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Bioactive long chain *N*-acylethanolamines in five species of edible bivalve molluscs
Possible implications for mollusc physiology and sea food industry

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Abstract

Several long chain *N*-acylethanolamines, including the proposed endogenous ligands of cannabinoid receptors, anandamide (*N*-arachidonoylethanolamine, C20:4 NAE) and *N*-palmitoylethanolamine (C16:0 NAE), as well as some of their putative biosynthetic precursors, the *N*-acyl-phosphatidylethanolamines, were found in lipid extracts of five species of bivalve molluscs, including *Mytilus galloprovincialis*, commonly used as sea food. The amounts of these metabolites, the most abundant being C16:0 NAE and *N*-stearoylethanolamine, appeared to increase considerably when mussels were extracted 24 h post-mortem, but were not significantly affected by boiling the tissue prior to extraction. In particulate fractions of homogenates from *Mytilus*, where the existence of a highly selective cannabinoid receptor with an immunomodulatory function has been previously described, an enzymatic activity capable of catalyzing the hydrolysis of C20:4 NAE amide bond, and displaying similar pH dependency and inhibitor sensitivity profiles as the recently characterized 'fatty acid amide hydrolase' was found. The enzyme K_m and V_{max} for C20:4 NAE were 29.6 μM and 73 pmol/mg protein/min, respectively. These findings support the hypothesis that C20:4 NAE, never reported before in the phylum Mollusca, may be a mollusc physiological mediator, and suggest that edible bivalves may be a dietary, albeit limited, source of C16:0 NAE, whose anti-inflammatory properties, when administered orally in amounts higher than those reported here, have been previously reported. © 1998 Elsevier Science B.V.

Keywords: Anandamide; Palmitoylethanolamide; Marine eicosanoid; Cannabinoid receptor; Fatty acid amide hydrolase; *N*-acylethanolamine

1. Introduction

Long chain *N*-fatty acid ethanolamines (NAEs) represent a well known class of bioactive natural products whose presence and possible phospholipid origin in several species of both animal and plant kingdoms have been investigated since the 1960s (for a review see [1]). Recently, this class of compounds has attracted renewed interest due to the finding that some of its polyunsaturated members, such as *N*-arachidonoylethanolamine (anandamide, C20:4 NAE), may be endogenous neuromodulators acting at cannabinoid receptors in mammalian brain ([2,3] and

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for reviews [4,5]) and capable of mimicking most of the CNS pharmacological actions previously ascribed to psychoactive cannabinoids. More recently, a saturated, and, therefore, non-psychotropic, *N*-acylethanolamine, i.e. *N*-palmitoylethanolamine (C16:0 NAE), previously known for its powerful in vivo anti-inflammatory and anti-anaphylactic actions [1], was shown, together with synthetic cannabinoids, to down-modulate the release of serotonin from basophils and mast cells expressing the peripheral CB2, but not the central CB1, cannabinoid receptor subtype [6]. A mast-cell dependent action against substance P-induced plasma extravasation and carrageenan-induced hindpaw edema and hyperalgesia has also been described upon oral administration of C16:0 NAE to rodents [7]. In the immune system, C20:4 NAE, which counteracts C16:0 NAE-induced down-regulation of basophils/mast cells [6], has been found to exert anti-proliferative and immunosuppressive actions on lymphocytes and macrophages [8,9] and to induce arachidonate release from macrophages [10]. Whether they exert similar, different or opposing actions, C20:4 NAE and C16:0 NAE have been found to share the same biosynthetic and degradative mechanisms in all tissues studied so far. In neurons as well as testes and leukocytes, the two compounds were shown to be produced, together with other AEs, from the hydrolysis of the corresponding *N*-acylphosphatidylethanolamines (NaPEs, [11–13]). Indeed, a phospholipase D-like enzyme capable of converting NaPEs with saturated and monounsaturated *N*-fatty acid chain into the corresponding NAEs had been previously characterized from rat heart [14] and dog brain [15]. C20:4 NAE and C16:0 NAE are inactivated through the hydrolysis of the amide bond [13,16–18], catalyzed by the recently cloned and characterized 'fatty acid amide hydrolase' (FAAH) [18], an enzyme present in both neuronal and peripheral cell types, capable of recognizing also long chain fatty acid primary amides [16–18] and likely to be identical to the amidohydrolase previously described to catalyze the hydrolysis of saturated and monounsaturated NAEs [19].

In invertebrates, C20:4 NAE inhibits the egg jelly-induced acrosome reaction of sperm cells with oocytes in sea urchins [20], and stimulates nitric oxide (NO) release from immunocytes of the mussel *Mytilus edulis*, thereby inducing their rounding and subsequent down-modulation [21]. Both effects were suggested to be mediated by cannabinoid receptors, since they were induced also by synthetic cannabinoids, and selective and high affinity cannabinoid binding sites were found in both sea urchin spermatozoa and *M. edulis* immunocytes [22,21]. However, while the hypothesis that C20:4 NAE may be an endogenous modulator of sea urchin reproductive functions has recently found support with the finding, in sea urchin ovaries, of C20:4 NAE, C16:0 NAE and *N*-stearoylethanolamine (C18:0 NAE), as well as of the enzymatic activities responsible for their biosynthesis and degradation [23], no evidence has been reported so far on the presence of NAEs in *Mytilus* nor in other bivalve genera (*Lamellibranchia*).

