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Anandamide metabolism by *Tetrahymena pyriformis* in vitro. Characterization and identification of a 66 kDa fatty acid amidohydrolase

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Abstract

Fatty acid amidohydrolase, a membrane-bound enzyme found in a variety of mammalian cells, is responsible for the catabolism of neuromodulatory fatty acid amides, including anandamide. In an earlier study we reported that *Tetrahymena pyriformis* was able to secrete a FAAH-like activity in starvation medium (Karava V., Fasia L., Siafaka-Kapadai A., FEBS Lett. 508 (2001) 327–331). In this study the endocannabinoid anandamide, was found to be metabolized by *T. pyriformis* homogenate by the action of a FAAH-like enzyme, in a time- and concentration-dependent manner. The main metabolic products of [^{3}H]anandamide hydrolysis were [^{3}H]arachidonic acid and ethanolamine. Amidohydrolase activity was maximal at pH 9–10, it was inhibited by phenylmethylsulfonyl fluoride and arachidonyltrifluoromethyl ketone and was Ca^{2+} and Mg^{2+}-independent. Kinetic experiments demonstrated that the enzyme had an apparent K_m of 2.5 μM and V_{max} of 20.6 nmol/min mg. Subcellular fractionation of *T. pyriformis* homogenate showed that the activity was present in every subcellular fraction with highest specific activity in the microsomal as well as in non-microsomal membrane fraction. Immunoblot analysis of selected subcellular fractions, using an anti-FAAH polyclonal antibody, revealed the presence of an immunoreactive protein with a molecular mass ~66 kDa similar to the molecular mass of the mammalian enzyme. In conclusion, this study demonstrates that a FAAH similar to the mammalian enzyme is present in a unicellular eukaryote, indicating the importance of FAAH activity throughout evolution. It also supports the notion that *Tetrahymena* species may be a suitable model for metabolic studies on endocannabinoids, as well as for the study of drugs targeted towards FAAH.

Keywords: Anandamide; *Tetrahymena pyriformis*; Fatty acid amidohydrolase

Enzyme: Fatty acid amide hydrolase (arachidonoylethanolamide amidohydrolase; EC 3.5.1.4)

1. Introduction

The existence of an endogenous cannabinoid signaling system has been established and recent studies provided evidence that the major physiological effect of anandamide and other endogenous cannabinoids in the brain is the regulation of neurotransmitter release through the activation of presynaptic CB_1 receptors. In particular, it has been demonstrated that endocannabinoids are involved in retrograde signaling at GABAergic and glutamergic synapses (for a recent review see Ref. [1]).

Anandamide was first isolated from porcine brain in 1992 [2]. Since then it has been isolated from tissues of various mammalian species, such as rat, bovine and human brain, rat kidney, testis, skin and spleen, blood plasma and human spleen and heart [3–7].

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAAH, fatty acid amidohydrolase; NAPE, *N*-acylphosphatidylethanolamine; NL, neutral lipids; PL, polar lipids; PMSF, phenylmethylsulfonyl fluoride; POPOP, 1,4-di[2-(5-phenyloxazole)] benzene; PPO, 2,5-diphenyloxazole.

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The production and release of anandamide is induced through a mechanism involving the hydrolysis of *N*-arachidonoyl-phospatidylethanolamine (NAPE) by the action of a NAPE-specific phospholipase D [8]. Anandamide is inactivated through sequential re-uptake and enzymatic hydrolysis. The uptake of anandamide is rapid, temperature dependent, selective for anandamide over other *N*-acylethanolamines and saturable, suggesting the presence of facilitated transport mechanism rather than an active co-transport system or passive diffusion [9]. Yet the absence of transporter has been recently reported by Glaser et al. [10] and also by our laboratory in rabbit platelets [11]. Anandamide is then hydrolyzed, and therefore inactivated, to arachidonic acid (AA) and ethanolamine by the action of an enzyme called fatty acid amidohydrolase (FAAH), which has been found in brain, liver, kidney and cell lines derived from various mammalian tissues [12,13]. Another pathway has also been proposed for the metabolism of anandamide, consisting of its enzymatic oxidation by enzymes of the arachidonate cascade such as lipoxygenases, cycloxygenases and cytochrome P450 oxygenases [14–16].

