-ethanolamide), [^{14}C]linoleoyl($C_{18:2,\Delta 9,12}$)-ethanolamide, [^{14}C]*cis*-eicosadienoyl($C_{20:2,\Delta 11,14}$)-ethanolamide, [^{14}C]erucoyl($C_{22:1,\Delta 11}$)-ethanolamide, [^{14}C]palmitoyl($C_{16:0}$)-ethanolamide, [^{14}C]oleoyl ($C_{18:1,\Delta 9}$)-amide or [^{14}C]linoleoyl($C_{18:2,\Delta 9,12}$)-amide, synthesised as described in section 2. Apparent K_m and V_{max} values were obtained from Lineweaver–Burk profiles, and are means of at least two separate experiments conducted in duplicate.

0.750 nmol·min^{-1}·mg protein^{-1} in 10,000 × g pellets). More importantly, both 10,000 × g pellet and microsomal (but not cytosolic) fractions catalysed also the hydrolysis of [^{14}C]oleoyl-amide, although the former fraction displayed the lowest apparent K_m (9.0 ± 2.2 μM vs. 14.4 ± 2.5 μM) and the highest V_{max} (0.941 ± 0.248 vs. 0.340 ± 0.080 nmol·min^{-1}·mg protein^{-1}) for the primary amide. These data prompted the use of N18TG2 cell 10,000 × g pellet fractions for further experiments aimed at assessing whether [^{14}C]anandamide - and [^{14}C]oleoyl-amide-hydrolysing activities are associated to one or more enzymes in these cells.

[^{14}C]Oleoyl-amide hydrolysis to oleic acid in the presence of N18TG2 cell 10,000 × g pellets exhibited temperature- and pH-dependency as well as inhibition profiles identical to those observed for [^{14}C]anandamide hydrolysis (Fig. 2a–c, Table 2). As with the anandamide amidohydrolase activity described above, this 'oleoyl-amide hydrolase' was still prevalently (40%) active after heating at 60 °C, while 90% and 100% of the activity was lost after heating at 63 °C and 65 °C, respectively (Fig. 2a). It is worth mentioning that this thermal stability profile is identical also to that previously reported for porcine brain anandamide amidohydrolase [7]. Oleoyl-amide hydrolase displayed its optimal pH at 9.5 (Fig. 2b), a value very close to that reported here (Fig. 1a) and elsewhere [7] for anandamide amidohydrolase. The rate of hydrolysis of both amides was dependent upon protein concentration in the range 6–96 μg/ml, and was maximal at 192 μg/ml in both cases (not shown). The inhibition profile by $AACOCF_3$ of [^{14}C]oleoyl-amide hydrolysis (Fig. 2c) was superimposable to that observed for the inhibition by the same compound of 10,000 × g pellet anandamide amidohydrolase (this study) and almost identical to that reported for partially purified porcine brain anandamide amidohydrolase [7] (IC_{50} = 3 μM, 3 μM and 1 μM, respectively). This inhibition was competitive, as determined from Lineweaver–Burk profiles (not shown) obtained at different [^{14}C]anandamide and [^{14}C]oleoyl-amide concentrations with and without 1 μM $AACOCF_3$ (which determined a shift in the apparent K_m values from 15.0 and 9.0 μM to 40.0 and 37.0 μM, respectively). Finally, the profiles for the sensitivity to several agents of both oleoyl-amide and anandamide hydrolysis by 10,000 × g pellet fractions were strikingly similar (Table 2). In particular, the two activities were: (a) inhibited by the alkylating agents phenyl-methyl-sulphonyl-fluoride and *p*-bromo-phenacyl-bromide as well as by the sulphydryl reagent *p*-hydroxy-mercuri-benzoate; (b) activated by dithiothreitol; (c) not sensitive to the specific peptidase inhibitors *o*-phenanthroline and benzamidine, nor to the divalent ion-chelating agent EDTA; (d) inhibited by both anandamide and oleoyl-amide. As determined from Lineweaver–Burk graphs, anandamide inhibition of [^{14}C]oleoyl-amide hydrolysis (Fig. 3b) and oleoyl-amide inhibition of [^{14}C]anandamide hydrolysis (Fig. 3a) were both competitive. From this finding, it appears that the two amides compete for the same site on the same amidohydrolase enzyme(s).

Taken together, these data support the hypothesis that, at least in N18TG2 cells, the activities responsible for the hydrolysis of either anandamide (and, with slower rates, of other long chain fatty acid ethanolamides) or oleoyl-amide are associated to the same enzyme or to the same class of enzymes. In principle, rigorous evidence substantiating further this hypothesis should be looked for by comparing the enzymatic, chromatographic and physico-chemical properties of the pure protein(s) catalysing anandamide and oleoyl-amide hydrolysis. However,

Table 2
Effect of various substances on [^{14}C]oleoyl-amide and [^{14}C]anandamide hydrolysis by N_{18} neuroblastoma 10,000 × g pellet fractions