The finding of NAEs, and/or of their putative biosynthetic precursors NaPEs, in soybean, egg yolk and cocoa powder [1,24,25], raised the possibility that plant and animal tissues commonly used in the food industry could owe some of their not simply nutritional properties to the presence of such bioactive compounds [25]. With the aim, at once, of (a) providing biochemical support to the previous hypothesis [21] that the 'endogenous cannabinoid signalling system' may operate also in bivalve molluscs, and (b) addressing the question of whether typical shell fish may represent a significant dietary source of bioactive NAEs, we have undertaken the present study. We show that C20:4 NAE and/or C16:0 NAE, as well as some of their congeners and the corresponding NaPEs, are present in five species of edible bivalve molluscs. We also present evidence for the occurrence, in a mussel of the genus *Mytilus*, of a FAAH-like enzyme responsible for the inactivation of endogenous C20:4 NAE with a possible role as an immunomodulatory mediator.

2. Materials and methods

2.1. Materials

The mussel *Mytilus galloprovincialis*, the clams *Venus verrucosa*, *Tapes decussatus* and *Callista chione* and the oyster *Crassostrea* sp. were purchased from a local mariculture center. Synthetic C20:4 NAE, C16:0 NAE and other NAEs, either radiolabeled or unlabeled, were synthesized by the di-isopropyl-carbodiimide method described previ-

ously [26], starting from either [^{14}C]ethanolamine (Amersham, UK, 53–57 mCi/mmol) or unlabeled ethanolamine plus the corresponding free fatty acids (Sigma). The resulting synthetic [^{14}C]C20:4 NAE had a specific radioactivity of 5.3–5.7 mCi/mmol. Synthetic *N*-arachidonoylphosphatidylethanolamine was obtained likewise but starting from 1,2-*sn*-dipalmitoylphosphatidylethanolamine (Sigma).

2.2. Tissue extraction and NAE and NaPE purification

Molluscs were killed by breaking the adductor muscles, and the shell free tissue (50–100 g wet tissue weight) immediately extracted with chloroform/methanol 2:1 (v:v). In some cases, the extraction was carried out after 6 or 24 h at 8–10°C. In order to purify and characterize the NAEs and NaPEs therein contained, the organic phase, after addition of 1 nmol of *N*-heptadecanoyl (C17:0)-ethanolamine, *N*-*cis*-eicosadienoyl (C20:2)-ethanolamine, $^{2}H_{8}$-C20:4 NAE and $^{2}H_{3}$-C16:0 NAE as internal standards, was brought to dryness and then submitted to a series of chromatographic steps extensively described in previous reports [13,23,26,27], including SiO_2 open bed chromatography (eluted with increasing concentrations of methanol in chloroform), thin layer chromatography (TLC, for column fractions containing NaPEs, on semi-preparative plates developed with chloroform/methanol/NH_4OH 85:15:1 [v:v]) and normal phase high pressure liquid chromatography (NP-HPLC, for column fractions containing NAEs, eluted with increasing concentrations of iso-propanol in *n*-hexane under conditions that do not discriminate among different NAEs [26]). NaPEs were characterized further by digestion with phospholipase D from *Streptomyces chromofuscus* [13,23,27], which released the corresponding NAEs, followed by NP-HPLC [26,27]. Spots and bands on TLC plates were visualized by exposure to iodine vapours. Yields of NAEs on a sample to sample basis were obtained from the area of GC–MS peaks (see below) relative to the internal standards and were not significantly different from those reported previously [13,23,26,27].

2.3. GC–MS analysis of NAEs

Identification and quantitation of NAEs, purified from either lipid extracts or incubates of phospholipase D digestion of NaPE fractions, was obtained by means of gas chromatography–electron impact mass spectrometry (GC–EIMS) of the corresponding acetoxy-derivatives [26]. HPLC fractions with the same retention time as synthetic NAEs were acetylated with 100 μl acetic anhydride in 400 μl of anhydrous pyridine. The reaction product was analyzed by GC–MS carried out on a Hewlett–Packard instrument consisting of a HP-GC 5890 series II apparatus equipped with a HP-5MS capillary column (30 × 0.25 mm, 0.25 μm film thickness, crosslinked 5% HP ME siloxane), and of a HP-MS 5989B quadrupole mass analyzer equipped with an electron impact (EI) source operating at 70 eV and 250°C. In order to improve sensitivity, in some cases acquisition was performed in the selected ion monitoring (SIM) mode which consists in monitoring the presence in the GC eluate of only few selected ion fragments. GC was carried out by using a 3 min isotherm step at 200°C followed by a temperature gradient from 200°C to 300°C at a rate of 5°C/min (elium flow 1 ml/min, injector and transfer line temperature 260°C). The quantities of C20:4 NAE and C16:0 NAE were determined by constructing standard curves for stable isotope dilution GC–EIMS measurement of the two NAEs [13]. 1 nmol of $^{2}H_{8}$-C20:4 NAE and $^{2}H_{3}$-C16:0 NAE plus varied amounts (0, 0.1, 0.5, 1.0, 5.0 and 10.0 nmol) of the two non-deuterated compounds were derivatized and analyzed by GC–MS as described above. Ion current ratios, which represent the ion current peak area for the unlabeled derivative divided by that of the deuterated derivative, were calculated for the molecular ions as well as the fragment ions corresponding to the loss of acetic acid (Section 3). Standard curves were obtained by reporting on the abscissa axis the amount of NAE and on the ordinate axis the ion current ratios. The amounts, in GC–EIMS analyses of mollusc extracts, of acetoxy-C20:4 NAE and -C16:0 NAE, which, under the above elution conditions, were eluted after 17.85 and 13.6 min, respectively, were calculated by interpolation of the corresponding ion current ratios on the standard curves described above. The amounts of *N*-myristoyl-, *N*-stearoyl-, *N*-oleoyl- and *N*-linoleoyl-ethanolamines, eluted respectively, after 10.62, 16.50, 16.10 and 16.00 min were calculated by interpolation of the molecular ion fragment peak areas against external standard calibra-