FAAH is mainly localized in microsomal membranes, but it has also been found in non-microsomal membrane fractions from rat and porcine brain as well as from mouse neuroblastoma and glioma cells [12,17,18]. The primary structure of FAAH displays significant homology with the "amidase signature family" of enzymes [19,20].

FAAH activity has also been found in invertebrates such as mussels, leech and snails [21,22]. In addition, cannabinoid receptors and endocannabinoids have been identified in various species of invertebrates. These signal molecules seem to have multiple roles in invertebrates, by diminishing sensor input, and by controlling reproduction, feeding behavior, neurotransmission and anti-inflammatory actions (for a comprehensive review, see Ref. [23]).

Tetrahymena species are widely used models for studies in diverse fields of biochemistry and molecular biology. It has been reported that delta-9-tetrahydrocannabinol elicited an effect on movement, cellular growth and division in *Tetrahymena pyriformis* [24,25]. *Tetrahymena* releases large amounts of proteases, nucleases, glucosidases, lipases and phospholipases in the medium, and in dilute salt solutions [26]. It has been recently shown by our laboratory that *T. pyriformis* is capable of hydrolyzing anandamide in vivo and releasing amidohydrolase activity in starvation medium. The released enzyme causes the hydrolysis of anandamide to AA and ethanolamine, in a time- and concentration-dependent manner. In addition, it has alkaline maximal pH (9–10) and apparent K_m of 3.7 μM and V_{max} of 278 pmol/min × mg [27].

The aim of the present study was to investigate the in vitro metabolism of [^{3}H]anandamide and to identify the metabolites thereof, as well as to identify and characterize the main enzyme involved, namely FAAH.

2. Materials and methods

2.1. Materials

[^{3}H]Anandamide (200 Ci/mmol), radiolabeled at the AA moiety, and [^{3}H]AA (30 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 5,5′-dithio-di(2-nitrobenzoic-acid) (DTNB), arachidonoyl-trifluoromethyl ketone ($AACOCF_3$), caffeic acid, acetylsalicylic acid, palmitoyl chloride, oleoyl chloride, PPO, POPOP, Ponceau S solution and diaminobenzidine were from Sigma Chemicals Co. (St Louis, MO, USA). Other chemicals were of the highest purity available.

2.2. Cell cultures

T. pyriformis, strain W was cultured at 25 °C under constant stirring in a medium consisting of 2% "proteose"-peptone, 0.5% dextrose, 0.2% yeast extract and 1% Fe^{2+}–9 mM EDTA (pH 5.5). At the end of the logarithmic phase (approximately 60 h), cells were harvested by centrifugation at 500 × *g* for 10 min, the cell pellet was washed using 5 mM Tris–HCl pH 9 (or 0.9% NaCl) and resuspended in the same solution (2.5–3.0 × 10^6 cells per ml).

2.3. In vitro metabolism of [^{3}H]anandamide

T. pyriformis cells (2.5–3.0 × 10^6 cells per ml), harvested as described above, were resuspended in 5 mM Tris–HCl buffer (pH 9) and homogenized with a sonicator (Vibra Cell Sonics and Materials, Newtown, CT, USA). Homogenate fractions were incubated with [^{3}H]anandamide (5 μM, 1 μCi/ml) in 1 ml final volume at 37 °C for 10 min, unless stated otherwise. The lipids were extracted according to the Bligh and Dyer [28] method, separated by TLC on heat activated silica gel G plates, using a solvent system of $CHCl_3$/CH_3OH/NH_4OH (28%) 80:20:2 (v/v/v), and were visualized by exposure to iodine vapors. Bands corresponding to anandamide and its metabolic products (free AA, or AA incorporated into phospholipids, PL, or into neutral lipids, NL) were scraped off the plate and the radioactivity was measured by liquid scintillation counting using a toluene-based scintillation fluid (5 g PPO and 0.3 g POPOP in 1 l toluene). The radioactivity of the methanol–water phase was also measured using a dioxan based scintillation fluid (100 g naphthalene, 7 g PPO, 0.3 g POPOP, 200 ml water in 1 l dioxan) in a liquid scintillation counter (Wallac 1209 Rackbeta of Pharmacia, Wallac, UK). The influence of metal ions and inhibitors on the hydrolytic activity was investigated after preincubation of the cell homogenate with the appropriate concentrations of the reagent solution at 37 °C for 5 min. Other FAAH substrates (*N*-oleoylethanolamine, *N*-palmitoylethanolamine) were added simultaneously with [^{3}H]anandamide.