Substance (concentration)	Rate of [^{14}C]-anandamide hydrolysis (%)	Rate of [^{14}C]-oleoyl-amide hydrolysis (%)
None	100	100
Arachidonoyl-trifluoro-methylketone (10 μM)	15.2 ± 2.2	14.0 ± 4.4
Phenyl-methyl-sulphonyl-fluoride (100 μM)	2.8 ± 0.3	2.2 ± 0.3
p-Bromo-phenacyl-bromide (100 μM)	32.8 ± 15.4	14.2 ± 0.3
p-Hydroxy-mercuri-benzonate (100 μM)	10.6 ± 0.6	11.0 ± 3.2
o-Phenanthroline (100 μM)	97.9 ± 5.6	117.5 ± 10.0
Benzamidin (100 μM)	102.7 ± 10.0	91.3 ± 12.0
EDTA (5 mM)	99.9 ± 0.7	100.5 ± 0.6
Dithiothreitol (1 mM)	130.6 ± 0.5	123.9 ± 0.6
Anandamide (100 μM)	9.0 ± 2.5	6.1 ± 0.5
Oleoyl-ethanolamide (100 μM)	13.9 ± 5.1	13.4 ± 2.3
Palmitoyl-ethanolamide (100 μM)	96.7 ± 5.4	77.6 ± 6.9
Oleoyl-amide (100 μM)	27.2 ± 13.1	24.0 ± 6.9

Data (means ± S.D., n = 3) are expressed as percent of the rate of [^{14}C]anandamide and [^{14}C]oleoyl-amide hydrolysis with no substance added (554 ± 19 and 361 ± 2 pmol-min^{-1}-mg protein^{-1}, respectively). Membranes (30 μg) were incubated at 37°C for 20 min in 0.5 ml of buffer containing 30,000 cpm of either [^{14}C]anandamide or [^{14}C]oleoyl-amide (5.3 mCi/mmol, final concentration 7.2 μM).

since this(these) enzyme(s) is(are) probably an integral membrane protein(s), its(their) purification to homogeneity can be predicted to require still a long time. Moreover, it must be pointed out that when temperature- and pH-dependency studies, as well as inhibition experiments with $AACOCF_3$, have been conducted with a 20-fold purified anandamide amidohydrolase fraction from porcine brain [7], results strikingly similar to the ones reported here for anandamide amidohydrolase activity in N18TG2 cell $10,000 \times g$ pellets were obtained. This implies that, at least for the system used in this study (where anandamide amidohydrolase activity has been associated for the first time to a single neuronal cell line), accurate (albeit preliminary) information on the properties of anandamide amidohydrolase can be gained already in non-purified proteins from $10,000 \times g$ pellet fractions. These properties, judging from the data presented above, cannot be distinguished from those of the enzyme, characterised here for the first time, responsible for the hydrolysis of the novel sleep-inducing factor, oleoyl-amide. However, in order to provide further support to this suggestion, we treated N18TG2 cell $10,000 \times g$ pellets with Triton X-100 at a concentration (1%) successfully used in previous studies to prepare anandamide amidohydrolase from porcine brain microsomes [7], and directly analysed the solubilised proteins by isoelectrofocusing. The latter was carried out in the pH interval 5–8, using gels prepared in Triton X-100, since pilot experiments had shown that SDS inactivates both anandamide amidohydrolase and oleoyl-amide hydrolase activities. The proteins eluted from 0.5 cm gel slices were assayed for their capability of catalysing, in separate assays, the hydrolysis of both [^{14}C]anandamide and [^{14}C]oleoyl-amide. When using either substrate, the same fatty acid amide hydrolase bands were found in correspondence with the gel well (pH $\geq$ 8.13, possibly due to overloading or to protein precipitation) and at pH 6.99 $\pm$ 0.14 (mean $\pm$ S.E.M., $n = 7$). This final result provides further evidence that anandamide - and oleoyl-amide-hydrolysing activities are due to the same enzyme(s) or, at least, to enzymes possessing very similar pI's as well as substrate sites capable of recognizing either amide. Apart from conclusively confirming this finding, the purification to homogeneity of this(ese) enzyme(s) is now needed in order to assess two other long debated issues (reviewed in [13,14]), i.e. (i) whether anandamide amidohydrolase can also catalyse, in the presence of high mM concentrations of ethanolamine, the energy-free condensation of the latter with long chain fatty acids (including arachidonic acid), as suggested by a recent study [7], and (ii) whether anandamide amidohydrolase is identical to the acyl-ethanolamide-hydrolysing enzyme partially characterised in several mammalian tissues in the mid 1980's (for review see [15]).

In conclusion, this study has provided some physico-chemical data on anandamide amidohydrolase from $10,000 \times g$ pellet fractions of a single neuronal cell line. More importantly, strong evidence to the fact that this enzyme might be responsible also for the breakdown of the novel sleep-inducing factor, oleoyl-amide, has been presented herein. Signal-terminating enzymes, such as monoamino oxidase and neutral endopeptidases, are often used for the inactivation of more than a primary messenger [16]. Current and future studies are likely to confirm a physiological role for long chain fatty acid ethanolamides and/or primary amides as modulators of neuron [2,5], astrocyte [17] and inflammatory cell [4,18,19] functionality. In this case, our data will predict a more general function for 'anandamide amidohydrolase' (which might be eventually renamed 'long chain fatty acid amidohydrolase') as regulatory enzyme for more than one bioactive fatty acid amide.

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Note added during revision

During the refereeing process of this paper, and in agreement with the data presented herein, a microsomal anandamide amidohydrolase, partially purified from porcine brain, has been reported to catalyse also the hydrolysis of oleoyl-amide (Ueda, N., Kurahashi, Y. and Yamamoto, S., Oral communication at the 4th International Conference on 'Eicosanoids and other bioactive lipids in cancer, inflammation and radiation injury', 4–7 October, 1995).