tion curves constructed using the molecular ion fragment peak areas of known amounts of the corresponding non-deuterated synthetic compounds. In all cases, NAE amounts were corrected keeping into account the yields of the purification/quantitation procedures on a sample to sample basis (see above). Using the SIM acquisition mode, amounts of acetoxy-AE as little as 10 pmol can be detected. NAE amounts lower than 100 pmol (30–40 ng) cannot be measured accurately with the GC–MS quantitation methods described above, as they lay outside the calibration curves used.

2.4. Preliminary characterization of Mytilus FAAH

Shell-free specimen of *M. galloprovincialis* were homogenated in 50 mM Tris–HCl, pH 7.4 using an Ultra-Turrax homogenizer. The homogenate was centrifuged sequentially at 800 × *g* (5 min), 10 000 × *g* (15 min) and 100 000 × *g* (30 min). Pellets from the last two centrifugations (in aliquots of 100–200 μg total proteins) 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 100 000 cpm (24 μM) of [^{14}C]C20:4 NAE (5.3 mCi/mmol, labeled on the

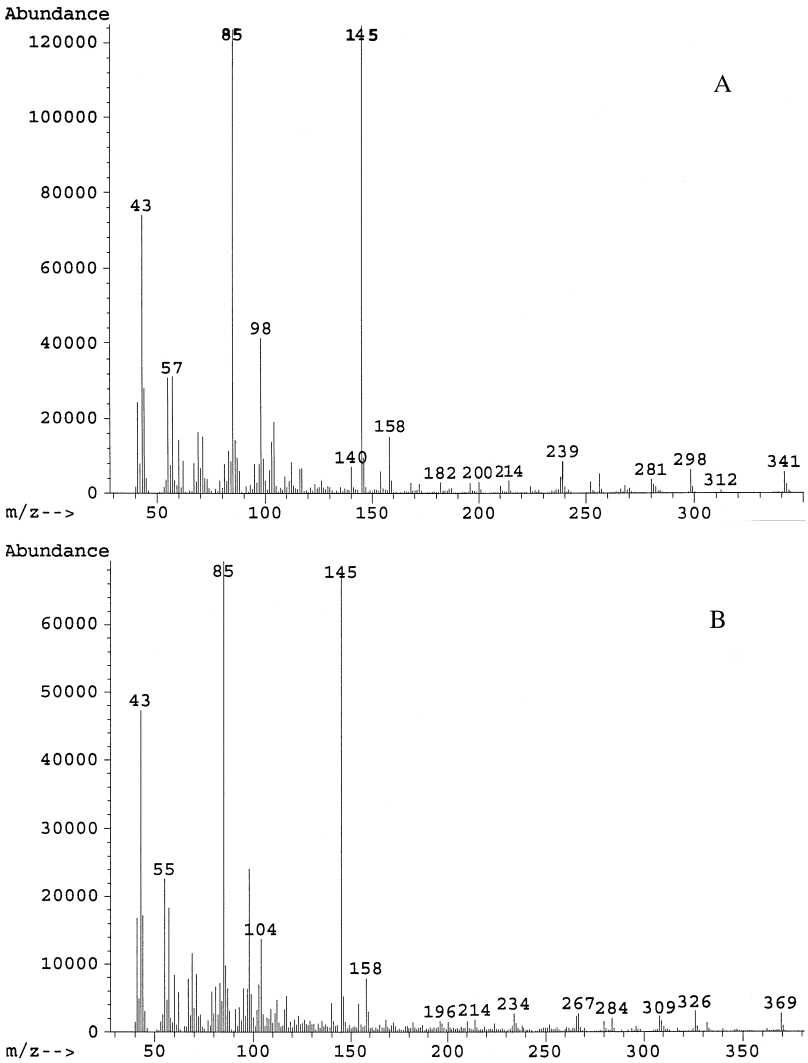


Fig. 1. EIMS spectra of the acetoxy-derivatives of C16:0 NAE (A) and C18:0 NAE (B) from *M. galloprovincialis*. Spectra were run on fractions eluting from a GC column during GC–MS analyses of NP-HPLC-purified, derivatized fractions. The other four bivalve species under study yielded two NAEs with identical fragmentation patterns as those of C16:0 NAE and C18:0 NAE (Table 1).

