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Research report

Evidence for an endocannabinoid system in the central nervous system of the leech *Hirudo medicinalis*

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Abstract

In invertebrates, like Hydra and sea urchins, evidence for a functional cannabinoid system was described. The partial characterization of a putative CB1 cannabinoid receptor in the leech *Hirudo medicinalis* led us to investigate the presence of a complete endogenous cannabinoid system in this organism. By using gas chromatography–mass spectrometry, we demonstrate the presence of the endocannabinoids anandamide (*N*-arachidonoylethanolamine, 21.5±0.7 pmol/g) and 2-arachidonoyl-glycerol (147.4±42.7 pmol/g), and of the biosynthetic precursor of anandamide, *N*-arachidonylphosphatidyl-ethanolamine (16.5±3.3 pmol/g), in the leech central nervous system (CNS). Anandamide-related molecules such as *N*-palmitoylethanolamine (32.4±1.6 pmol/g) and *N*-linolenoylethanolamine (5.8 pmol/g) were also detected. We also found an anandamide amidase activity in the leech CNS cytosolic fraction with a maximal activity at pH 7 and little sensitivity to typical fatty acid amide hydrolase (FAAH) inhibitors. Using an antiserum directed against the amidase signature sequence, we focused on the identification and the localization of the leech amidase. Firstly, leech nervous system protein extract was subjected to Western blot analysis, which showed three immunoreactive bands at ca. ~42, ~46 and ~66 kDa. The former and latter bands were very faint and were also detected in whole homogenates from the coelenterate *Hydra vulgaris*, where the presence of CB1-like receptors, endocannabinoids and a FAAH-like activity was reported previously. Secondly, amidase immunocytochemical detection revealed numerous immunoreactive neurons in the CNS of three species of leeches. In addition, we observed that leech amidase-like immunoreactivity matches to a certain extent with CB1-like immunoreactivity. Finally, we also found that stimulation by anandamide of this receptor leads, as in mammals, to inhibition of cAMP formation, although this effect appeared to be occurring through the previously described anandamide-induced and CB1-mediated activation of nitric oxide release. Taken together, these results suggest the existence of a complete and functional cannabinoid system in leeches.

Theme: Neuromodulators, modulators, transporters, and receptors

Topic: Transmitters in invertebrate

Keywords: Leech; Central nervous system; Endocannabinoid; Anandamide amidase; Cannabinoid receptor; Localization

Abbreviations: 2-AG, 2-arachidonoylglycerol; CNS, Central nervous system; FAAH, Fatty acid amide hydrolase; GC-EIMS, Gas chromatography–electron impact mass spectrometry; L-NAME, *N*-omega-nitro-L-arginine methyl ester; NArPE, *N*-arachidonoyl-phosphatidyl-ethanolamine; NO, Nitric oxide; NP-HPLC, Normal phase high pressure liquid chromatography; PLD, Phospholipase D; SNAP, *S*-nitroso-*N*-acetyl-penicillamine.

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1. Introduction

For centuries marijuana, derived from the Indian hemp *Cannabis sativa*, has been used for its medicinal as well as its psychotropic effects [28]. Trans-Δ^9-tetrahydrocannabinol (THC) is the major active psychotropic component of cannabis [19]. Central nervous system responses to THC and synthetic cannabimetic drugs include the therapeutically beneficial analgesia, attenuation of nausea and vomiting in cancer chemotherapy, appetite stimulation in wasting syndromes, and reduction of intestinal motility [2]. These effects are mediated by receptors, two of which have been identified: the CB1 and CB2 receptors. The CB1 receptor was cloned in 1990 from the rat [35], and later from human [20] and mouse [6]. Additionally, a splice variant of CB1, named CB1a, has been discovered in the rat [48], and this latter appears to be a relatively minor component of the total CB1 message. CB1 is distributed primarily in the CNS [18], in peripheral neurons and in gonads [27,43]. The CB2 receptor was cloned by Munro et al. from a human promyelocytic leukemia cell HL60 cDNA library [39]. It exhibits only 44% homology with CB1. Both receptors belong to the G-coupled seven transmembrane domain family. It was demonstrated that activation of these receptors is coupled to inhibition of adenylate cyclase [29] and to nitric oxide release [51]. The first endogenous cannabinoid was isolated from porcine brain by Mechoulam and co-workers [11] and was found to be an unsaturated fatty acid ethanolamide, *N*-arachidonoylethanolamine (AEA or anandamide). A second 'endocannabinoid', 2-arachidonoylglycerol (2-AG), was found in rat brain [52] and in canine gut [37]. The biosynthesis of anandamide and 2-AG in the brain is accompanied by anandamide-related molecules such as *N*-palmitoylethanolamine, *N*-oleoylethanolamine, *N*-linolenoyl-ethanolamine [12,25], and by non-cannabimimetic monoacylglycerols [14,31].

The endocannabinoids have been proposed to act as neuromodulators [16]. Anandamide has a short duration of action in vivo [56] because of its facilitated diffusion through the cell membranes of neurons and astrocytes [17] via a selective transporter [26]. It is not clear yet whether 2-AG also uses the same transporter [13,44]. Furthermore, anandamide is inactivated by enzymatic breakdown [10]. The enzyme responsible for anandamide hydrolysis was first named anandamide amidase and later fatty acid amide hydrolase (FAAH). This enzyme was cloned from rat [7], mouse and human [21] liver. FAAH, a serine hydrolase [40] and a member of the amidase family [30,42], is an integral membrane protein that hydrolyzes anandamide [1,32] and 2-arachidonoylglycerol [15,22,55]. FAAH was detected in large neurons of brain [53], preferentially in the hippocampus [54], but also in the liver [53], retina [57], vascular tissues [3] and uterus [41]. This distribution overlaps to a large extent with the localization of cannabinoid receptors [54].

The endocannabinoid system comprises the ligands (anandamide, 2-AG), the anandamide precursor (*N*-arachidonylphosphatidyl-ethanolamine, NArPE), the cannabinoid receptors (CB1, CB1a, CB2), the anandamide membrane transporter and FAAH. Recently, the question of the conservation in the course of evolution of such system has been examined. In invertebrates, there is evidence for the existence of an endocannabinoid system, in particular, in sea urchins [4,46], in the coelenterate *Hydravulgaris* [8] and in the mollusk *Mytilus edulis* [47,51]. Together with cannabinoid binding sites and endogenous agonists, enzymatic activities capable of catalyzing the hydrolysis of anandamide were detected in these animals. Finally, we have partially characterized a putative CB1-like receptor in the CNS of the leech *Hirudo medicinalis* and have provided preliminary evidence of a hydrolytic enzyme for anandamide in leech brain [49,50].

Here, we demonstrate the presence in leech CNS of the endogenous agonists of the CB1-like receptor and of their biosynthetic precursors, as well as of an amidase enzyme distinct from FAAH. Moreover, by using an antiserum raised against the amidase signature sequence we describe a strong amidase immunoreactivity in the leech *H. medicinalis* and in two others species, which partially matches with CB1-like immunoreactivity. Finally, we demonstrate that anandamide stimulation cause NO release, thereby leading to the inhibition of adenylate cyclase.

2. Experimental procedures

2.1. Animals

Leeches of three species were used in this study: (i) *Hirudo medicinalis*, purchased from Ricardimpex (Bordeaux); (ii) *Erpobdella octoculata*, collected at Harchies (Belgium); and (iii) *Theromyzon tessulatum* reared in our laboratory and whose life cycle is subdivided in stages defined by taking as indicators the three blood meals and sex maturation [34]. *Hydra vulgaris* polyps were equilibrated at room temperature in physiological solution (1 mM $CaCl_2$, 0.1 mM $NaHCO_3$, buffered with 1 mM Tris–HCl, pH 7.35) before the test. Adult male Wistar rats (animal use accreditation by the French Ministry of the Agriculture No. 04860) were used in this study and maintained under standard care.

2.2. Dissections and surgical procedures

Leeches were anesthetized with chloretone (0.01%) and pinned flat ventral side up in leech saline solution [38]. CNS (brain and complete nervous cord) were dissected and placed in liquid nitrogen. Rats were anesthetized before their liver was removed and placed in nitrogen.