Protein was measured by the method of Lowry et al. [29].

2.4. Preparation of subcellular fractions

T. pyriformis cells were washed using 20 mM Tris–HCl buffer, pH 7.2, 3 mM EDTA and 0.1 M NaCl, and homogenized in the same buffer containing 0.25 M sucrose [30]. The homogenate was layered on a buffered discontinuous sucrose gradient (0.34 M, 10 ml; 1.00 M, 15 ml; 1.46 M, 15 ml) and centrifuged at 4080 × *g* for 5 min. Three major bands were separated: a top band down through the 0.34 M layer, a discrete band of pellicles (membranes) at the interface between the 1.00 M and the 1.46 M layers and a very small pellet of unbroken cells at the bottom. The pellicle fraction, which contained the cell membranes, was removed carefully by a syringe. The top band, composed of mitochondria and less dense material, was diluted with buffer by one third of its volume. The suspension was centrifuged at 27,000 × *g* for 20 min, in order to pellet mitochondria. The supernatant was further subjected to centrifugation at 100,000 × *g* for 60 min, yielding a pellet comprised of microsomes and cytosol. All subcellular fractions were tested for amidohydrolase activity.

2.5. SDS-PAGE—immunoblot analysis

SDS-PAGE (12% polyacrylamide) was performed under reducing conditions in a Mini Protean II apparatus (BioRad Laboratories, Hercules, CA, USA), using 0.75 mm spacer arms. SDS-PAGE Molecular Weight Standards (BioRad Laboratories) were phosphorylase b, BSA, ovalbumin and carbonic anhydrase (97.4, 66.2, 45.0, 31.0 kDa, respectively). Total protein from selected subcellular fractions were transferred onto nitrocellulose filters (Shleicher and Schuell, Keene, NH, USA) using a Semi-dry blotter (Phase, Mölln, Germany). After blocking in TBS/3% BSA, the blot was incubated with the primary and the secondary antibody and finally was detected by ECL chemiluminescence procedure. Specific anti-FAAH polyclonal antibodies elicited in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR conjugated to ovalbumin (Primm, Milan, Italy) were used as primary antibody [20,31]; goat anti-rabbit IgG horseradish peroxidase conjugate (AlterChem, Athens, Greece) was used as a secondary antibody. Immunoreactive bands were stained using ECL chemiluscence according to the manufacturer's instructions (Chemicon International, Temecula, CA, USA). Non-immune rabbit serum was from Primm, and purified FAAH was a kind gift of Dr. Benjamin F. Cravatt (The Scripps Research Institute, La jolla, CA).

2.6. Synthesis of N-oleoylethanolamine and N-palmitoylethanolamine

Oleoyl/palmitoyl chloride was dissolved in methylene chloride and a 10-fold molar excess of ethanolamine was added. The reaction took place at 0–4 °C, under nitrogen, for 15 min and was terminated by the addition of water. The organic phase was dried under vacuum and the product was purified by preparative TLC using $CHCl_3/CH_3OH/NH_4OH$ (28%) 80:20:2 (v/v/v) as solvent (Rf = 0.7) [2].

The structure of *N*-oleoylethanolamine was verified by ^{1}H-NMR at 300 MHz, using a Varian NMR Unity Plus, 300 MHz. The main peaks of ^{1}H-NMR were a single at 5.9–6 ppm (–NH–C=O), a multiple at 5.4 ppm (–CH=CH–), a triple at 0.9 ppm ($–CH_3$) and a single at 1.3–1.4 ppm. In addition, ^{13}C-NMR analysis (data not shown) confirmed the results obtained with ^{1}H-NMR (not shown).

The structure of *N*-palmitoylethanolamine was verified with electrospray mass spectrometry using a Finnigan Quadruple MAT SSQ700. There was one main peak at *m*/*z* 300.3 which corresponds to the $[M + H]^+$ (not shown).