Table 1
Amounts (ng/g wet tissue weight) of NAEs in lipid extracts from five species of lamellibranch molluscs

Species	14:0	16:0	18:0	18:1	18:2	20:1	20:4	22:2
M. galloprovincialis	2.0 ± 1.0	21.0 ± 3.0	10.6 ± 4.8	2.8 ± 0.8	2.8 ± 0.6	Trace	1.8 ± 0.2	n.d.
Crassostrea sp.	n.d.	16.4 ± 1.6	12.6 ± 1.4	2.6 ± 0.2	n.d.	n.d.	Trace	n.d.
T. decussatus	0.6 ± 0.4	58.8 ± 6.2	35.4 ± 2.7	1.6 ± 1.0	1.6 ± 1.2	n.d.	2.0 ± 0.9	Trace
C. chione	n.d.	28.4 ± 4.5	16.8 ± 3.9	Trace	Trace	n.d.	n.d.	n.d.
V. verrucosa	n.d.	39.2 + 6.1	11.8 ± 2.0	2.7 ± 0.6	2.8 ± 0.8	n.d.	n.d.	Trace

NAE species are indicated by the type of their *N*-fatty acid chain. The amounts were calculated by means of GC–EIMS analyses of the acetoxy-derivatives in comparison with the appropriate internal and/or external standards as described Section 2, and are means ± S.D. of two separate deteminations. n.d. = not detectable; trace = detectable but < 0.3 ng/g wet tissue weight.

ethanolamine moiety). 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]C20:4 NAE was determined by measuring the radioactivity of the aqueous phase by liquid scintillation counting. Experiments were carried out also with different amounts of total membrane proteins (0–500 μg) or with different concentrations (2.4, 5.6, 11.2, 22.5, 34 μM) of [^{14}C]C20:4 NAE, for K_m and V_{max} determination, or in different buffers at different pH values, as described previously [16]. The effect of the specific FAAH inhibitors [16,28] arachidonoyl-trifluoromethyl-ketone (ATFMK, 200 μM), arachidonoyl-diazomethyl-ketone (ADMK, 200 μM), arachidonoyl-chloromethyl-ketone (ACMK, 200 μM), and methyl-arachidonoyl-fluoro-phosphonate (MAFP, 1 μM), the former three compounds synthesized as described previously [28] and the latter purchased from Biomol, was also determined by conducting the assay in identical aliquots of the same 10 000 × *g* pellet suspension and incubating as usual with and without the inhibitors. The effect of C16:0 NAE (100 μM) and oleoyl-amide (100 μM, synthesized as described previously [16]), and of 5 mM EDTA, 0.25 mM PMSF and 0.25 mM *p*-hydroxy-mercuri-benzoate (*p*-HMB), all purchased from Sigma, was likewise studied.

3. Results

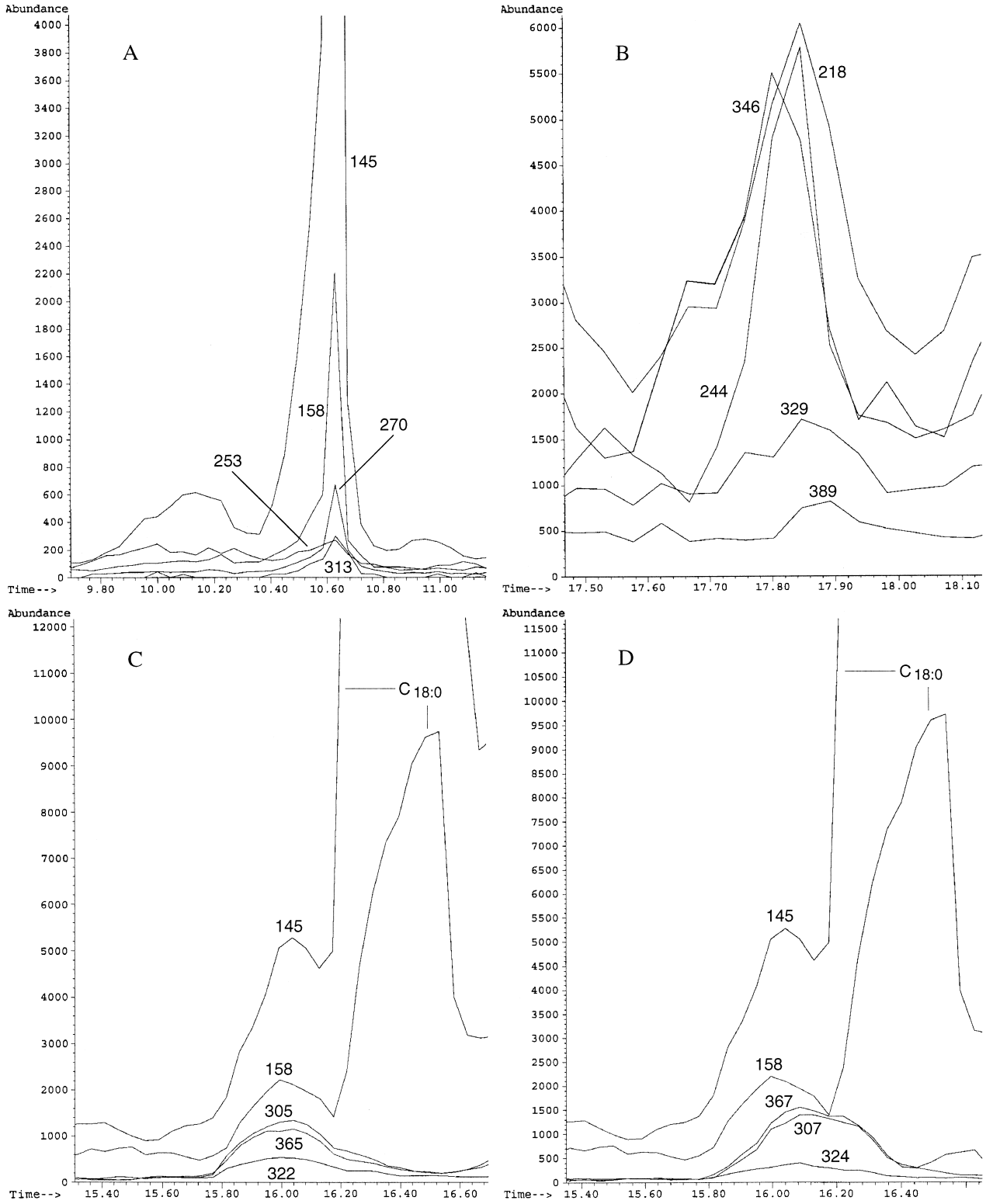
3.1. Edible bivalve molluscs contain NAEs and NaPEs