2.3. Materials

Anandamide, [^{14}C]anandamide (5.7 μCi/mmol), *N*-palmitoylethanolamine and [^{14}C]*N*-palmitoylethanolamine (5.5 μCi/mmol) were synthesized as described by Fontana et al. [24]. [^{3}H]*N*-palmitoylphosphatidyletanolamine, deuterated anandamide and 2-AG were synthesized from [^{3}H]palmitic acid or [^{2}H]$_{8}$arachidonic acid as described previously [5,24]. *p*-Hydroxymercuribenzoate was purchased from Sigma, and methylarachidonoyl-fluorophosphonate and arachidonoyltrifluoromethylketone from Biomol.

2.4. Purification and quantification of endocannabinoids

The extraction, purification and quantification of anandamide, 2-AG and NArPE from dissected nervous system of *Hirudo medicinalis* require a set of different biochemical steps [5]. First, the total lipids were extracted with chloroform/methanol/Tris buffer pH 7.5 (2:1:1, v/v) containing internal standard ([^{2}H]$_{8}$anandamide, [^{2}H]$_{8}$2-AG, and [^{3}H]N-palmitoylphosphatidylethanolamine) and dried under nitrogen. Lipids fractionations were made using a SiO_2 open bed chromatography and resultant fractions were obtained by eluting the column with 9:1 and 1:1 (by vol.) chloroform/methanol. The NArPE-containing fraction (1:1 by vol.) was digested with phospholipase D (PLD) from *S. chromofuscus* in order to release anandamide [5]. The 2-AG/anandamide-containing fraction (9:1 by vol.) and lipids obtained from the PLD digestion of the NArPE-containing fraction were both subjected to normal phase high pressure chromatography (NP-HPLC) eluted with increasing concentrations of iso-propanol in *N*-hexane. The amounts of NArPE were calculated from the amounts of anandamide produced from PLD digestion and corrected by taking into account the recovery of radioactivity in HPLC fractions corresponding to [^{3}H]*N*-palmitoylethanolamine (i.e. the product of the PLD digestion of [^{3}H]*N*-palmitoylphosphatidylethanolamine used as internal standard). NP-HPLC fractions from incubates of PLD digestion of NArPE-containing fraction or from 2-AG/anandamide-containing fraction, with the same retention time of synthetic anandamide (35 min) and synthetic 2-AG (21 min), were lyophilized and derivatized with 20 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide containing 1% of trimethylchlorosilane for 2 h at room temperature, prior to analysis by gas chromatography–electron impact mass spectrometry (GC–EIMS) in the selected ion monitoring mode. Ions were selected at m/z = 427, 419, 412 and 404 for anandamide (corresponding to the molecular ions and the −15 mass unit fragment ions of [^{2}H]$_{8}$anandamide and non-deuterated anandamide, respectively), and at m/z = 530, 522, 515 and 507 for 2-AG (corresponding to the molecular ions and the −15 mass unit fragment ions of [^{2}H]$_{8}$2-AG and nondeuterated 2-AG, respectively). The amount of 2-AG and anandamide were calculated from the area peak ratios of the −15 fragment for the non-deuterated versus the deuterated compounds. Anandamide from PLD digestion of NArPE was also quantitated by comparison with the corresponding deuterated standard added after the digestion.

2.5. Anandamide and N-palmitoylethanolamine hydrolase assay

Briefly, CNS dissected from *Hirudo medicinalis* was crushed and homogenized in 50 mM Tris–HCl (pH 7.5) by using a Dounce homogenizer. The whole homogenate was first centrifuged at 800 *g* (5 min) in order to eliminate the non-crushed tissue. The whole homogenate was then centrifuged at 10,000 *g* and 100,000 *g*. Whole homogenate or supernatant from the last centrifugation corresponding to the cytosolic fraction (~300 μg total proteins) were incubated in 50 mM Tris–HCl (pH 9.0) at 37°C in the presence of [^{14}C]anandamide (25,000 c.p.m.) or [^{14}C]palmitoylethanolamide (25,000 c.p.m.). The formation of [^{14}C]ethanolamine, quantitated as described previously [9], was taken as a measure of anandamide hydrolysis or *N*-palmitoylethanolamine hydrolysis. Incubations were also carried out in different buffers at different pH values or in the presence of FAAH inhibitors. N18TG2 cell membranes, which contain FAAH, were used as a positive control for the effect of the inhibitors [36].

2.6. Peptide synthesis

The peptide corresponding to the catalytic site of the amidases (amidase signature sequence: GGSSGGEGALI) was synthesized according to classical Fmoc chemistry on *p*-alkoxybenzyl alcohol resin on a 25-μmol scale with a ABI 432 A. Conventional side chain-protecting groups were used 2,3,5,7,8-pentamethylchroman 6-sulfonyl (Arg), triphenylmethyl (Cys, Asn and Glu), *t*-butoxycarbonyl (Lys) and *t*-butyl (Ser and Tye). Briefly, a standard Fmoc deprotection was used in conjunction with benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate/*N*-hydroybenzotriazole/diisopropyethylamine. Coupling reactions were allowed to proceed for 15 min. After two dimethylformamide washings, a second coupling with the same excess of reagents was routinely performed. At the end of the synthesis, the resin was washed with dichloromethane and ether and dried under nitrogen. The final trifluoroacetic acid cleavage was performed in the same reaction vessel with 5 ml of reacting buffer (100 μl trisopropylsilane, 100 μl ethanedithiol and 1.8 ml trifluoroacetic acid) during 150 min. At the end, the peptide was drained in a 40-ml polypropylene centrifuge tube previously filled with 25 ml of cold ether. The peptide was then centrifuged, and the pellet was washed twice with ether. After the last centrifugation, the pellet containing the reduced peptide was taken up in 0.1 M ammonium acetate buffer (pH 8.5) at a concentration of 35 mg/l and was

allowed to refold via air oxidation for 17 h at room temperature with constant stirring. The refolded peptide was purified by semi-preparative reversed-phase chromatography (Aquapore RP300 column, 250×7.0 mm) with a linear gradient of 1%/min in acidified water (0.1%) at a flow rate of 1 ml/min.

2.7. Antibodies

Polyclonal amidase antiserum raised against a consensus signature sequence of vertebrate and invertebrate FAAH, corresponding to the catalytic site of the amidases (Amidase Signature Family) [30], was obtained in our laboratory by immunizing one rabbit with the synthetic peptide GGSSGGEGALI coupled with glutaraldehyde to thyroglobulin according to procedure described elsewhere [45]. Control of specificities was performed by preadsorbing the antibody by the homologous antigen at a concentration of 350 μg/ml of pure serum. Rabbit anti-human CB1 polyclonal antibody was purchased from Chemicon (Temecula, USA). The antibody was raised against the *N*-terminal region of human CB1 (^{23}NKSLSSFKENEENIQC^{38}). This sequence is upstream of the leech-CB1 like partially cloned by Stefano et al. [50]. The specificity of this antibody was described in Dove Petit et al. [18].

2.8. Production of recombinant rat FAAH

To confirm that the polyclonal antiserum against the catalytic site of vertebrate and invertebrate FAAH recognizes effectively the fatty acid amide hydrolase, an eukaryotic expression vector containing rat FAAH (pcDNA3-FAAH, kindly provided by Dr Dale G. Deutsch, Department of Biochemistry and Cell Biology, State University of New York at Stony Brook), was expressed transiently in COS-7 cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, and L-glutamine (complete DMEM) at 37°C and 5% CO_2 atmosphere to 70% confluency, and then washed with serum free medium. Cells (2.10^5 cells/100-mn dish) were incubated in a DNA/DEAE–dextran–chloroquine mixture (1.6 μg/ml pcDNA3-FAAH, 10 mg/ml DEAE–dextran, 2.5 mM chloroquine in 5 ml of serum-free medium) for 4 h. The cells were then washed with 5 ml of a phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4) containing 10% of dimethyl sulfoxide (DMSO) to increase DNA uptake. After 2 min of incubation, complete medium was added and cells were incubated for 72 h with one change of medium at 24 h. The COS-7 cells were washed two times with PBS and with PBS plus 0.5 mM EDTA, harvested with the aid of trypsin–EDTA and suspended in 0.1 ml of 250 mM Tris–HCl buffer (pH 7.4) after centrifugation at 2000 *g* for 10 min. The cells were submitted to heat shock (−70°C/+37°C for 5 min each three times) and sonicated for 3 s. The lysate was centrifuged at 8000 *g* for 10 min at 4°C. The supernatant was cleaned on a 0.22 μm filter and successively filtered on a cut-off (Pall Filtron) of 100 kDa and 30 kDa. The yielded supernatant was used as enzyme source.

