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FINDING OF THE ENDOCANNABINOID SIGNALLING SYSTEM IN *HYDRA*, A VERY PRIMITIVE ORGANISM: POSSIBLE ROLE IN THE FEEDING RESPONSE

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Abstract—*Hydra* (Cnidaria) is the first animal organism to have developed a neural network, which has been proposed to control, *inter alia*, the "feeding response", i.e. a mechanism through which the coelenterate opens and then closes its mouth in the presence of prey and/or glutathione. Here, we report that *Hydra* contains: (i) selective cannabinoid binding sites; (ii) the endogenous cannabinoid receptor ligand, anandamide (arachidonoylethanolamide); (iii) a fatty acid amide hydrolase-like activity catalysing anandamide hydrolysis; and (iv) the putative biosynthetic precursor of anandamide, *N*-arachidonoylphosphatidylethanolamine. We suggest that this "endogenous cannabinoid system" is involved in the modulation of the "feeding response". Anandamide (1 nM–1 μM) potently inhibited (up to 45%) the glutathione-induced "feeding response" by accelerating *Hydra vulgaris* mouth closure. The effect was maximal at 100 nM anandamide and was reversed by the selective antagonist of the CB_1 subtype of mammalian cannabinoid receptors, SR 141716A (50–100 nM). Specific cannabinoid binding sites were detected in membranes from *Hydra* polyps by using [^{3}H]SR 141716A ($K_d = 1.87$ nM, $B_{max} = 26.7$ fmol/mg protein), and increasing anandamide concentrations were found to displace the binding of [^{3}H]SR 141716A to these membranes ($K_i = 505$ nM). *Hydra* polyps were also found to contain amounts of anandamide (15.6 pmol/g) and *N*-arachidonoylphosphatidylethanolamine (32.4 pmol/g), as well as the other "endocannabinoid" 2-arachidonoylglycerol (11.2 nmol/g), comparable to those described previously for mammalian brain. Finally, a fatty acid amide hydrolase activity ($V_{max} = 3.4$ nmol/min/mg protein), with subcellular distribution, pH dependency and sensitivity to inhibitors similar to those reported for the mammalian enzyme, but with a lower affinity for anandamide ($K_m = 400$ μM), was also detected in *Hydra* polyps.

These data suggest that the endocannabinoid signalling system plays a physiological role in *Hydra* that is to control the feeding response. *Hydra* is the simplest living organism described so far to use this recently discovered regulatory system. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: *Hydra*, cannabinoids, endocannabinoids, anandamide, 2-arachidonoylglycerol, GABA.

The neural network of the freshwater coelenterate *Hydra* (Cnidaria, Hydrozoa) is considered one of the most primitive nervous systems to have evolved.[34] Neurons are generally dispersed in the epithelial layers and are either inter-connected into a functional network or form uni- and bi-directional synapses with non-nervous cells.[49] This rather simple system is homogeneously spread over the coelenterate body, but is developed to a higher degree of complexity near the head region, where the fibres are condensed to form a circular ring. This rudimentary cephalization is probably responsible for the control of a behavioural response typical of hydrozoans, the "feeding response",[25,37,48] during which polyps stimulate with glutathione, released from the prey, respond with tentacle writhing and mouth opening, eventually followed by mouth closure.[28] Despite its simplicity, the *Hydra* nervous system shows neurophysiological and cellular features as sophisticated as those of higher invertebrates in terms of electrogenesis, plasticity, development and growth regulation.[29] Neurotransmission in *Hydra* was originally thought to be essentially peptidergic, due to the presence of several neuropeptides in the coelenterate,[23,24] but evidence has been obtained for the participation also of amine and amino acid neurotransmitters, such as dopamine and GABA, particularly in the control of the feeding response.[37,48] In particular, it was shown recently that GABA, by acting at selective binding sites identified in *Hydra vulgaris* membrane preparations, prolongs the glutathione-induced feeding response, an effect that is reduced by the $GABA_A$ receptor antagonist SR 95531 and mimicked by the $GABA_A$ receptor agonist muscimol.[9,38,39] Further evidence for the presence of $GABA_A$-like receptors in *Hydra*, and for their involvement in the feeding response, came from the findings that: (i) neurosteroids and benzodiazepines potentiated both the

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Abbreviations: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; EDTA, ethylenediaminetetra-acetate; FAAH, fatty acid amide hydrolase; GC–(EI)MS, gas chromatography–(electron impact) mass spectrometry; HPLC, high-pressure liquid chromatography; NArPE, *N*-arachidonoylphosphatidylethanolamine; PLD, phospholipase D.

binding of [^{3}H]GABA to membrane preparations and the effect of GABA on the glutathione-induced feeding response;[9] and (ii) the Cl^- channel blockers t-butylbicyclophosphorothionate and picrotoxin decreased the feeding response duration.[9,40] Some progress has also been made in the understanding of the possible intracellular signalling events underlying the feeding response. For example, it was found that inhibitors of the nitric oxide/cyclic-GMP pathway prolong the response duration.[8] We recently suggested that activation of arachidonic acid (AA) release from *H. vulgaris* phospholipids and, possibly, the subsequent formation of AA metabolites with inhibitory action on the feeding response, could be stimulated by glutathione.[39] AA and, to a lesser extent, α-linolenic acid, as well as their metabolites obtained by the action of a ω10(*R*)-lipoxygenase characterized previously by us in *H. vulgaris*,[18] inhibited glutathione-induced feeding response in the 1–100 μM range of concentrations, the metabolites being at least 10-fold more potent than the precursor fatty acids.[39,40] We hypothesized that these lipids were produced following activation of glutathione chemoreceptors in order to terminate the feeding response through a negative feedback interaction with endogenous GABA action at the level of GABA-gated Cl^- channels.[39] Our data also raised the possibility that other AA metabolites, through different mechanisms, could intervene in this process.[40]