3. Results

3.1. In vitro metabolism of [^{3}H]anandamide

T. pyriformis cells were harvested, homogenated and tested for their ability to metabolize [^{3}H]anandamide. As shown in Fig. 1, [^{3}H]anandamide was rapidly metabolized: ~10% was hydrolyzed after approximately 2 min, ~30% and ~60% after 5 and 10 min, respectively. The catabolism reached ~100% after 40 min. The main metabolic product was a compound comigrating with free fatty acids, apparently AA (~60% of total radioactivity after 10 min incubation), while the proportion of radioactivity corresponding to polar lipids was very low. It should be mentioned that ~20% was found in the water–methanol phase of the Bligh–Dyer extraction after 60 min incubation with [^{3}H]anandamide. The water soluble products could derive either from enzymatic or non-enzymatic oxidation of [^{3}H]anandamide and their identification is under investigation.

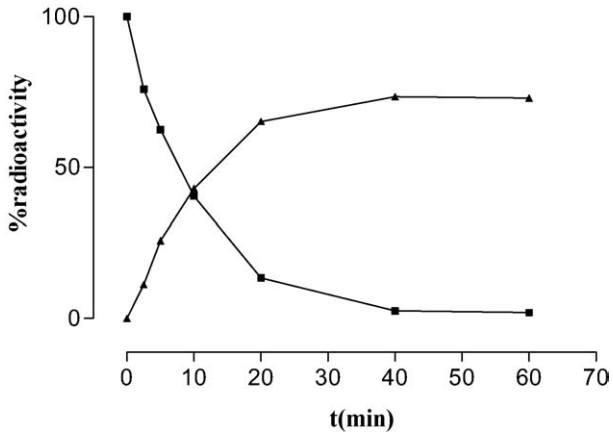

Fig. 1. Fatty acid amide hydrolase activity in the homogenate of *T. pyriformis* cells. The homogenate (10 μg protein per ml) was incubated at 37 °C in 50 mM Tris–HCl buffer, pH 9, containing [^{3}H]anandamide (5 μM). At the indicated times (0 corresponds to ~2 min) aliquots of the incubation mixture were collected. Lipids were then extracted from the mixture, were separated on TLC and their radioactivity was measured. (▲) Free AA (■) anandamide. Values are the means of two experiments performed in duplicate.

3.2. Characterization of FAAH-like activity

As shown in Fig. 2, anandamide hydrolysis followed first order kinetics and the rate of hydrolysis was dependent on protein concentration. The extent of metabolism was a linear function of protein concentration between 0 and 10 μg/ml (Fig. 2 inset), while a plateau was reached at approximately 50 μg. The metabolism of anandamide was also dependent on the substrate concentration. The apparent K_m of hydrolysis was 2.5 ± 1.3 μM and the V_{max} was 20.64 ± 2 nmol/min mg protein (Fig. 3).

In addition, anandamide hydrolysis was pH-dependent, exhibiting a pH optimum at 9–10 (Fig. 4), and was also dependent on the temperature with an optimum between 30 and 40 °C (not shown).

As shown in Table 1, the serine protease inhibitor PMSF inhibited almost completely the hydrolysis suggesting the presence of amidohydrolase activity. Also $AACOCF_3$, a potent inhibitor of amidohydrolase, inhibited anandamide hydrolysis to a great extent, indicating the action of an amidohydrolase. Anandamide metabolism was not affected by the presence of DTT and DTNB, suggesting that sulfhydryl groups do not play important role in the activity. It was also unaffected by the addition of EGTA, EDTA and $MgCl_2$, showing that the activity was not dependent on Ca^{2+} or Mg^{2+}. In our hands, preincubation with caffeic acid and indomethacin ruled out the possibility that anandamide could be metabolized by lipoxygenase (LOX) or cyclooxygenase (COX), since anandamide hydrolysis was not affected.