2.9. Western immunoblotting

Analytical SDS–polyacrylamide gel electrophoresis (SDS–PAGE 10%) was performed as described previously [33] on lysates from *H. medicinalis* dissected nervous system, whole *Hydra vulgaris*, rat liver and on recombinant FAAH enzyme source. Western blot analysis was then carried out with the amidase antiserum. Briefly, rat liver or *H. medicinalis* dissected nervous system and whole *Hydra vulgaris* were homogenized using a Dounce homogenizer in lysis buffer (1 mM EDTA, 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM Na-ortovanadate, 1 mM Na-fluoronate, 1% NP-40, 0.1% SDS, 1% Triton, 0.25% Na-desoxycholate, 1 mM PMSF, 1 mg/ml serine proteases inhibitors), incubated at 4°C for 30 min and finally centrifuged at 10,000 *g* for 20 min. The amount of proteins in each resulting supernatant and in recombinant FAAH enzyme source was titrated by Biorad assay. Supernatants were mixed 4:1 (v/v) with sample buffer (300 mM Tris–HCl pH 6.8, 50% glycerol, 500 mM dithiotreïtol, 0.05% Bromophenol Blue, 10% SDS) and boiled for 5 min prior to loading on a 0.75-mm-thick gel. Samples were subjected to electrophoresis (150 V) for 2 h under reducing conditions, and separated proteins on the gel were transferred onto Immobilon Protein Transfer at 65 mA overnight at 4°C. The membrane was preincubated with 5% non-fat dry milk in Tris-buffered saline (TBS: 10 mM Tris–HCl pH 8, 150 mM NaCl) for 30 min to block non-specific binding. The membrane was incubated for 1 h in amidase antiserum at a dilution of 1:3000 and a control was made in the same conditions using the amidase antiserum preabsorbed with the homologous antigen (45 μg/μl of pure antiserum). Then, the membrane was washed 3×10 min in TBS containing 0.05% Tween-20 (TBST) and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (dilution 1:3000) for 1 h. The membrane was again washed 3×10 min in TBST and rinsed in TBS. Signals were detected with an ECL kit (Biorad). Control of specificities was performed by preabsorbing the antibody by the homologous antigen at a concentration of 140 μg/ml of pure serum

2.10. Immunocytochemical procedure

Anterior parts of mature leeches (*H. medicinalis*, *Theromyzon tessulatum*, and *Erpobdella octoculata*) were fixed overnight at 4°C in Bouin Hollande fixative supplemented with 10% of mercuric chloride, embedded in Paraplast and serially sectioned at 7 μm. After removal of Paraplast with toluene, sections were subjected to an indirect immunoperoxidase method as described elsewhere

[34,45]. In short, sections of anterior parts were incubated overnight at room temperature with either anti-amidase (1:200) or anti-CB1 (16 μg/ml) sera. They were then treated for 1 h at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:50). Immunostaining was revealed with a solution of 4-chloro-1-naphtol (0.4 mg/ml in 0.1 M Tris buffer, pH 7.6) containing 0.01% H_2O_2. Controls of specificity were performed by incubation of the primary antiserum saturated with the homologous antigen (1 mg synthetic peptide/ml of pure antiserum) prior to application. Sections were examined with a Zeiss Axioskop microscope.

2.11. Cyclic AMP assay

Nervous system of *H. medicinalis* (10 ganglia) was placed in 2 ml Tris/malate buffer (2 mM, pH 7.4) containing EGTA (0.8 mM) and homogenized using a ground glass homogenizer. Following homogenization, the sample was maintained at 4°C. The material was deproteinized in acidic ethanol (1 ml, 1 M HCl/100 ml ethanol), and centrifuged. The supernatant was washed three times with four volumes of water-saturated ether, and the residue was then evaluated for cAMP using the Amersham/cAmp-[^{3}H] kit RIA TrK432 instructions (Arlington Heights, IL, USA). Triplicate incubations for each test condition were run for each experiment. The results are corrected for recoveries and expressed as mean values±S.D. (means±S.D., $n=3$). Protein concentration determinations were made according to the Biorad method.

3. Results

3.1. Endocannabinoid characterization and quantification

After a lipid extraction in chloroform/methanol, a separation was conducted using SiO_2 open bed chromatography. The separated lipids (9:1 fraction) were then submitted to normal phase high-pressure chromatography. Elution was performed by a linear gradient of iso-propanol in *N*-hexane. In these conditions, synthetic 2-AG and anandamide elute from the column at a retention time of, respectively, 21 min and 35 min. Leech material collected at these retention times were then derivatized and subjected to GC–MS analysis (Fig. 1). As seen in Fig. 1, GC–MS chromatograms obtained in ion selected monitoring mode, show peaks at *m/z* 427, 419, 412 and 404 for the material collected at 35 min (Fig. 1A) and peaks at *m/z* 530, 522, 515 and 507 for the material collected at 21 min (Fig. 1B). This corresponds to the molecular ions and the −15 mass ions of $[^2H]_8$anandamide and non-deuterated anandamide, respectively, and similarly to the molecular ions and the −15 mass ions of $[^2H]_8$2-AG and non-deuterated 2-AG, respectively. The amounts were 21.5±0.7 pmol/g and 147.4±42.7 pmol/g of wet weight of tissue, for anandamide and 2-AG, respectively (means±S.D., $n=2$).

Anandamide precursor NArPE was also quantified as the anandamide released from PLD digestion of the 1:1 fraction from SiO_2 chromatography and estimated at 16.4±3.2 pmol/g of wet weight of tissue (means±S.D., $n=2$). We also demonstrate that leech anandamide and 2-AG are accompanied by anandamide-related molecules like *N*-palmitoylethanolamine, at an amount of 32.3±1.5 pmol/g of wet weight of tissue (means±S.D., $n=2$), and its precursor, *N*-palmitoylphosphatidylethanolamine (348.9±99.3 pmol/g of wet weight of tissue means±S.D., $n=2$), and *N*-linolenoylethanolamine (5.8 pmol/g of wet weight of tissue, $n=1$).

3.2. Evidence for anandamide amidase enzyme(s) in leech CNS

Whole homogenate and subcellular fractions obtained from *H. medicinalis* CNS were analyzed for the presence of [^{14}C]anandamide-hydrolyzing activity (Table 1). The enzymatic formation of [^{14}C]ethanolamine by whole homogenate was essentially due to the cytosolic fraction. As a matter of fact the enzymatic formation of [^{14}C]ethanolamide in the membrane and 'microsomal' (100,000 *g* pellet) fraction was very weak (Table 1). The anandamide congener [^{14}C]palmitoylethanolamide was also hydrolyzed by the whole homogenate (Table 1). Like for the [^{14}C]anandamide amidase activity, the [^{14}C]*N*-palmitoylethanolamine amidase activity was most abundant in the cytosolic fraction .

The anandamide amidase activity from whole homogenate was further characterized and found to exhibit a pH dependency profile different from that of mammalian FAAH, with a maximal activity at pH 7 (Fig. 2). The typical FAAH inhibitors, *p*-hydroxymercuribenzoate, methylarachidonoylfluorophosphonate and arachidonoyltrifluoromethylketone, at concentrations causing almost 100% inhibition of N18TG2 cell FAAH (control), were not effective on the leech [^{14}C]anandamide-hydrolyzing activity (Fig. 3). Furthermore, most of this activity was not selective for anandamide or 2-AG because these compounds exhibited only a small inhibition of [^{14}C]anandamide hydrolysis.