Two different subtypes of G-protein-coupled receptors for marijuana's active principle, (−)-Δ^9-tetrahydrocannabinol, have been characterized in mammalian tissues.[30,33] One of these, the CB_1 receptor, is the most abundant in the CNS and has been implicated in the modulation of the release, action and re-uptake of several neurotransmitters, including dopamine, glutamate and GABA (see Refs 12, 19 and 35 for recent reviews). A selective antagonist for this receptor, SR 141716A, was synthesized and its pharmacological properties characterized *in vivo* and *in vitro*.[41] In 1992, anandamide, the first endogenous ligand of "central" cannabinoid receptors, was isolated from porcine brain. This compound is the amide of AA with ethanolamine and, since its discovery, several pharmacological studies[15,19,21,26,32] have recognized its likely role as a cannabimimetic neuromodulator. This suggestion was also supported by the finding of molecular mechanisms for the synthesis, release and inactivation of anandamide by intact neurons.[17] It was proposed that anandamide was: (i) synthesized from a phospholipid precursor, *N*-arachidonoylphosphatidylethanolamine (NArPE), through the action of a specific phospholipase D (PLD); and (ii) inactivated via carrier-mediated facilitated diffusion into cells followed by enzymatic hydrolysis.[17] The enzyme responsible for anandamide hydrolysis, originally named "anandamide amidohydrolase", was characterized, purified, cloned, overexpressed into host cells and found to also recognize other fatty acid amides and esters,[11,15] including the other putative "endocannabinoid", 2-arachidonoylglycerol (2-AG), whose cannabimimetic properties were recognized in 1995.[31] The enzyme was therefore renamed "fatty acid amide hydrolase" (FAAH).[11]

Since the discovery of cannabinoid receptors in mammals, several studies have been aimed at assessing whether these proteins could also be found in organisms representing early phases of animal evolution. Cannabinoid receptors with some homology with CB_1 receptors have been cloned from the puffer fish[50] and the leech.[20,45] Selective cannabinoid binding sites have also been detected in cells from simpler organisms, i.e. sea urchin sperm[7] and *Mytilus* immunocytes,[44] and were suggested, respectively, to mediate inhibition of oocyte fertilization and monocyte function. Accordingly, anandamide and NArPE were isolated from sea urcin eggs and *Mytilus* together with FAAH-like activities,[4,43] thus suggesting that the "endocannabinoid signalling system", formed by cannabinoid receptors and their endogenous agonists, may have been highly conserved through evolution. In the present study, we examined whether, and for what physiological function, this recently discovered neuromodulatory system is expressed in animals with a very primitive nervous system, such as hydrozoans.

EXPERIMENTAL PROCEDURES

Materials

Anandamide and palmitoylethanolamide were synthesized as described.[22] Deuterated anandamide and 2-AG were synthesized from [^{2}H]$_8$AA, as described previously.[3,22] [^{3}H]*N*-Palmitoylphosphatidylethanolamine, [^{14}C]anandamide and [^{14}C]oleoylethanolamide (5 mCi/mmol) were synthesized as described previously.[22] SR 141716A was a gift from Sanofi Recherche (Montpellier, France). [^{3}H]SR 141716A (60 Ci/mmol) was purchased from Amersham (U.K.). Phenylmethylsulphonyl fluoride and *p*-hydroxymercuribenzoate were purchased from Sigma, and methylarachidonoylfluorophosphonate and arachidonoyltrifluoromethyl ketone from Biomol. Arachidonoyldiazomethyl ketone and arachidonoylchloromethyl ketone were synthesized as described elsewhere.[14]

Feeding response

The feeding reaction was studied as follows. *Hydra vulgaris* polyps, starved for at least three days before the test, were equilibrated at room temperature in 3.5-cm-diameter Falcon dishes containing 1 ml of physiological solution (1 mM $CaCl_2$, 0.1 mM $NaHCO_3$, buffered with 1 mM Tris–HCl, pH 7.35); the dishes were divided into four chambers by glass partitions, in order to allow simultaneous testing and recording of individual mouth opening (t_i) and closing (t_f) times for each animal. Drugs were added immediately before the test. Lipophilic molecules were dissolved in small amounts of dimethyl sulphoxide and diluted to working concentrations in physiological solution, so that the final dimethyl sulphoxide content in the test solution did not exceed 1 μl/ml. Equal amounts of dimethyl sulphoxide were added to control solutions. Drugs were prepared before use to avoid degradation of the molecules.

The test was started by adding reduced glutathione at different concentrations (1–10 μM); six to eight animals were tested per group and per glutathione concentration. Scoring of mouth opening and closing was performed on a cold light stereo microscope and times recorded. In every experiment, a control series was effected; for each substance tested the experiments were repeated at least three times. All the experiments were carried out in a conditioned environment at 22°C. [Glutathione]/($t_f - t_i$) ratios were then calculated and plotted as a function of glutathione concentration.[39] The duration of the response to different glutathione doses in the absence or presence of different drugs was measured and the kinetics of response analysed by linear regression. When different regression curves were obtained, percentages of increase (or decrease) versus respective control samples were calculated for a given glutathione concentration (10 μM). Statistical significance of differences was evaluated by ANOVA followed by Scheffé's test. A P value < 0.05 was considered statistically significant.

Binding assays

Membranes were prepared from homogenates of *H. vulgaris* polyps obtained following the procedure described previously.[9,37] The binding of increasing concentrations of [^{3}H]SR 141716A to aliquots (0.6 mg total proteins) of these membranes and the displacement of a fixed concentration (600 pM) of [^{3}H]SR 141716A by increasing concentrations of anandamide were measured in equilibrium assays, carried out as described.[1,13] Bound and unbound [^{3}H]SR 141716A were separated by means of the filtration method described elsewhere.[1,13] SR 141716A (10 μM) was used to determine non-specific binding, which in displacement assays was between 25% and 30% of total binding. Receptor binding results were analysed with GraphPad software (San Diego, CA). Saturation curves were fitted with a rectangular hyperbola ($r^2 = 0.98$). Scatchard curves for the binding of [^{3}H]SR 141716A were used to calculate B_{max} and K_d values for this ligand by using non-linear regression, and one- and two-site analyses were compared to determine better fit ($r^2 = 0.89$ for one-site binding). Displacement curves (calculated by means of Pharm/PCS software) were used to calculate the K_i value for anandamide by inserting the corresponding IC_{50} value from the best fitting curve ($r^2 = 0.97$) into the Cheng–Prusoff equation.