When NP-HPLC fractions from lipid extracts of the five bivalve species under study and corresponding to C20:4 NAE standard were acetylated and analyzed by GC–MS, several components with the fragmentation typical of NAEs were found. The presence of ion fragments at m/z = 85, 145 and m/z = 98 and 158 were indicative of *O*-acetyl-AEs as a class of compounds [26], and were due to β-clea-

Table 2
Amounts (ng/g wet tissue weight) of NAEs in lipid extracts of three species of lamellibranch molluscs submitted to the treatment shown

Species	16:0	18:0	18:1	18:2	20:4
M. galloprovincialis (24 h post-mortem)	53.0 ± 3.9	32.8 ± 2.6	3.6 ± 0.7	3.5 ± 0.6	3.0 ± 0.4
Crassostrea sp. (6 h post-mortem)	39.8 ± 5.7	11.0 ± 1.9	3.5 ± 0.5	Trace	2.6 ± 0.9
Crassostrea sp. (24 h post-mortem)	45.7 ± 1.0	23.4 ± 8.0	3.9 ± 0.4	Trace	4.9 ± 1.8
T. decussatus 20 min boiling	51.6 ± 7.8	23.4 ± 9.6	1.3 ± 1.0	1.1 ± 0.2	3.8 ± 0.8

NAE species are indicated by the type of their *N*-fatty acid chain. The amounts were calculated by means of GC–EIMS analyses of the acetoxy-derivatives in comparison with the appropriate internal and/or external standards as described in the Section 3, and are means ± S.D. of two separate deteminations. n.d. = not detectable; trace = detectable but < 0.3 ng/g wet tissue weight.


A
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270
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313
B
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218
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244
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389
C
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C 18:0
145
158
305
365
322
D
Abundance
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C 18:0
145
158
367
307
324

Table 3
Amounts (pmol/g wet tissue weight) of NaPEs from lipid extracts from five species of lamellibranch molluscs. NaPEs were quantitated as the corresponding NAEs released from the digestion with *S. chromofuscus* phospholipase D [13,24] of NaPE-like fractions purified from lipid extracts as described in the Section 3 and previously [13,23]. NaPE species are indicated by the type of their *N*-fatty acid chain. The amounts were calculated by means of GC–EIMS analysis of the corresponding acetoxy-NAE in comparison with the appropriate internal and/or external standards as described in the Section 3, and are means ± S.D. of two separate deteminations

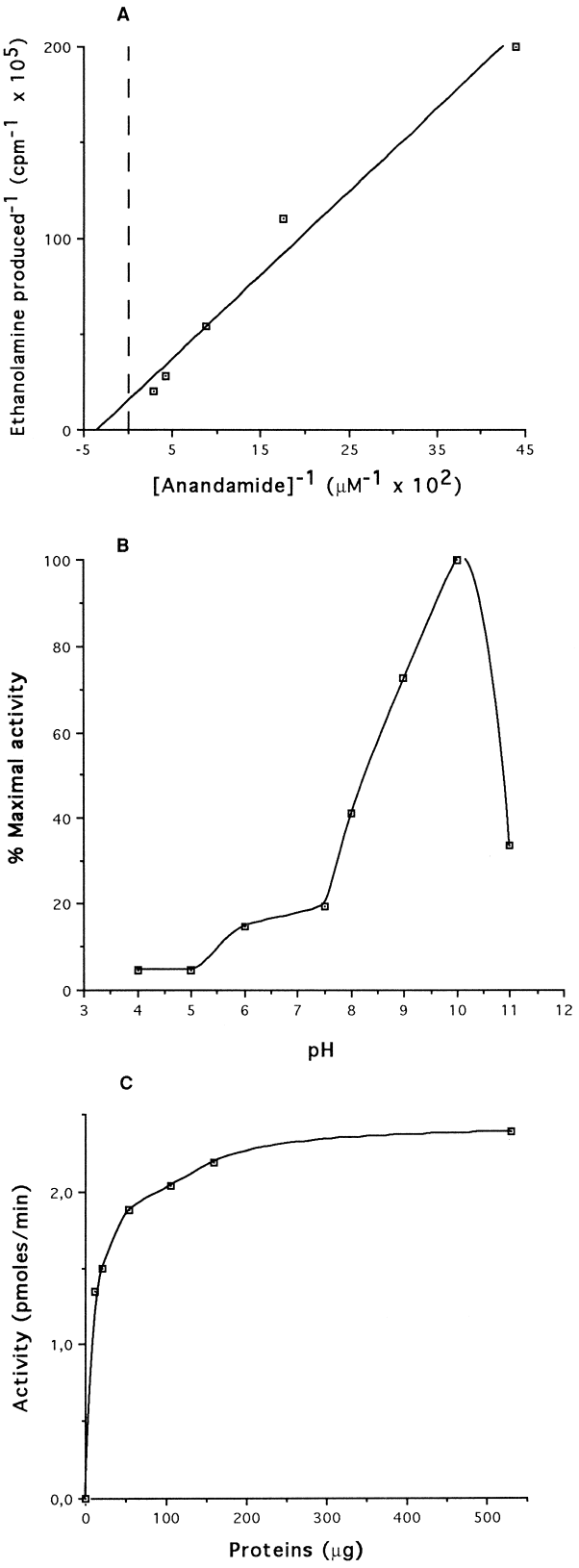
Species	16:0	18:0	18:1	18:2	20:4
M. galloprovincialis	27.6 ± 5.7	9.9 ± 1.2	Trace	n.d.	1.9 ± 0.9
(24 h post-mortem)	(200.5 ± 37.7)	(109.7 ± 15.6)	(11.0 ± 2.3)	–	(3.6 ± 0.4)
Crassostrea sp.	6.0 ± 3.5	4.0 ± 1.9	n.d.	n.d.	n.d.
(24 h post-mortem)	(95.5 ± 9.7)	(59.7 ± 7.9)	–	–	–
T. decussatus	7.0 ± 2.7	2.7 ± 0.5	n.d.	n.d.	n.d.
C. chione	7.0 ± 1.8	3.0 ± 1.0	n.d.	n.d.	n.d.
V. verrucosa	11.4 ± 2.1	2.9 ± 0.9	n.d.	1.4 ± 0.7	n.d.