The predicted size of rat FAAH protein, based on its amino acid sequence following extrapolation from its corresponding cDNA, is 63.4 kDa. Western immunobloting is performed with the anti-amidase serum that is able to recognize proteins containing the amidase consensus sequence, including FAAH or other amidases. Western immunobloting of recombinant rat FAAH reveals an immunoreactive band at ~64 kDa (Fig. 4, lane c). The same analysis performed on rat liver homogenate confirms the specificity of the anti-amidase serum for the native enzyme (data not shown). In leech CNS, the immunoblot

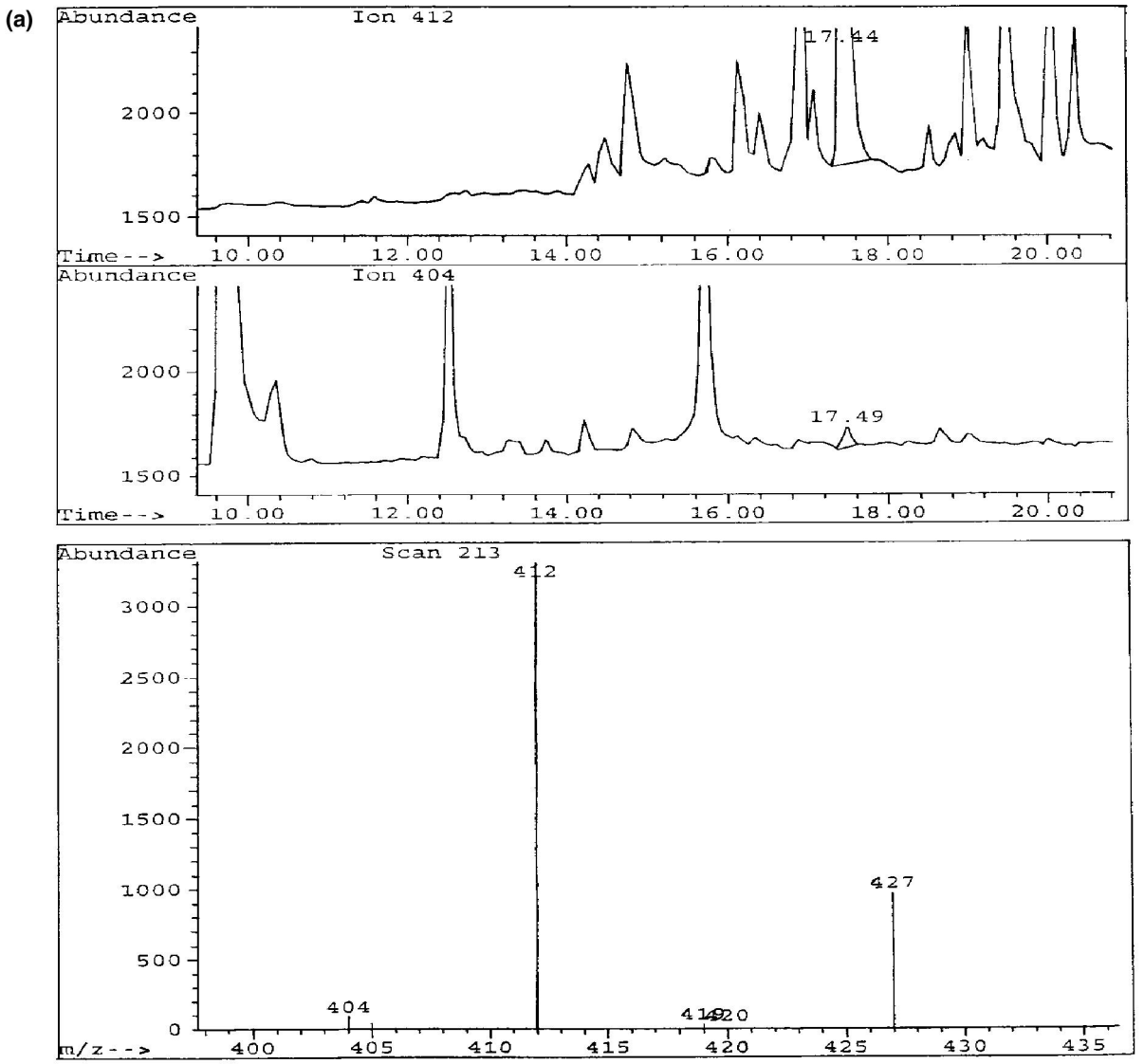

Fig. 1. GC–MS chromatograms of bio-active lipids in the leech *Hirudo medicinalis* CNS. (A) Selected ion monitoring GC–MS chromatogram of anandamide (middle panel, selected ion $m/z=404$) in the presence of deuterated internal standard (upper panel, selected ion $m/z=412$), and selected ion monitoring fragmentogram of the peak at 17.40–17.50 min (lower panel). (B) Selected ion monitoring GC–MS chromatogram of 2-AG (upper panel, selected ion $m/z=522$; middle panel, selected ion $m/z=507$) in the presence of deuterated internal standard (data not shown) and total ion monitoring fragmentogram of the peak at 17.78 min (lower panel).

analysis revealed three specific immunoreactive bands at ca. ~42, ~46 and ~66 kDa (Fig. 4, lane a). The most abundant band was the one at ~46 kDa, which was present in both total homogenate and cytosolic fractions, and was absent in Hydra homogenate (data not shown). The band at ~42 kDa was less intense and was present also in Hydra. The ~66 kDa band was very faint in leech homogenate but quite intense in Hydra and could correspond to a FAAH-like enzyme. Control of specificity was carried out by pre-adsorbing the anti-amidase serum with its antigen (Fig. 4, lanes b and d).

3.3. Localization of amidase in leech CNS

Immunocytochemical studies were performed with both anti-amidase serum raised against the consensus sequence of amidases (Signature Amidase Family) and anti-CB1 serum in different species of leeches. The anti-amidase serum is able to localize in principle proteins containing the amidase consensus sequence, including FAAH or other amidases.

As seen in Figs. 5, 6 and 7, immunoreactivity to anti-amidase was observed in three different species of leeches. In *H. medicinalis* numerous perikaria exhibit an intense immunolabeling throughout the supra-esophageal ganglia (Figs. 5A, B). The immunostained cells correspond to neurons and the labeling is found on nerve cell bodies. In *E. octoculata,* amidase-like immunoreactivity was mainly detected in the brain (Fig. 6A) but immunoreactive cells were also seen in the segmental ganglia (Fig. 6C). In

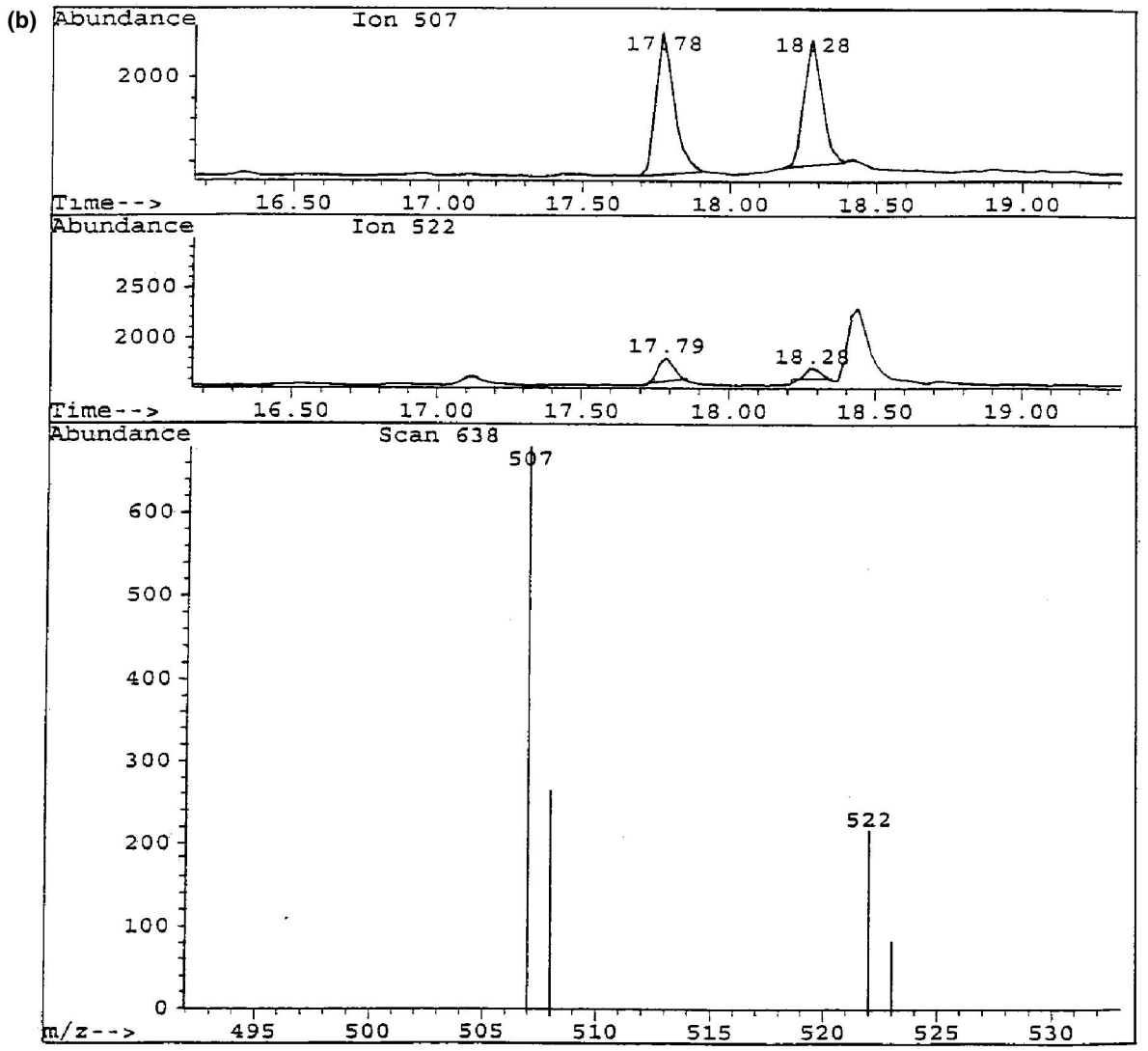

Fig. 1. (*continued*)