Purification and quantitation of endocannabinoids

Extraction, purification and quantitation of anandamide, 2-AG and NArPE was performed as described previously.[2,3] Briefly, starved *Hydra* live polyps were extracted with chloroform/methanol (2:1, v/v) containing [^{2}H]$_8$anandamide and [^{2}H]$_8$2-AG or [^{3}H]*N*-palmitoylphosphatidylethanolamine as internal standards. In order to purify and characterize the anandamide, NArPEs and 2-AG contained therein, the organic phase was brought to dryness and submitted to a series of chromatographic steps, including SiO_2 open bed chromatography, thin layer chromatography (for column fractions containing NArPE, on plates developed with chloroform:methanol:NH_4OH, 85:15:1) and normal phase high-pressure liquid chromatography (HPLC; for column fractions containing anandamide and 2-AG, eluted with increasing concentrations of iso-propanol in *n*-hexane as described previously).[3] NArPE-containing fractions (which also contain other *N*-acylphosphatidylethanolamines) were digested with PLD from *S. chromofuscus*, which released anandamide and other *N*-acylethanolamines,[2,22] followed by thin layer chromatography and normal phase HPLC.[2] 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]palmitoylethanolamide (i.e. the product of the PLD digestion of [^{3}H]*N*-palmitoylphosphatidylethanolamine used as internal standard). Quantitation of anandamide, purified either from lipid extracts or incubates of PLD digestion of NArPE fractions, and 2-AG was obtained by means of gas chromatography–electron impact mass spectrometry (GC–EIMS) of the corresponding trimethylsilyl ether derivatives.[2] HPLC fractions with the same retention time as synthetic anandamide and 2-AG were derivatized with 20 μl *N*-methyl-*N*-trimethylsylyltrifluoroacetamide + 1% trimethylchlorosylan for 2 h at room temperature, and analysed by GC–EIMS carried out in the selected ion monitoring mode, as described elsewhere.[2,3] 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 non-deuterated 2-AG, respectively). The sensitivity of the measurement was of about 1 pmol. When allowed by the amounts of metabolite, GC–EIMS was also run in the total ion current mode, thus yielding whole electron impact mass spectra. Amounts of anandamide and 2-AG were quantitated by comparison with the corresponding and co-eluting deuterated standards by calculating the ratios between the areas of the peaks obtained at the m/z of the −15 mass unit fragment ions.[2] Anandamide from the PLD digestion of NArPE was also quantitated by comparison with the corresponding deuterated standard which, in this case, was added after the digestion and prior to normal phase HPLC analysis.

Fatty acid amide hydrolase assay

This assay was performed as described previously.[14] Briefly, polyps were homogenized in 50 mM Tris–HCl (pH 7.4), containing 1 mM EDTA, using a Dounce homogenizer. The homogenate was centrifuged sequentially at $800 \times g$ (5 min), $10{,}000 \times g$ (15 min) and $100{,}000 \times g$ (30 min). Pellets from the three centrifugations or the supernatant from the last centrifugation (100–200 μg total proteins) were incubated in 50 mM Tris–HCl (pH 7.4) at 37°C in the presence of [^{14}C]anandamide or [^{14}C]oleoylethanolamide (50,000 c.p.m., 8 μM). The formation of [^{14}C]ethanolamine, quantitated as described previously,[14] was taken as a measure of anandamide hydrolysis. Incubations were also carried out with different concentrations of [^{14}C]anandamide, for K_m and V_{max} determination, or in different buffers at different pH values,[14] or in the presence of FAAH inhibitors. The statistical significance of the effect of the latter was tested by ANOVA followed by Fisher's test (threshold for significance, $P < 0.05$).

RESULTS

Effect of anandamide on glutathione-induced feeding response

Anandamide potently and dose-dependently inhibited *Hydra* feeding response induced by a range of glutathione concentrations (1–10 μM; data not shown). With 10 μM glutathione, the effect was already marked with 10 nM and maximal at 100 nM anandamide (Fig. 1), with a 43% reduction of the response duration, obtained essentially by accelerating *Hydra* mouth closure (i.e. by reducing t_f). The anandamide analogue palmitoylethanolamide, which is inactive at CB_1 receptors,[26,35] was not active up to a 1 μM concentration. The inhibitory effect of 100 nM anandamide was comparable to that obtained with a 1000-fold higher concentration