n.d. = not detectable. As no NaPE internal standard was available, these values were not corrected for extraction and purification yields.

vages accompanied by McLafferty rearrangements and to γ-cleavages of the molecular ions after or before loss of acetic acid, respectively, (Figs. 1 and 2). The molecular ion peaks at m/z = 313, 341, 369, 367, 365, 389, 395 and 421, accompanied by fragment ions at −43 and/or −60 (due to loss of CH_3CO– or acetic acid) were used to identify the single *O*-acetyl-NAEs present in each GC peak, and indicated the presence of either *N*-myristoyl (C14:0)-, *N*-palmitoyl (C16:0)-, *N*-stearoyl (C18:0)-, *N*-oleoyl (C18:1)-, *N*-linoleoyl (C18:2)-, *N*-arachidonoyl (C20:4)-, *N*-eicosaenoyl (C20:1)- or *N*-docosadienoyl (C22:2)-ethanolamines, respectively, in the lipid extracts from each species (Figs. 1 and 2 and data not shown). The complete EIMS fragmentation patterns of acetoxy-NAEs have been extensively described previously [26]. In most cases, the presence of the less abundant NAEs, including C20:4 NAE, could be detected only by using the SIM mode of acquisition (Fig. 2(a)–(d)). In all cases, C16:0 NAE and C18:0 NAE were the most abundant metabolites, whereas C20:4 NAE, C18:1 NAE, C18:2 NAE, C14:0 NAE, C20:1 NAE and C22:2 NAE could be detected only in some of the five species under study (Table 1). The largest variety of NAEs was detected in the mussel *M. galloprovincialis* and in the clam *T. decussatus*; the three species of clams under study exhibited the highest total NAE contents, the highest levels (100.0 ng/g wet weight tissue) being observed in *T. decussatus* (Table 1).

Some of the NaPEs corresponding to the above NAEs were also found in lipid extracts of the bivalves under investigation. NaPEs were measured as NAEs after digestion of the purified phospholipids with *S. chromofuscus* phospholipase D. The yield of this quantitation technique, which allows conclusive structure identification of NaPEs, is lower than that of NAEs (25–35% vs. 45–55%, unpublished observations and [23,27]) and this might explain in part why not all the NaPEs corresponding to the NAEs

Fig. 2. GC–EIMS fragmentograms of the acetoxy-derivatives of C14:0 NAE (A), anandamide (C20:4 NAE) (B), C18:2 NAE (C), C18:1 NAE (D) from *M. galloprovincialis*. GC–EIMS analyses were run using the selected ion monitoring mode at m/z values corresponding to the molecular ions or to fragment ions due to β-cleavages accompanied by McLafferty rearrangements (m/z = 145), to γ-cleavages (m/z = 158), to loss of acetic acid (molecular ion −60) or of an acetyl group (molecular ion −43). In (C) and (D) peaks at m/z = 158 and 145 eluting after 16.50 min are shown also for *N*-stearoylethanolamine (indicated as C18:0). Peaks at these m/z values are common to C18:1 NAE and C18:2 NAE, which are eluted after similar retention times (16.1 and 16.0 min) under the chromatographic conditions used. In (B), peaks at m/z = 244 and 218 are, respectively, due to β-cleavage (corresponding to loss of 145) and δ-cleavage followed by rearrangement [23]. The other four bivalve species under study yielded analogous fragmentograms for the four NAEs shown here, except for C18:2 NAE, which was not detected in *Crassostrea* sp., and anandamide (C20:4 NAE), which was not detected in *C. chione* and *V. verrucosa* (Table 1).



found in bivalve molluscs were detected. *N*-palmitoyl- and *N*-stearoyl-phosphatidylethanolamine were detected in all species. *N*-arachidonoyl- and *N*-oleoyl-phosphatidylethanolamine could be observed only in *M. galloprovincialis*, and *N*-linoleoyl-phosphatidylethanolamine only in *V. verrucosa* (Table 3).