Theromyzon tessulatum, a much larger distribution of amidase-like signal was observed. Indeed, intense immunostaining was seen in different compartments of the supra-esophageal ganglia (compartments 4 and 5, Fig. 7A) and in ganglia of the nerve cord (Fig. 7D). Coelomocytes (Fig. 7E) and parts of genital tracts (Fig. 7G) exhibited also a low but specific immunostaining. Pre-absorption of amidase antiserum with the homologous antigen abolished the positive staining, reflecting the specificity of the immunolabelling in the three species (Figs. 5C; 6B, D; 7B, C, F, H). Labeling of glial cells was never observed.

3.4. Evidence for a functional CB1-like receptor

Ganglia of *H. medicinalis* were exposed to either anandamide (10^{-6} M), the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) or the NO synthase inhibitor *N*-omega-nitro-L-arginine methyl ester (L-NAME) (Table 2). Anandamide alone was not able to induce the cyclic AMP release even though forskolin was able to initiate this release. By contrast, anandamide is able to inhibit the forskolin-induced formation of cyclic AMP and this effect was abolished by the NO synthase inhibitor L-NAME. The

Table 1
Distribution of the amidase activity in leech CNS

	Whole homogenate	Cytosol fraction	Membrane fraction
[^{14}C]AEA (pmol/min/mg of protein)	14.34±1.45	12.48	1.55±0.55
[^{14}C]PEA (pmol/min/mg of protein)	4.96±0.56	3.1±0.33	0.385±0.003

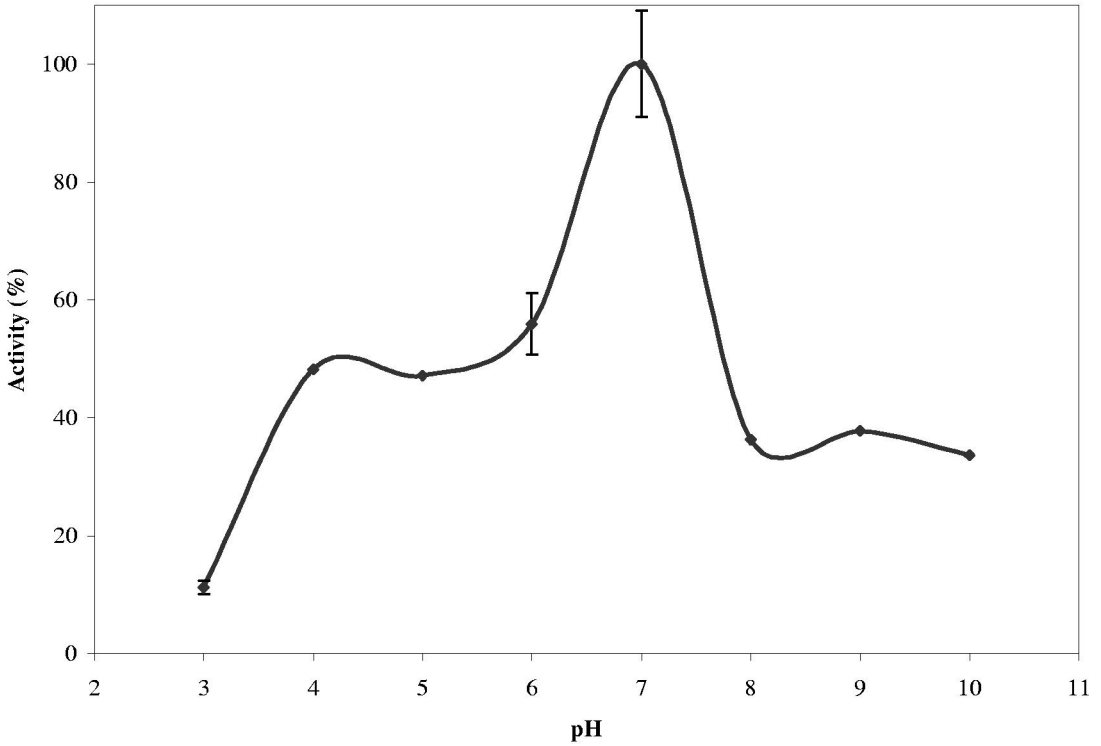

Fig. 2. pH dependency of the [^{14}C]anandamide-hydrolyzing activity from *Hirudo medicinalis* CNS whole homogenate expressed as a percentage of the activity at pH 7.0 (maximal activity) (means S.D., $n=2$).

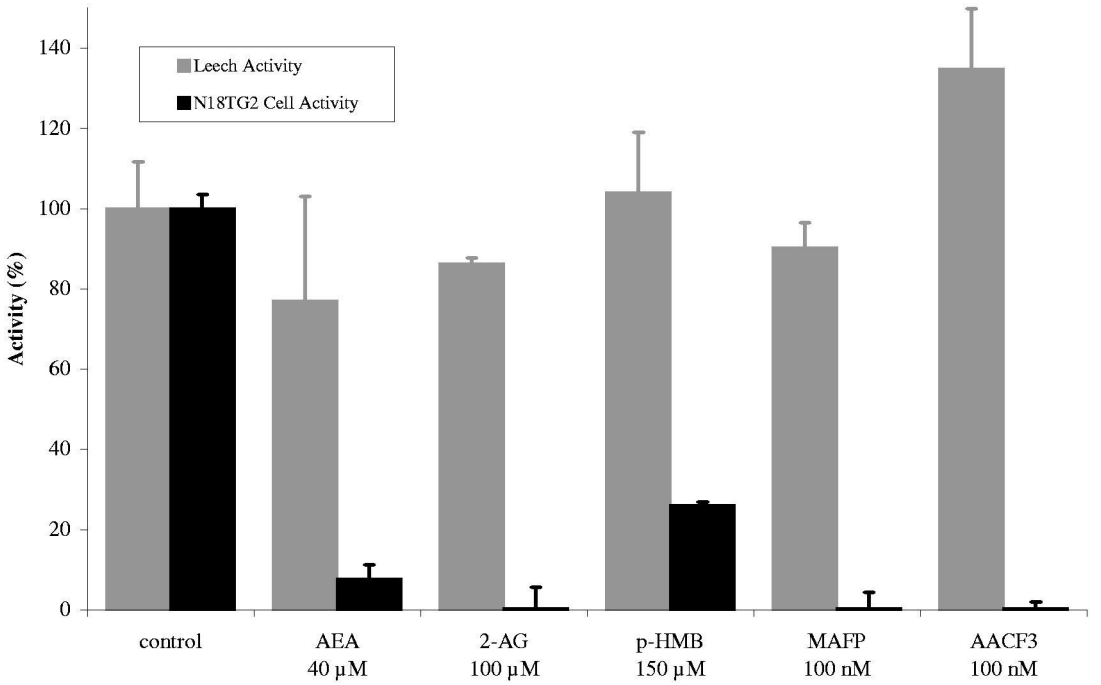

Fig. 3. Effect of inhibitors of mammalian FAAH on the [^{14}C]anandamide-hydrolyzing activity from *Hirudo medicinalis* CNS whole homogenate. The effect is expressed as a percentage of the activity in the absence of inhibitors (and with vehicle) and compared with the effect on N18TG2 cell membrane activity. AEA, anandamide; 2-AG, 2-arachidonoylglycerol; *p*-HMB, *p*-hydroxymercuribenzoate; MAFP, methylarachidonoyl-fluorophosphonate; AACF3, arachidonoyltrifluoromethylketone (mean±S.D., $n=2$).