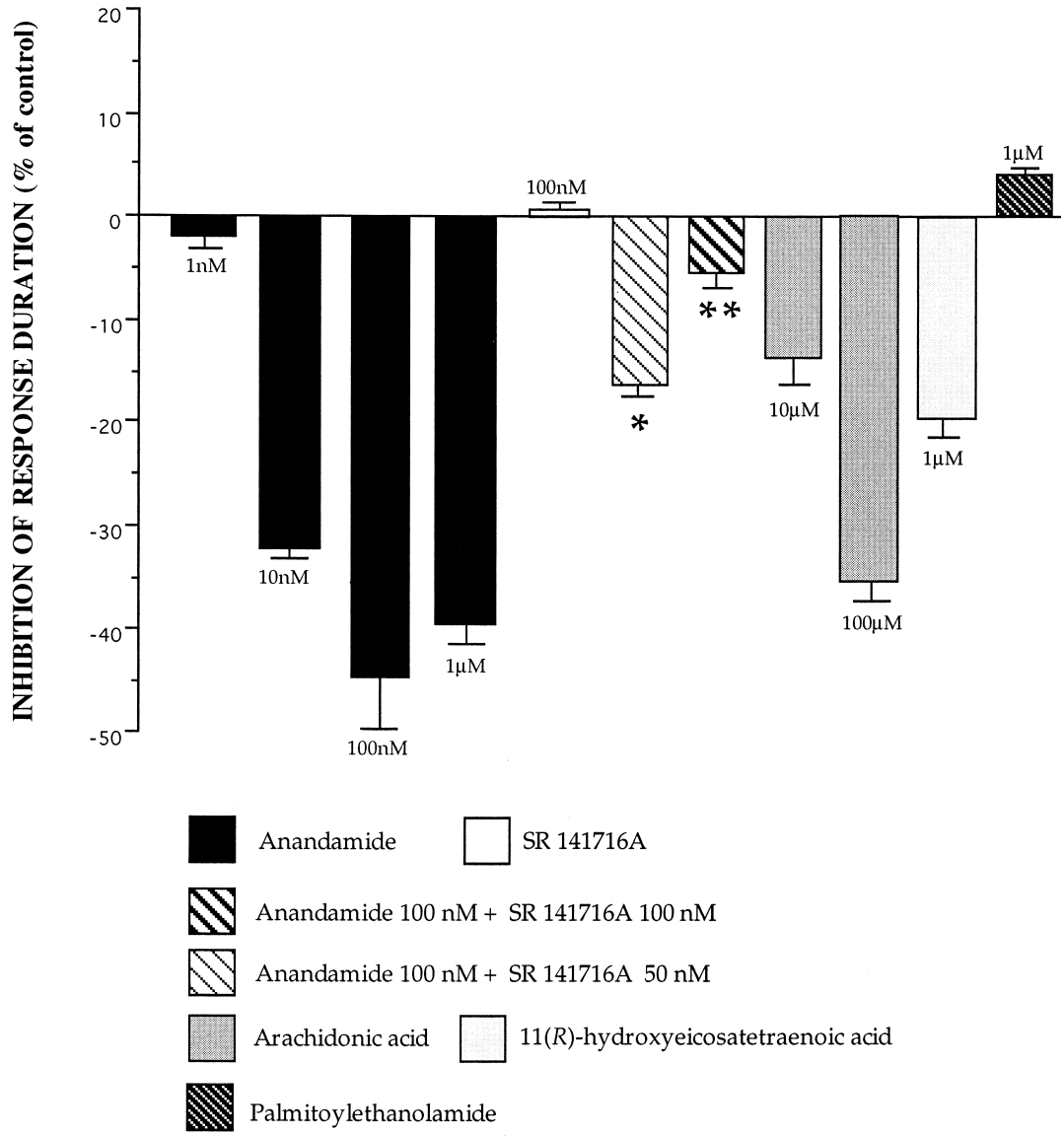

Fig. 1. Effect of anandamide on *Hydra* feeding response. Anandamide dose-dependently reduces the duration ($t_f - t_i$) of the feeding response induced by 10 μM glutathione measured as a percentage of the duration of the response in animals treated with vehicle only (1500 s at 10 μM glutathione; for a recent representation of the duration of the feeding response at various glutathione concentrations, see Ref. 39). The effect of anandamide was exerted by accelerating mouth closure (i.e. by reducing t_i; not shown). The effects of arachidonic acid and 11(*R*)-hydroxyeicosatetraenoic acid, as reported previously,[39] and of palmitoylethanolamide are shown for a comparison. The effect of administration of 100 nM anandamide plus SR 141716A (50 and 100 nM) or SR 141716A alone (100 nM) is also shown. $^*P < 0.05$, $^{**}P < 0.01$ vs 100 nM anandamide alone, calculated by ANOVA followed by Scheffé's test.

of AA, whereas the effect of a 10 nM dose was slightly higher than that observed previously with a 100-fold higher concentration of the major oxidation product of AA in *H. vulgaris*, i.e. 11(*R*)-hydroxyeicosatetraenoic acid (Fig. 1). Furthermore, the effect of 100 nM anandamide was attenuated by co-incubation of polyps with increasing concentrations (50 and 100 nM) of the CB_1 receptor antagonist SR 141716A, which alone did not exhibit any significant effect (Fig. 1).

CB_1-like cannabinoid binding sites in Hydra vulgaris

In agreement with the antagonist action exerted by SR 141716A against anandamide inhibition of the feeding response, we found that increasing concentrations of [^{3}H]SR 141716A bound to membrane preparations from *H. vulgaris* polyps specifically and in a saturable fashion (Fig. 2A). Scatchard analysis (Fig. 2B) revealed B_{max} and K_d

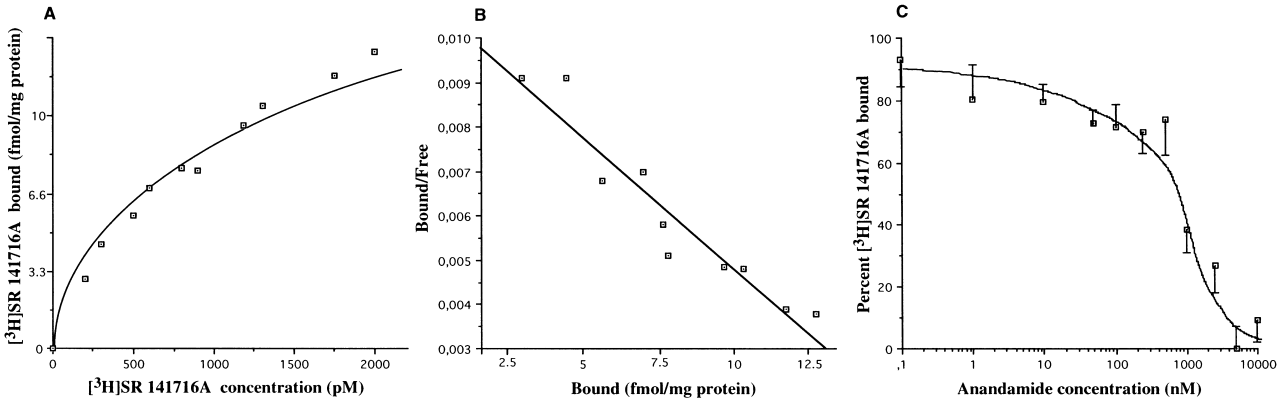

Fig. 2. *Hydra* contains selective cannabinoid binding sites. (A, B) Saturation (A) and Scatchard (B) curves for the specific binding of [^{3}H]SR 141716A to membrane preparations from *H. vulgaris* polyps. (C) Displacement by anandamide of [^{3}H]SR 141716A bound to membrane preparations from *H. vulgaris* polyps.

values, respectively, of 26.7 ± 12.6 fmol/mg protein and 1.87 ± 0.87 nM (means ± S.D., $n = 3$). Anandamide dose-dependently displaced the binding of [^{3}H]SR 141716A to these sites with a K_i value of 505 ± 69 nM (mean ± S.D., $n = 3$; Fig. 2C).