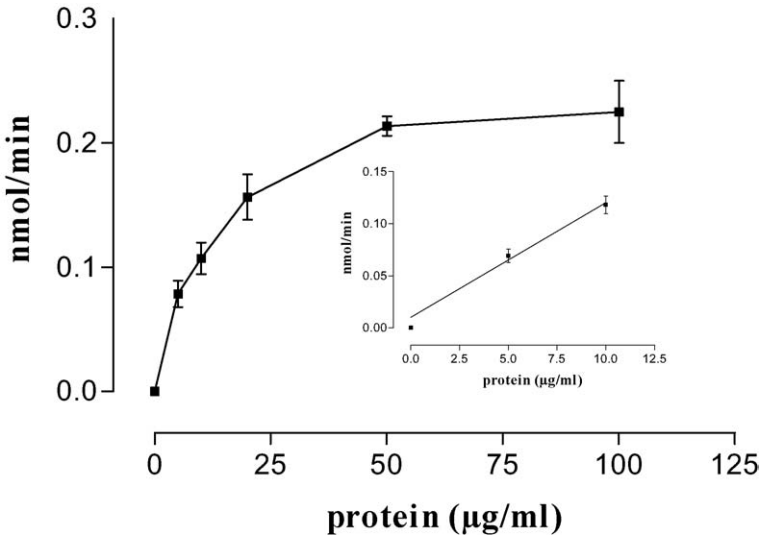

Fig. 2. FAAH activity as a function of protein concentration. *T. pyriformis* homogenate containing different amounts of protein was incubated with [^{3}H]anandamide (5 μM) for 10 min. For other experimental details see Fig. 1 legend. Values are the mean ± S.E.M. of three experiments performed in duplicate. Inset: Metabolism of anandamide from 0 to 10 μg protein.

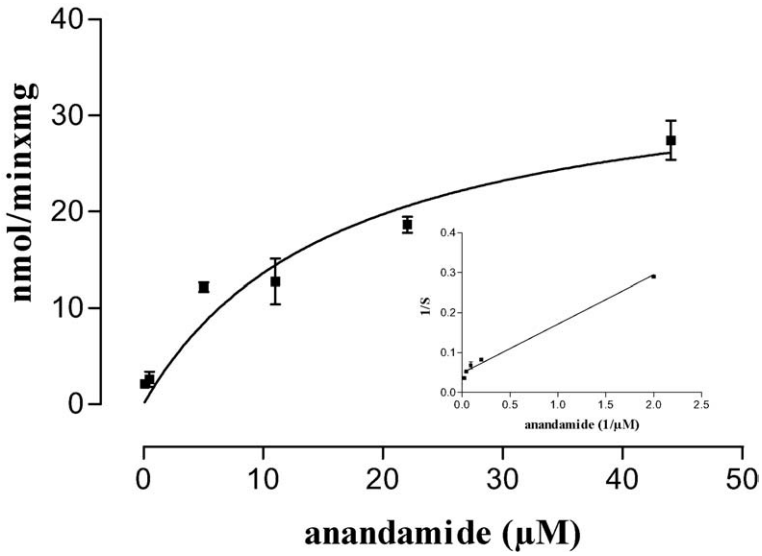

Fig. 3. FAAH activity as a function of substrate concentration. *T. pyriformis* homogenate (10 μg/ml) was incubated with different concentrations of [^{3}H]anandamide at pH 9 for 10 min. Values are the mean ± S.E.M. of six experiments. Inset: Lineweaver–Burk plot.

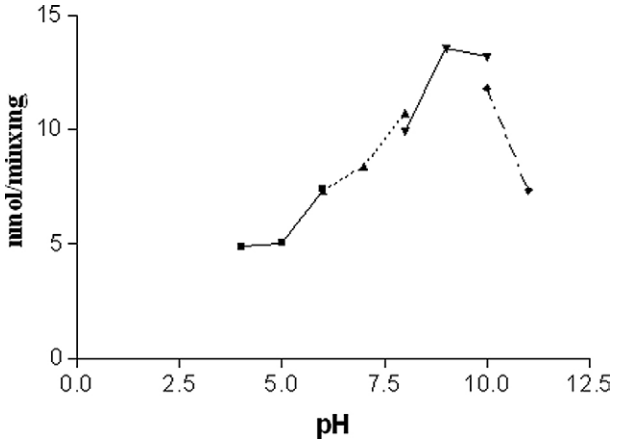

Fig. 4. pH-dependence of the hydrolysis of anandamide. The homogenate (10 μg/ml) was incubated with 5 μM [^{3}H]anandamide at the indicated pH for 10 min. The buffers (50 mM) used were: citrate at pH 4.0, 5.0, 6.0(■), phosphate at pH 6.0, 7.0 , 8.0 (▲), Tris–HCl at pH 8.0, 9.0, 10.0 (▼) and bicarbonate at pH 10.0, 11.0 (♦). Lipids were extracted, separated on TLC and radioactivity was measured. Results are from one representative experiment performed in duplicate.