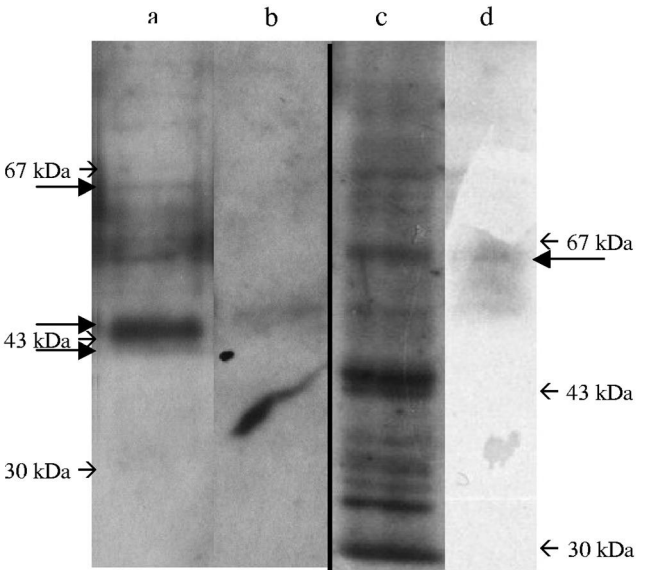

Fig. 4. Western blot analysis of the putative leech amidase. Nervous system of *Hiudo medicinal*is (50 μg of proteins, lane a) and recombinant FAAH (50 μg of proteins, lane c) were submitted to SDS–polyacrylamide gel electrophoresis and Western immunobloting using rabbit antiserum against a peptide corresponding to amino acids 215–226 of the rat FAAH enzyme, pre-treated with the homologous antigen (lanes b and d) or not (lanes a and c). The three arrows on the left indicate the position of ca. ~42, ~46 and ~66 kDa leech FAAH. The arrow on the right indicates the migration of the recombinant FAAH (64 kDa).

NO donor, SNAP, is also capable to inhibit the forskolin-induced formation of cyclic AMP, suggesting that the action of anandamide occurs through NO release.

As regards the localization of the CB1-like cannabinoid receptor in *H. medicinalis*, specific immunostaining was detected in neurons of the supra-esophageal ganglion (Fig. 8A). *T. tessulatum* also reveals a few immunoreactive neurons in median compartments of the sub-esophageal ganglia (Fig. 9A, C) and in the supra-esophageal ganglion (data not show). Pre-adsorption of the primary antibody with the synthetic peptide completely abolished the signal (Figs. 8B, D; 9B, D).

4. Discussion

By using a combination of techniques including GC–MS, we have demonstrated, for the first time, the presence of anandamide, 2-AG, NArPE, *N*-palmitoylethanolamine and *N*-linolenoylethanolamine in the CNS of the leech *H. medicinalis*. These results are in line with previous observations reported for *Hydra vulgaris* [8], *Mytilus edulis* [47] and sea urchins [4].

The leech anandamide amount was comparable to the one detected in the rat, where the compound was proposed to act as a neuromodulator [12]. Leech 2-AG amount was lower than that found in vertebrates and other invertebrates

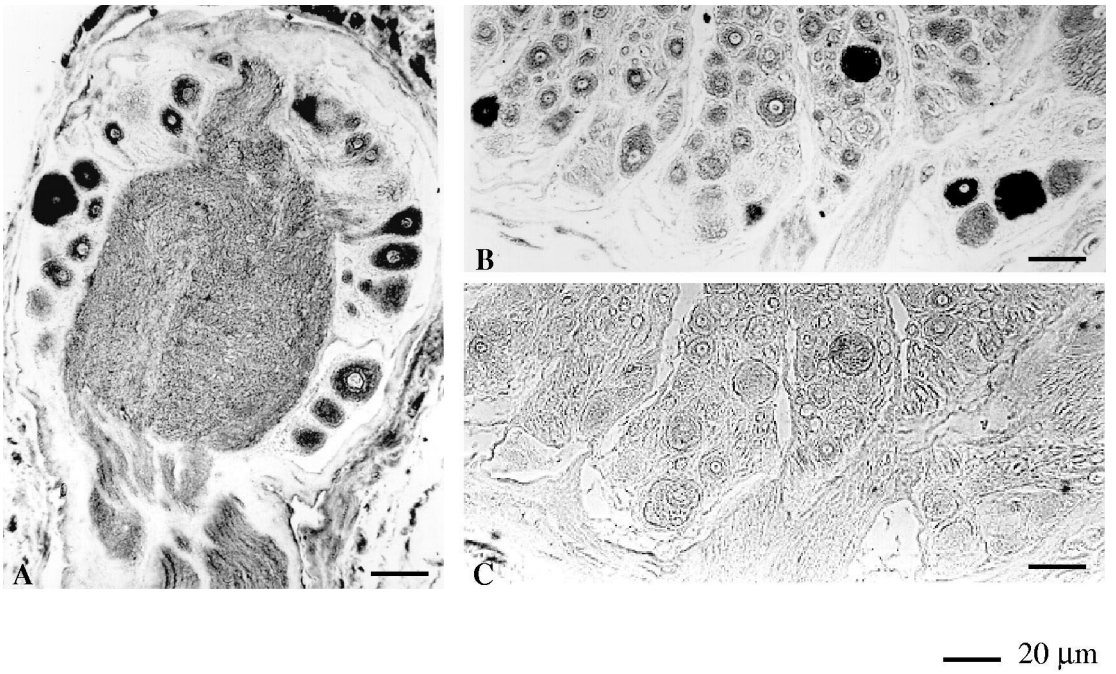

Fig. 5. Immunohistochemical detection (indirect peroxidase) of an amidase-like protein in frontal sections of *H. medicinalis*. (A) Immunoreactive neurons are detected in the supra-esophageal ganglia. (B, C) Adjacent sections of different compartments of the supra-esophageal ganglion treated either with anti-amidase serum (B) or with anti-amidase serum preadsorbed with the homologous antigen (C). Pre-adsorption of the serum with the corresponding peptide abolished the immunostaining.