Anandamide and its biosynthetic precursor are produced in Hydra vulgaris

We have analysed, by gas chromatography–mass spectrometry (GC–MS), run in the selected ion monitoring mode, the purified lipids extracted from *H. vulgaris* polyps and found that they contain measurable levels of "endocannabinoids". These were detected as peaks in the gas chromatogram co-eluting with either deuterated anandamide or 2-AG, and possessing the same mass spectral peaks, i.e. the ones at $m/z = 427$ and 419 (molecular ions for [^{2}H]$_8$anandamide and undeuterated anandamide), 412 and 404 (loss of 15 mass units, corresponding to a methyl group, from [^{2}H]$_8$anandamide and undeuterated anandamide), 530 and 522 (molecular ions for [^{2}H]$_8$2-AG and undeuterated 2-AG) and 515 and 507 (loss of 15 mass units, corresponding to a methyl group, from [^{2}H]$_8$2-AG and undeuterated 2-AG) (Fig. 3 and data not shown).[2] Amounts of anandamide and 2-AG were 15.6 ± 1.5 pmol/g and 11.2 ± 1.9 nmol/g wet tissue weight, respectively (means ± S.D., $n = 2$), comparable to the amounts described previously in mammalian tissues.[5,46] We also detected measurable amounts of anandamide in fractions of NArPE, the proposed biosynthetic precursor of anandamide in the mammalian CNS, that had been digested with PLD. Also, the amounts of NArPE were similar to those measured in rat brain (32.4 ± 8.1 pmol/g, mean ± S.D., $n = 2$).[5,46] Anandamide and 2-AG were accompanied by several congeners (whose exact quantities were not calculated), including palmitoyl- and oleoylethanolamide, and the $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{20:3}$, $C_{20:5}$, $C_{22:5}$ and $C_{22:6}$ analogues of 2-AG (data not shown). These were detected in aliquots of normal phase HPLC fractions containing, respectively, anandamide or 2-AG,[2,3,22] after derivatization and analysis by GC–MS run in the total ion current mode.

Fatty acid amide hydrolase activity in Hydra vulgaris

Subcellular fractions obtained from *H. vulgaris* homogenates were analysed for the presence of [^{14}C]anandamide-hydrolysing activity. As with mammalian FAAH,[15] the enzymatic formation of [^{14}C]ethanolamine was most abundant in the "microsomal" (100,000 × *g* pellet) fraction (Fig. 4A). The activity of the two pooled membrane fractions was further characterized and found to exhibit a pH dependency profile typical of mammalian FAAH,[15] with maximal activity between pH 9 and 10, and another apparent active-site pK_a value at pH 6 (Fig. 4B). Although the enzyme seemed to be rather abundant (apparent $V_{max} = 3.4 \pm 0.9$ nmol/min/mg protein), its affinity for anandamide was considerably lower than that described previously for mammalian FAAH,[15] with an apparent $K_m = 400 \pm 88$ μM (means ± S.D., $n = 3$; Fig. 4C). Accordingly, the typical FAAH inhibitors and AA derivatives arachidonoyltrifluoromethyl ketone, methylarachidonoylfluorophosphonate, arachidonoyldiazomethyl ketone and arachidonoylchloromethyl ketone, at concentrations shown previously to cause 100% inhibition of mammalian FAAH,[14,15] caused a sub-maximal, albeit statistically significant, inhibition of *H. vulgaris* [^{14}C]anandamide-hydrolysing activity (Fig. 4D), while the anandamide congener [^{14}C]oleoylethanolamide was also efficiently hydrolysed by *Hydra* membranes (data not shown).

DISCUSSION

The data presented here show that: (i) the endogenous cannabinoid receptor ligand anandamide inhibits the glutathione-induced feeding response,