To test the substrate specificity of the enzyme in the homogenate of *T. pyriformis* cells, *N*-palmitoylethanolamine and *N*-oleoylethanolamine were included in the reaction mixture. The hydrolysis of [^{3}H]anandamide was inhibited at high doses only, e.g. 100 μM *N*-oleoylethanolamine caused 87.9% inhibition (Table 1). Therefore, these results suggest that amidohydrolase was rather specific for anandamide.

3.3. Subcellular distribution of FAAH

The subcellular distribution of FAAH activity in *T. pyriformis* cell homogenate was investigated. The homogenate was fractionated as described under Materials and Methods. Each fraction was incubated with [^{3}H]anandamide and FAAH activity was determined. As shown in Table 2, the specific activity of the anandamide hydrolysis was highest in the non-microsomal membrane fraction (44 nmol/min × mg protein) followed by the 100,000 × *g* pellet (microsomal fraction) and the 27,000 × *g* pellet. It is worth noting that FAAH activity was also detected in the 100,000 × *g* supernatant (cytosolic fraction) (see Fig. 5).

Table 1
Effects of various compounds on FAAH activity (substrate was 5 μM [^{3}H] anandamide)

Inhibitor	Activity nmol/min mg protein	Percent inhibition of anandamide degradation
–	24.0 ± 1.0	–
PMSF (0.2 mM)	2.9 ± 0.1	87.5 ± 0.5
$AACOCF_3$ (8 μM)	5.5 ± 2.5	77.0 ± 0.0
N-palmitoylethanolamine (100 μM)	8.5 ± 2.2	64.5 ± 9.2
N-oleoylethanolamine (10 μM)	7.8 ± 1.7	67 ± 7.6
N-oleoylethanolamine (100 μM)	2.9 ± 0.4	87.9 ± 1.7
EGTA (2 mM)	23.5 ± 1.5	–
EDTA (2 mM)	21.5 ± 0.5	10.0 ± 2.0
DTT (0.33 mM)	21.5 ± 0.5	10.0 ± 2.0
DTNB (0.1 mM)	26.5 ± 1.5	–
Cafeic acid (0.3 mM)	28.0 ± 3.0	–
Indomethacin (10 mM)	22.5 ± 1.5	–
Mg^{2+} (1 mM)	27.5 ± 1.5	–

Table 2
Subcellular distribution of the anandamide amidohydrolase activity in *T. pyriformis* cells (substrate was 5 μM [^{3}H] anandamide; results are from a representative experiment)

Cell fraction	Specific activity (nmol/min mg protein)	Total activity (%)
Homogenate	13	100
* Membranes (pellicles)	44	33
* 27,000 × *g* pellet	40	3
*100,000 × *g* pellet	33	1
* 100,000 × *g* supernatant	9	6

* The sum of total activity of these fractions is < 100%, because only selected subcellular fractions are shown in the table (see Section 3).

It should be mentioned here that only selected subcellular fractions are shown in Table 2. The fraction corresponding to the pellet of the first centrifugation (4080 × *g* for 5 min) contained ~30% of total activity, but was not further investigated since it could contain unbroken cells. Also the band between the pellicle and the above mentioned fractions contained ~12% of total activity and was not further fractionated (not shown in Table 2).

3.4. Immunodetection of FAAH activity

Selected subcellular fractions were subjected to SDS-PAGE electrophoresis (50 μg protein per lane) followed my immunoblot analysis. As shown in Fig. 5, two main protein bands were detected: one corresponding to a ~66 kDa protein (non-microsomal membrane fraction, lane 1) and a second one corresponding to a ~45 kDa protein (microsomal mem-

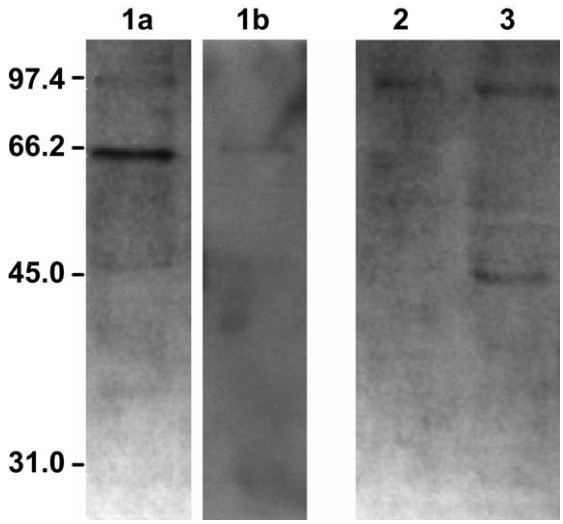

Fig. 5. Immunoblot analysis. Western blot analysis of non-microsomal membranes (lane 1a), microsomes (lane 2) and cytosol (lane 3), 50 μg protein per lane, separated by 12% SDS-PAGE and reacted with specific anti-FAAH polyclonal antibodies. Lane 1b: non-microsomal membranes (lane 1a) after incubation with anti-FAAH polyclonal antibodies that had been preincubated with pure FAAH (1:1 mixture). Results are from one representative experiment (n = 3–8).