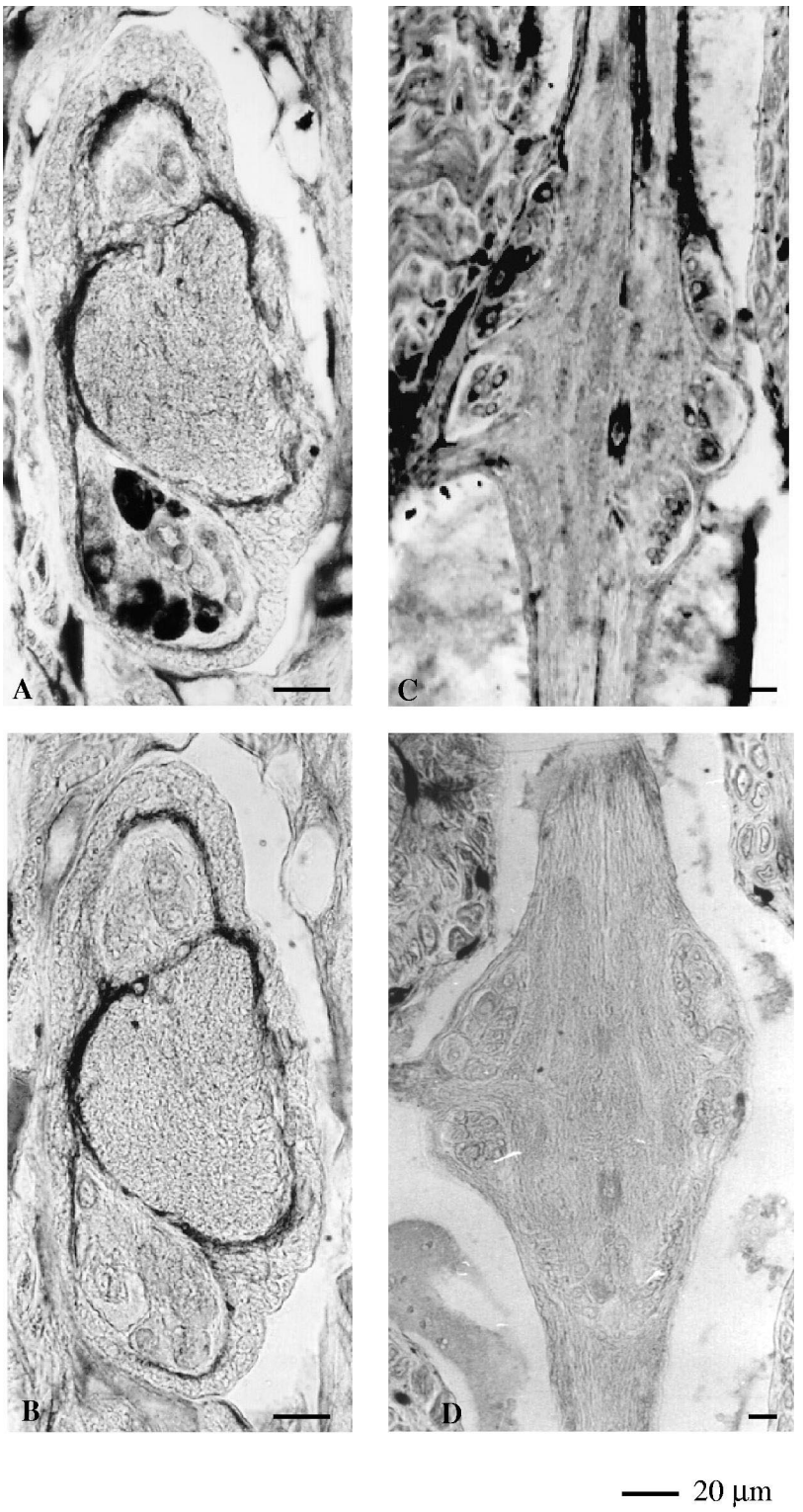

Fig. 6. Immunohistochemical detection (indirect peroxidase) of an amidase-like protein in frontal sections of *E. octoculata*. (A) Immunoreactive neurons are detected in the supra-esophageal ganglia. (A, B) Adjacent sections of different compartments of the supra-esophageal ganglion treated either with anti-amidase serum (A) or with anti-amidase serum pre-adsorbed with the homologous antigen (B). The amidase-staining capacity is abolished after adsorption with the synthetic peptide. (C) Immunoreactive neurons are also detected in ganglia of the nerve cord. Pre-absorption of the antiserum with the corresponding peptide abolished the immunostaining (D).

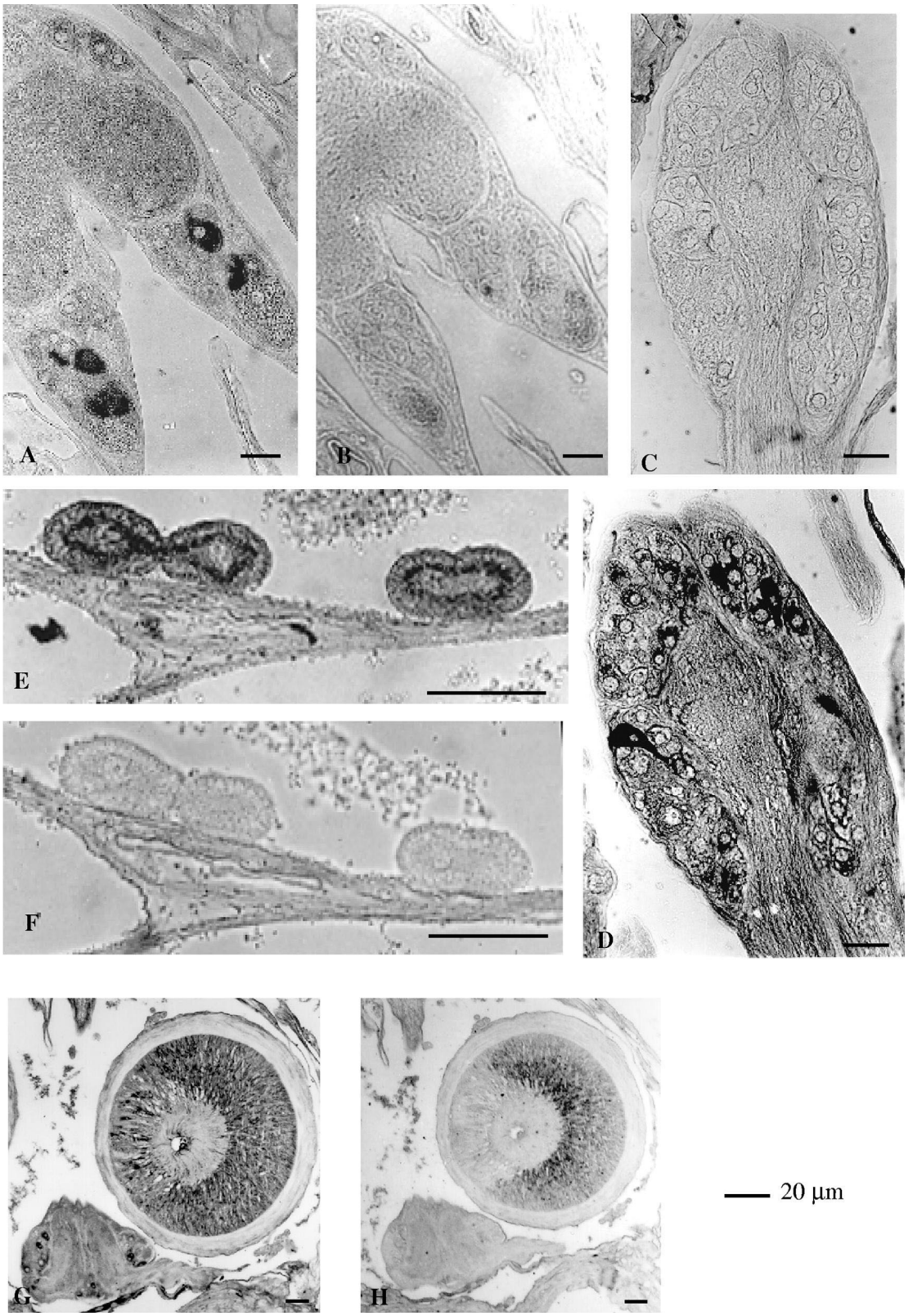

Fig. 7. Immunohistochemical detection (indirect peroxidase) of an amidase-like protein in frontal sections of *T. tessulatum*. (A) Immunoreactive neurons are detected in compartments 4 and 5 of the supra-esophageal ganglia. (D) Numerous neurons are detected in segmental ganglia. A moderate immunoreactivity is detected in coelomocytes (E) and in genital tract (G). Pre-absorption of the anti-amidase serum with the corresponding peptide abolished the immunostaining (B, C, F, H).

Table 2
Inhibition of forskolin-induced cAMP formation by anandamide through NO production[a]

Substances	cAMP levels pmol/mg protein/min
Anandamide (10^{-6} M)	1.6±0.8
Forskolin (10^{-6} M)	52.2±15.4
SNAP (10^{-6} M)+Forskolin (10^{-6} M)	2.6±0.95
Anandamide (10^{-6} M)+Forskolin (10^{-6} M)	5.3±1.3
Anandamide (10^{-6} M)+L-NAME (10^{-6} M)+Forskolin (10^{-6} M)	33.8±18.4

[a] (mean±S.D., $n=3$).

[12], but still seemingly more abundant than anandamide, analogous to others animals. In view of the finding of the putative anandamide biosynthetic precursor (NArPE), we can suggest that the formation of anandamide occurs via the phospholipase D pathway suggested for mammals [12,16]. As for *N*-palmitoylethanolamine, this anandamide congener is more abundant than anandamide in leech as in mammals.