3.2. NAE levels increase in post-mortem but not in boiled bivalve tissue

In the mussel *M. galloprovincialis* and the oyster *Crassostrea* sp., when lipids were not extracted immediately after opening the shell but 6 and/or 24 h post-mortem, a considerable increase of NAE levels could be detected (Table 3). Conversely, no statistically significant variation was observed in NAE levels of the clam *T. decussatus* when specimens of this species were extracted after a 20 min boiling in water (Table 2). Interestingly, NaPE levels in both *M. galloprovincialis* and *Crassostrea* sp. were also found to increase sensibly in 24 h post-mortem tissue (Table 3).

3.3. M. galloprovincialis contains a FAAH-like enzyme

When examined for their capacity to catalyze the hydrolysis of [^{14}C]C20:4 NAE to [^{14}C]ethanolamine, homogenates from whole, shell-free *M. galloprovincialis* specimens were found to contain measurable levels of an amidohydrolase enzymatic activity. Like FAAH from neuronal and peripheral murine and porcine cells [13,16–19], this activity was present in fractions obtained from both 10 000 × *g* and 100 000 × *g* pellets (microsomes), but was very low

Fig. 3. Preliminary characterization of [^{14}C]C20:4 NAE hydrolyzing activity from 10 000 × *g* pellets of *M. galloprovincialis* whole homogenates. (A) Lineweaver–Burk profile obtained at different concentrations of [^{14}C]C20:4 NAE; (B) pH-dependency profile; (C) dependency on the total amount of proteins. In (A) enzyme activity is expressed as radioactivity associated with ethanolamine released from of [^{14}C]C20:4 NAE hydrolysis. The enzyme assay was carried out as described in the Section 2. Data are representative of three separate experiments carried out in duplicates. S.E.M. bars are not shown for simplicity and were never higher than 10%.

Table 4
Effect of various substances on [^{14}C]C20:4 NAE hydrolyzing activity from 10 000 × *g* pellets of *M. galloprovincialis* whole homogenates. The effects are expressed as percent of the activity with no substance added, and are means ± S.D. of three separate experiments

None	EDTA 5 mM	Oleamide 100 μM	C16:0 NAE 100 μM	ADMK 200 μM	ACMK 200 μM	ATFMK 200 μM	MAFP 1 μM	PMSF 250 μM	*p*-HMB 250 μM
100	101.8 ± 9.8	68.4 ± 3.2	89.9 ± 5.0	70.4 ± 2.5	68.8 ± 1.2	70.1 ± 1.6	21.6 ± 0.1	36.9 ± 2.3	80.5 ± 3.0

in the 100 000 × *g* supernatant (cytosol) from the homogenate (41.4 ± 0.6%, 21.1 ± 0.9% and 7.0 ± 0.1% of total activity, means ± s.d., $n = 3$, respectively). When using 10 000 × *g* preparations, apparent K_m and V_{max} values for C20:4 NAE were 29.6 ± 1.7 μM and 73 ± 2.1 pmol/mg protein/min, respectively (Fig. 3(a)). Moreover, the enzyme displayed a typical pH dependency profile (Fig. 3(b)), with apparent active site pK_a values of 6.0 and 10.0, very similar to those previously reported for FAAH enzymes [13,16–18]. The enzyme activity increased linearly with increasing amounts of total proteins up to 20 μg, and was maximal at 500 μg total proteins (Fig. 3(c)). Finally, the enzyme was sensitive to pre-treatment with the typical FAAH covalent inhibitors PMSF and MAFP [28], whereas the reversible FAAH inhibitors ADMK, ACMK and ATFMK [16,28] and the sulphydryl group reagent *p*-HMB were less effective (Table 4). The enzymatic hydrolysis of [^{14}C]C20:4 NAE was inhibited by 100 μM oleoyl-amide but not by 100 μM C16:0 NAE (Table 4), thus suggesting that *Mytilus* FAAH-like enzymatic activity, in analogy with FAAH from mouse neuroblastoma cells, porcine brain and rat liver [16–18], recognizes as a substrate oleoyl-amide more efficiently than C16:0 NAE.

4. Discussion

In this paper, we have provided evidence for the presence of several long chain fatty acid ethanolamides in lipid extracts from five species of edible bivalve (lamellibranch) molluscs. Among the NAEs detected, C16:0 NAE, whose possible physiological role as an autacoid anti-inflammatory mediator has been recently described [6,13], was found in all mussel, clam and oyster species studied, whereas C20:4 NAE, the proposed ligand for the central CB1 cannabinoid receptor, was present only in *M. galloprovincialis*, *T. decussatus* and *Crassostrea* sp. The amounts of single NAEs detected in each species ranged from > 0.3 to 58.8 ng/g wet tissue weight (i.e. up to 197 pmol/g wet tissue weight), were not sensibly different from those detected in sea urchin ovaries [23] or in murine brain [29], testes [12] and leukocytes [13], and 6–10 times lower than those reported for pig, sheep and cow brain [30]. Like for all mammalian tissues examined so far, C16:0 NAE and C18:0 NAE were the most abundant NAEs in all bivalve species examined. C20:4 NAE was a minor component and its levels were 5–18 times lower than those reported for human brain, although similar to those found in human spleen and heart [31]. In agreement with previous studies carried out with bovine and porcine brain [30], NAE levels were found to increase considerably when the molluscs were extracted 24 h post-mortem. Since NAE (and NaPE) biosynthesis has been correlated to cell injury [1], this phenomenon might be due to cell damage occurring after death. On the other hand, tissue boiling did not significantly affect NAE levels in *T. decussatus*, probably due to inactivation of both biosynthetic and degradative enzymes of NAE metabolism. C16:0 NAE, which does not activate the central CB1 cannabinoid receptor and is devoid of psychotropic cannabimimetic actions [4,5], has been described to exert anti-inflammatory and anti-anaphylactic actions in guinea pigs when administered intraperitoneally at doses as little as 60–300 ng/kg body weight [1]. These doses are comparable to those detected here in a few grams of *M. galloprovincialis* and *Crassostrea* sp. after 24 h post-mortem (46–53 ng/g wet tissue weight), or of *T. decussatus* even after 20 min boiling (51.6 ng/g wet tissue weight). However, a more recent study [7] showed that higher oral doses of C16:0 NAE (0.1–1 mg/kg body weight) are necessary to exert an anti-inflammatory action against