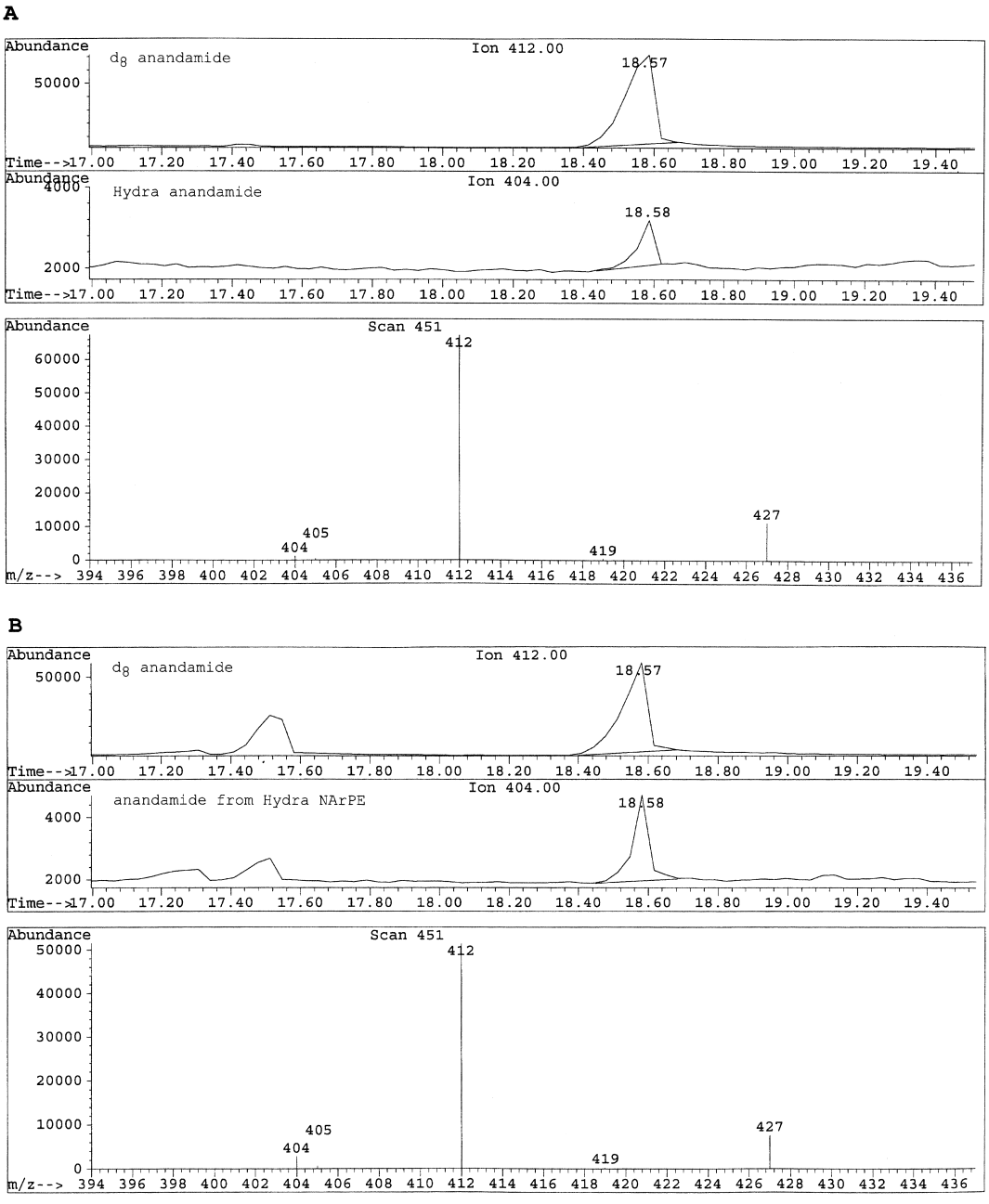

Fig. 3. *Hydra* contains anandamide and its putative precursor NArPE. Lipids from *H. vulgaris* polyps were purified and anandamide or 2-AG contained therein derivatized to form the tris-methylsilyl ethers. (A) Selected ion monitoring (SIM) GC–MS chromatogram of anandamide (middle panel, selected ion $m/z = 404$) in the presence of deuterated anandamide (upper panel, selected ion $m/z = 412$) as internal standard, and selected ion monitoring fragmentogram of the peak at 18.57–18.58 min (lower panel). (B) Selected ion monitoring GC–MS chromatogram of anandamide (middle panel, selected ion $m/z = 404$), obtained from PLD digestion of purified NArPE, in the presence of deuterated anandamide (upper panel, selected ion $m/z = 412$) as internal standard, and selected ion monitoring fragmentogram of the peak at 18.57–18.58 min (lower panel). (C) Selected ion monitoring (SIM) GC–MS chromatogram of 2-AG (upper panel, molecular ion at $m/z = 522$; middle panel, -15 ion at $m/z = 507$) in the presence of deuterated 2-AG (not shown) as internal standard. The full mass spectra of the peak at 20 min from a separate analysis run in the total ion current mode is shown in the lower panel. Another peak eluting after 20.36 min was also found to contain ions at $m/z = 522$ and 507 (not shown), and corresponds to 1(3)-arachidonoylglycerol.

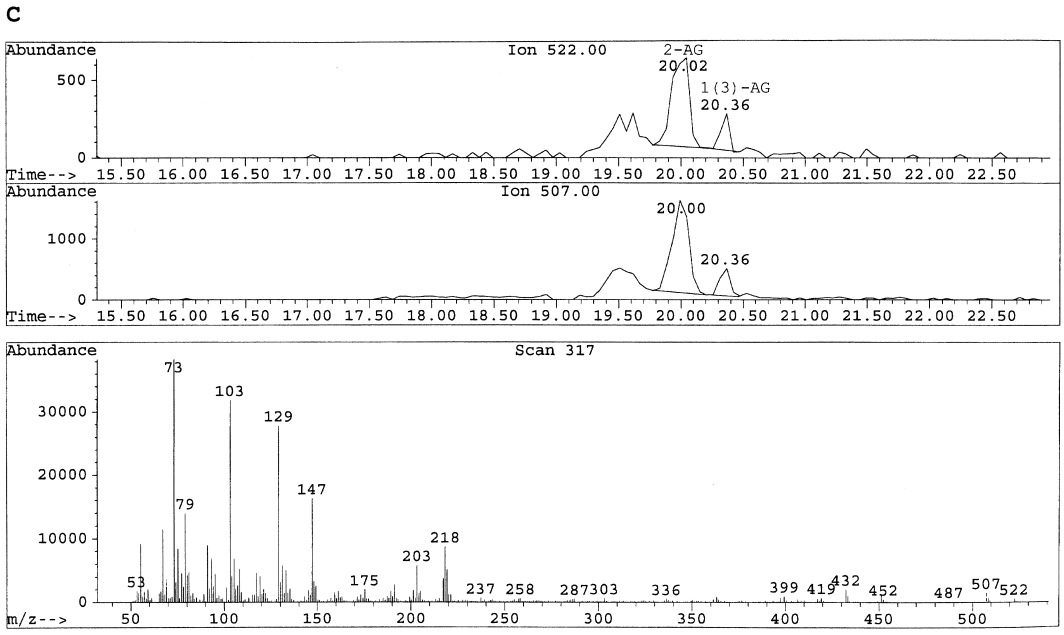

Fig. 3C.

the effect being blocked by a selective antagonist of mammalian CB_1 receptors; (ii) cannabinoid/anandamide receptors are present in *H. vulgaris*; (iii) *H. vulgaris* synthesizes anandamide and its putative biosynthetic precursor NArPE; (iv) an abundant anandamide-hydrolysing, FAAH-like activity, potentially responsible for the termination of the anandamide/cannabinoid receptor-mediated signal, is expressed in the coelenterate. *Hydra* represents, to the best of our knowledge, the simplest animal organism ever found to respond to anandamide and to possibly utilize the "endocannabinoid signalling system" for the control of a physiological response. Specific cannabinoid binding sites were detected by the use of a high-affinity selective ligand of the CB_1 mammalian cannabinoid receptor, SR 141716A, which behaves as an antagonist of CB_1-mediated functional responses.[41] In consideration of the selectivity of SR 141716A for CB_1 over CB_2 receptors,[41] the *Hydra* cannabinoid receptor could be regarded as CB_1-like, but, clearly, the full molecular characterization of this protein must be obtained to support this suggestion. It is worthwhile noting that the only two studies that have so far described the molecular characterization of cannabinoid receptors from an invertebrate species, the leech,[20,45] although reporting somewhat different results, agreed in pointing out a significant homology with the human CB_1 receptor.