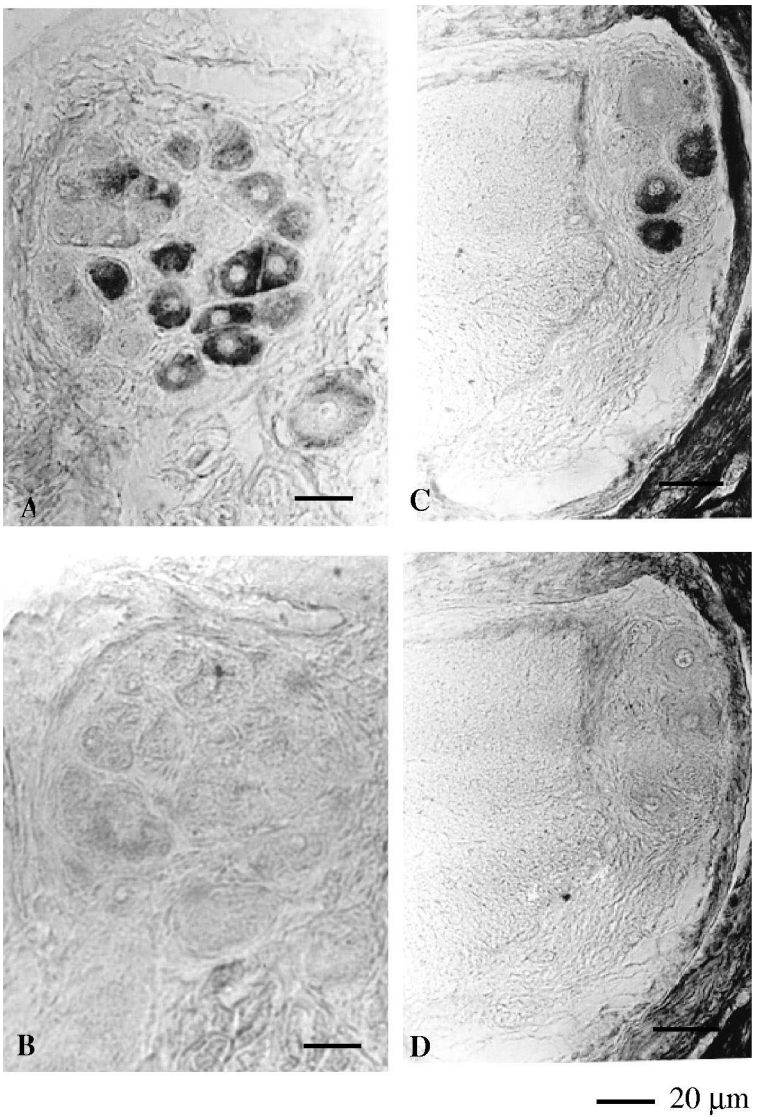

Fig. 8. Immunohistochemical detection (indirect peroxidase) of a CB1-like protein in frontal sections of *H. medicinalis*. (A, B) Numerous immunoreactive neurons are observed in the supra-esophageal ganglia. The amidase-staining capacity is abolished after adsorption with the synthetic peptide (B). (C, D) Adjacent sections of different compartments of the supra-esophageal ganglia treated either with anti-CB1 antibody (C) or with anti-CB1 antibody pre-absorbed with the homologous antigen (D). Pre-absorption of the antibody with the corresponding peptide abolished the immunostaining.

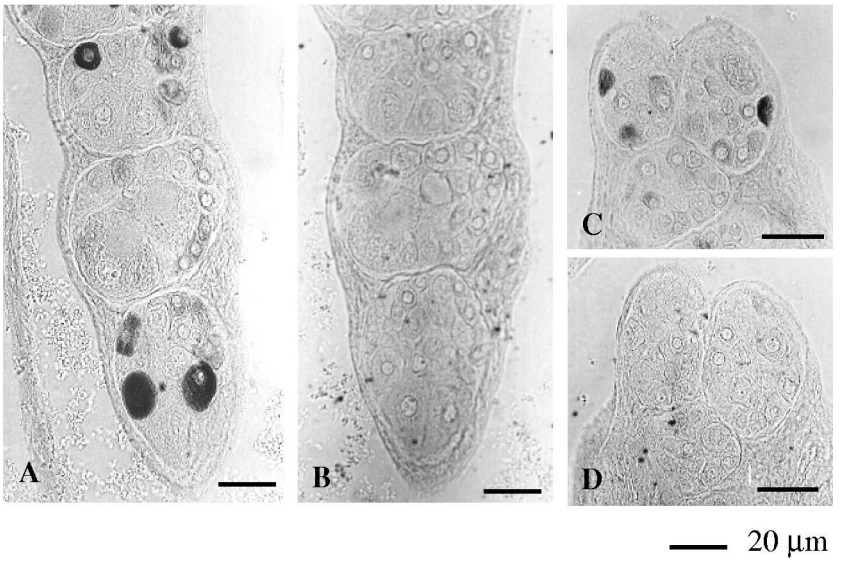

Fig. 9. Immunohistochemical detection (indirect peroxidase) of CB1-like protein in frontal sections of *T. tessulatum*. (A, C) Numerous immunoreactive neurons are observed in median compartments 14, 15, 16 (A) and 21 (C) of the sub-esophageal ganglia. Pre-adsorption of CB1 antiserum with the homologous antigen abolished the positive staining (B, D).

The discovery of these endocannabinoids in leech CNS, together with the previously described finding of CB1-like receptors in the same tissues [50], suggests that a complete endocannabinoid system exists in leeches. Therefore, the next step in our study was to assess the presence of an anandamide amidase in leech CNS, as indirect evidence for the existence of such enzyme had been provided in previous studies [49]. We found that [^{14}C]anandamide was enzymatically hydrolyzed by both total leech CNS homogenates and cytosolic fractions, and that very little activity was present in membrane fractions. This shows that this enzymatic activity has a distribution different from that reported for either mammalian or invertebrate FAAH [1,8,32]. In support of this hypothesis, we found that leech anandamide amidase activity was (i) optimal at pH = 7, instead of 9–10 for FAAH, (ii) not sensitive to typical FAAH inhibitors and (iii) inhibited only to a little extent by either AEA or 2-AG. We also found that [^{14}C]*N*-palmitoylethanolamine is hydrolyzed, to a lesser extent, by both homogenates and cytosolic fractions. These observations suggest that the anandamide amidase activity found here is, in fact, mostly not selective for anandamide, and that additional inactivating mechanisms more specific for endocannabinoids may exist in leech CNS.

Based on these data we decided to raise a non-selective antiserum against the amidase signature sequence [30]. This would have allowed us to identify in leech CNS also possible amidase enzymes different from FAAH, including the one responsible for the anandamide amidase activity detected in leech homogenates. By using this antiserum we found three immunoreactivity bands in Western immunoblot analyses. The two least abundant of these proteins were also present in *Hydra*, and the very minor of them exhibited an apparent molecular weight similar to that expected for mammalian FAAH enzymes. However, the major band at ca. 46 kDa was absent in *Hydra* and was very abundant in leech cytosolic fractions. These data, taken together with the enzyme activity data obtained here for the leech and previously for *Hydra* [8], suggest that, while in *Hydra* one of the amidase-immunoreactive bands could be due to a FAAH-like enzyme, in leech CNS most of the anandamide amidase activity is due to (an) enzyme(s) distinct from mammalian FAAH.

In the present study we utilized the anti-amidase antiserum for immunohistochemical localization of the enzyme in leech CNS, as compared to CB1 localization. We previously cloned a fragment of leech cannabinoid receptor cDNA [50]. The fragment presents, within its amino acid sequence, two highly conserved motifs — between amino acids 1–97 and 128–153 — which show 80% and 58% homology to human CB_1 receptors, respectively, while a third region is 98% identical to part of the bovine adrenocorticotropic hormone receptor. According to Elphick [23] this protein may therefore represent the putative ancestor of mammalian cannabinoid and melanocortin receptors. This hypothesis is supported by the high homology between the sequence of mammalian CB1 and melanocortin receptors, which contain domains, located in the central part of the two proteins, with more than 80% sequence identity. Here we found that CB1 immunoreactivity in both *H. medicinalis* and *T. tessulatum* is located in neurons of the CNS, more precisely in the supra-esophageal ganglia. More importantly, we observed that leech CB1 immunoreactivity matches to a certain extent with amidase immunostaining. This is in agreement with data obtained in the rat and showing that FAAH and CB1 distributions in the brain overlap to a great extent [53,54,57]. These data suggest that, even though the leech

amidase may not be selective for the endocannabinoids anandamide and 2-AG, this enzyme may still be deputed to the degradation of these metabolites since it is localized near their putative molecular target, i.e. the CB1 receptor. Moreover, in the leech, immunostaining with anti-CB2 was found in neurons and glial cells in the central neuropil of the ventral chain ganglia (Salzet et al., unpublished observations). This suggests the presence of different types of cannabinoid receptors in these simple animals, as previously described in mammals [6,20,35,39]. Finally, based on the information that in vertebrates as in mammals cannabinoid receptor stimulation by anandamide leads to the release of NO [51] and inhibition of cAMP formation [29], we wanted to assess whether the same phenomenon occurred in our model. Our data allow us to conclude that two of the secondary messengers mediating cannabinoid receptor signaling, e.g., NO and cAMP, are the same in leech as in mammals.

Taken together, our results demonstrate the existence of a complete endocannabinoid system in leech brain, and support the concept that this signaling system has been conserved throughout animal evolution.

Acknowledgements

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