mast-cell-mediated hyperreactivity in rodents, while the original report of the identification of C16:0 NAE from soybean lecithin stated that ethanolamine exhibited a similar anti-inflammatory action [32]. Therefore, further investigations are needed before drawing any conclusion on the possible effects of diet-derived bivalve C16:0 NAE. Pharmacological actions, such as an effect on fast sodium channels [33], have been described also for C18:0 NAE [1], but in this case no in vivo studies has ever been reported, and it is not possible to draw any conclusion as to whether the amounts of this NAE measured in edible bivalve molluscs can have any pharmaco-toxicological relevance. C18:1 NAE has been reported to exert platelet anti-aggregatory activity and to inhibit lipid peroxidation in vitro [1]. The finding in chocolate of this NAE as well as of C18:2 NAE, in levels higher than those reported here for edible bivalves [25], was hypothesized to result, upon chocolate ingestion, in a possible increase of endogenous C20:4 NAE levels, since these two NAEs inhibit the enzymatic hydrolysis of C20:4 NAE in both intact cells and cell-free homogenates [25]. Also in this case, however, no data are available on the pharmacological effects of orally administered C18:1 NAE and C18:2 NAE. Finally, the very low amounts (up to 5 ng/g wet tissue weight) of C20:4 NAE detected in this study, even in post-mortem tissue, and the previous observation that 50 mg/kg body weight of orally administered C20:4 NAE are necessary in order to observe its typical psychotropic actions [34], seem to rule out that edible bivalves may be a dietary source of this psychoactive cannabimimetic substance, particularly in view of the high levels of FAAH found in tissues of the gastrointestinal tract [35].

The presence in lipid extracts of some NAEs as both free ethanolamides and esters with phosphatidic acid suggests that these metabolites might be biosynthesized in bivalve molluscs through the same mechanism proposed for mammalian [1,11–15], sea urchin [23] and plant [24] tissues, i.e. the phospholipase D-catalyzed hydrolysis of NaPEs. This hypothesis is also supported by the finding that, like NAEs, NaPE levels also were found here to increase in post-mortem mussel and oyster tissue. Studies aimed at finding further experimental support to this suggestion were not within the scope of the present investigation, and must be awaited before drawing any conclusion as to whether NAEs are produced from the hydrolysis of NaPE precursors or via the energy-free condensation of the corresponding fatty acids with ethanolamine [1,5]. On the other hand, we have reported here the presence of an enzymatic activity in *M. galloprovincialis*, for the hydrolysis of C20:4 NAE to ethanolamine, whose sub-cellular distribution and catalytic properties are almost identical to those previously reported for FAAH, the enzyme responsible for C20:4 NAE hydrolysis and subsequent inactivation in mammalian tissues [13,16–18]. This finding, together with the presence in this mollusc of C20:4 NAE and of its putative biosynthetic precursor *N*-arachidonoyl-phosphatidylethanolamine, lends substantial support to the previous hypothesis [21] that this polyunsaturated NAE may be an endogenous cannabimimetic modulator of immune function in *Mytilus* sp. This hypothesis was based on the finding [21], in *M.edulis* immunocytes, of: (1) selective and high affinity C20:4 NAE binding sites, and (2) a stimulatory effect by exogenous C20:4 NAE on basal NO release from these cells. However, no evidence for the presence of either C20:4 NAE, its phospholipid precursor or the enzyme responsible for C20:4 NAE inactivation had been described, until the present report, in mussels or in any other species of the phylum Mollusca. The relatively low V_{max} value observed here for C20:4 NAE enzymatic hydrolysis by particulate fractions prepared from whole, shell-free molluscs, seems to suggest that *M. galloprovincialis* FAAH-like enzyme may be expressed only in select specialized tissues of the mussel. Therefore, further studies will be required in order to clarify the cellular source of both C20:4 NAE and FAAH in *Mytilus* as well as in other genera of bivalve molluscs, and to gain further support to the hypothesis of a bivalve immunomodulatory role for C20:4 NAE. Moreover, the biological function of C16:0 NAE as well as of other NAEs found here in edible lamellibranch molluscs also needs to be fully investigated in order to clarify the physiological meaning of their occurrence in these molluscs. In any event, the data reported in the present study, together with our previous finding of the NAE metabolic pathway in sea urchins [23], allow to conclusively indicate that the NAE/endogenous cannabinoid system is an ancient signalling system that has been conserved for several hundred million years [20–23].

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