The *Hydra* cannabinoid receptor described here for the first time is also an "anandamide receptor", since the endocannabinoid displaced the binding of [^{3}H]SR 141716A to *Hydra* membranes with a K_i value not far from those reported for the displacement of [^{3}H]SR 141716A, or other radiolabelled high-affinity cannabinoid receptor ligands, from mammalian brain membranes (reviewed in Refs 26 and 35). Furthermore, our data suggest that this receptor may be involved in the control of the glutathione-induced feeding response, since exogenous anandamide, at nanomolar concentrations, potently inhibited this behavioural response in a fashion that could be blocked by SR 141716A. The endocannabinoid is the most potent modulator of the *Hydra* feeding response described to date, since all the other drugs previously reported to influence this response are active at micromolar concentrations.[8,9,18,37–40] The estimated IC_{50} of anandamide (about 7 nM) for this effect is very similar to the possible concentration of the endocannabinoid in *H. vulgaris* polyps. This can be extrapolated from the amounts of anandamide measured in this study, i.e. 15.6 pmol/g wet tissue weight, which is likely to yield a 10–20 nM concentration *in vivo*, a value which could be increased further in feeding polyps. In fact, we showed previously[39] that glutathione stimulation of living *H. vulgaris* polyps leads to a twofold increase of free AA levels. Activation of AA release has been suggested to lead to anandamide biosynthesis in murine macrophages,[36] and a possible mechanism for this phenomenon is the remodelling of membrane phospholipids with subsequent increase in diarachidonoylphosphatidylcholine, which serves as a precursor for NArPE and anandamide biosynthesis[5,16,46] (also see Ref. 15 and references cited therein). It is possible that a similar pathway may also occur in glutathione-stimulated *Hydra*, leading to increased anandamide formation. Indeed, part of the previously reported[39,40] inhibitory action on the feeding

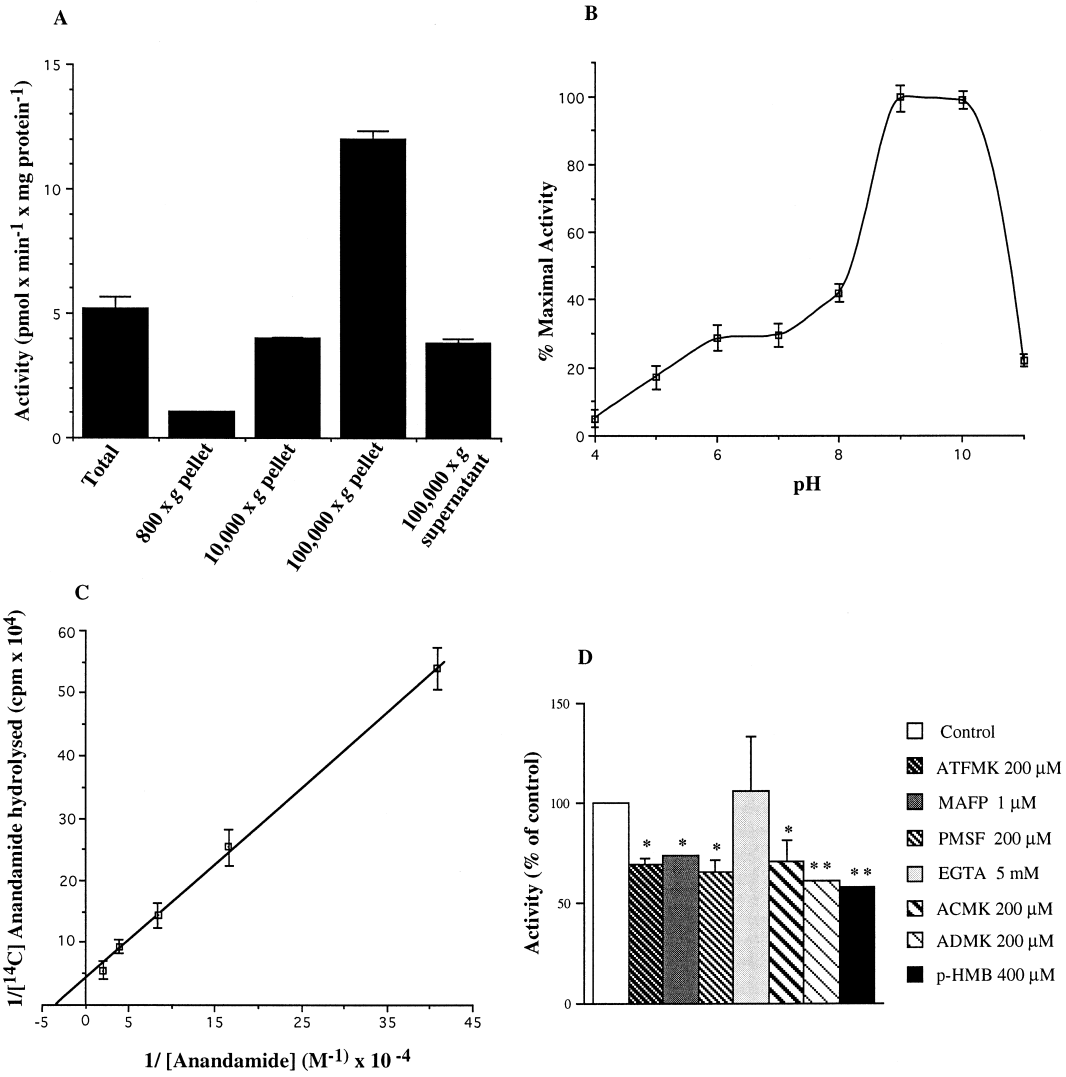

Fig. 4. *Hydra* contains an FAAH-like enzyme. Activity was calculated by measuring the formation of [^{14}C]ethanolamine from [^{14}C]anandamide. (A) Subcellular localization of the [^{14}C]anandamide-hydrolysing activity in *H. vulgaris* homogenates. (B) pH dependency of the [^{14}C]anandamide-hydrolysing activity from *H. vulgaris* membranes (pooled 10,000×*g* and 100,000×*g* pellets) expressed as a percentage of the activity at pH 9.0 (maximal activity). (C) Lineweaver–Burk profile of the [^{14}C]anandamide-hydrolysing activity from *H. vulgaris* membranes obtained when using different concentrations of [^{14}C]anandamide (2.4, 6.2, 12, 24 and 48 µM). (D) Effect of inhibitors of mammalian FAAH on the [^{14}C]anandamide-hydrolysing activity from *H. vulgaris* membranes. The effect is expressed as a percentage of the activity in the absence of inhibitors (and with vehicle). ACMK, arachidonoylchloromethyl ketone; ADMK, arachidonoyldiazomethyl ketone; ATFMK, arachidonoyltrifluoromethyl ketone; MAFP, methylarachidonoylfluorophosphonate; *p*-HMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethylsulphonyl fluoride. $*P < 0.05$, $**P < 0.01$ vs control, calculated by ANOVA followed by Fisher's test. Data are means ± S.D. of at least three independent experiments.

response exerted by high micromolar doses of AA (which are also likely to induce phospholipid remodelling and diarachidonoylphosphatidylcholine formation) may be due to the production of anandamide. Accordingly, both AA and anandamide inhibit the feeding response by selectively accelerating *Hydra* mouth closure[39] (and data reported here). However, given the lack of a stimulatory action on glutathione-induced feeding reaction by 100 nM SR 141716A, it is unlikely that endogenous anandamide alone controls this response. Other endogenous, possibly AA-derived mediators, whose action is not blocked by the CB_1 antagonist, may play a leading role *in vivo*. Also, anandamide congeners such as palmitoylethanolamide and oleoylethanolamide, found to accompany anandamide in *Hydra* as well as mammalian tissues and to exert weak cannabimimetic actions without activating CB_1 receptors,[32] may coadjuvate anandamide in the control of the feeding response. This latter hypothesis may explain why the FAAH-like activity detected in *Hydra* (see below) exhibits low affinity/selectivity for anandamide.

We have also shown here that *Hydra* contains mechanisms potentially responsible for anandamide biosynthesis and inactivation, and therefore have provided further biochemical grounds to the hypothesis that the anandamide/cannabinoid signalling system may play a physiological role in the coelenterate. NArPE was found in *H. vulgaris* extracts in a molar ratio with anandamide very similar to that described previously for tissues where the phospholipid behaves as the direct precursor of the endocannabinoid.[4,5,15,46] Moreover, we found an FAAH-like activity which may be responsible for the inactivation of both anandamide and 2-AG (which was also detected here in *H. vulgaris* lipid extracts), and therefore may play a role in the control of the feeding response. The kinetics as well as the pH dependency profile and sensitivity to inhibitors of this enzyme are very similar to those reported by us for analogous enzymes from invertebrate tissues.[4,43] However, we observed a low affinity and selectivity for anandamide and a reduced sensitivity to AA derivatives which potently inhibit mammalian FAAH,[15] suggesting that the invertebrate enzyme binding sites may have different structures from the equivalent mammalian protein, which has been cloned only recently.[11] Further studies will be needed to fully characterize FAAH enzymes from invertebrate organisms like *Hydra*.

An investigation of the possible mechanism(s) of action through which the activation of cannabinoid receptors causes inhibition of the feeding response was not among the aims of the present study. However, on the basis of the data existing in the literature on the molecular events that are triggered by cannabinoid receptors,[15,26,35] it is possible to make some speculations on this subject. At the intracellular level, CB_1 receptors are coupled, *inter alia*, with inhibition of both Ca^{2+} influx and cyclic-AMP formation,[15,26,35] and with activation of nitric oxide release.[44,45] These cannabinoid receptor-mediated effects might, in principle, all be responsible for anandamide shortening of the feeding response duration, since this behaviour has been shown to correlate with increases of intracellular Ca^{2+} or cyclic-AMP levels,[27] and to be inhibited by nitric oxide.[8] At the cellular level, the dopaminergic[48] and GABAergic[9,37] systems have been shown to be involved in the induction and/or prolongation of the feeding response. In view of the ever increasing number of publications documenting an inhibitory action by presynaptic cannabinoid receptors on the release of GABA[6,47] and dopamine in the mammalian CNS,[19] it is possible to hypothesize that anandamide inhibition of the feeding response is due to interaction with one or both of these two neurotransmitters. In particular, due to the fact that anandamide (or AA[39]) and GABA[37] act specifically, with opposing effects, on the timing of mouth closure rather than opening, it seems reasonable to suggest that endocannabinoid control of the feeding response is exerted at the level of GABA neurotransmission. However, it should be mentioned that the effects of cannabinoids on GABAergic transmission are still a matter of controversy, since these compounds have also been shown to potentiate the activity of GABA by inhibiting its re-uptake from nerve terminals.[10,42] Clearly, appropriate studies, requiring the co-ordinated effort of neurophysiologists and pharmacologists, will have to be designed to specifically address the possible interactions between anandamide and GABA in *Hydra*.

CONCLUSION

We have shown that signalling molecules (endogenous agonists and receptors) of the cannabinoid regulatory system exist in one of the most primitive animal organisms to have evolved a rudimentary nervous network, and may be involved in the control of a typical behaviour. This finding strongly supports the concept that the anandamide/cannabinoid receptor system has been preserved throughout animal evolution,[4,7,20,43–45] even though its physiological task may have evolved from the control of a very fundamental behaviour, i.e. feeding, in *Hydra* to more complex and finer regulatory roles in the central and peripheral nervous systems of higher vertebrates.[19]

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