brane fraction, lane 2 and cytosol, lane 3). Finally, an immunoreactive protein band of ~90 kDa was also observed (lane 2 and 3), possibly a dimer of the 45 kDa protein. In addition, when non-microsomal membrane fractions (lane 1a) were incubated with non-immune rabbit serum (not shown) or with anti-FAAH polyclonal antibodies that had been preincubated with pure FAAH (1:1 mixture) (Fig. 5, lane 1b) [31], the 66 kDa immunoreactive band (lane 1a) was almost undetectable.

4. Discussion

In this study we show evidence for the presence in a protozoon of a FAAH with characteristics very similar to the mammalian enzyme. *T. pyriformis* cell homogenate was able to rapidly metabolize [^{3}H]anandamide, the main metabolic product being identified as AA by TLC. It should be mentioned that in intact *T. pyriformis* cells [^{3}H]anandamide was rapidly incorporated and metabolized through the action of FAAH, although free fatty acid was a minor metabolic product [27]. Apparently, the AA produced was subsequently incorporated predominantly into phospholipids and to a lesser extent in neutral lipids [27]. In *T. pyriformis* it has been reported that only a small amount, if any, of AA is present [32]. However, in the case of cell homogenate, incorporation of AA produced by FAAH into other lipids (PL or NL) was not observed. Approximately 20% of radioactivity was found in the water–methanol phase (not shown) which could be due, at least in part, to small amounts of [^{3}H]anandamide or [^{3}H]AA produced by FAAH. In a control experiment, where [^{3}H]anandamide or [^{3}H]AA was extracted in the presence of boiled (and thus inactivated) cell homogenate, approximately 10% of radioactivity was found in the water–methanol phase (not shown).

The hydrolytic activity of *T. pyriformis* cell homogenate was PMSF sensitive, suggesting that the enzyme is a serine hydrolase. In addition, the potent inhibitor of amidohydrolase $AACOCF_3$, inhibited anandamide catabolism to a great extent. Instead, EGTA, EDTA and $MgCl_2$ had no effect on the enzymatic activity, suggesting that Ca^{2+} and Mg^{2+} do not play an important role in the catalytic mechanism. In addition, FAAH activity was not affected by DTT or DTNB, showing that it was not depended on sulfydryl groups (Table 1). Similar results have been reported for the secreted enzyme of *T. pyriformis* cells [27], as well as for a variety of mammalian tissues and cell lines [12,33–35]. Thus, we can conclude that [^{3}H]anandamide is metabolized by *T. pyriformis* cell homogenate through the action of an authentic FAAH.

In the presence of caffeic acid, a LOX inhibitor, and of indomethacin, a COX inhibitor, no reduction was observed, suggesting that LOX and COX were not involved in the metabolism of anandamide by FAAH. It is well known that [^{3}H]anandamide and [^{3}H]AA are metabolized by LOX and COX, leading to the formation of thromboxanes and leukotrienes [36]. We have recently reported that FAAH of rabbit platelets was inhibited by indomethacin indicating the influence COX, while caffeic acid had no effect, suggesting that LOX was not involved in anandamide metabolism [11].

Kinetic studies showed that anandamide hydrolysis followed Michaelis–Menten kinetics with apparent K_m of 2.5 ± 1.3 μM and V_{max} of 20.6 ± 2 nmol/min × mg. This apparent K_m value is very close to that (3.7 μM) of secreted enzyme from *T. pyriformis* cells [27].

A first attempt to determine the subcellular distribution of the enzyme showed its presence in membraneous fractions (Table 2). The enzymatic activity was higher in the non-microsomal membrane fraction (pellicles) (Table 2). It has been shown in a variety of cells that FAAH is a transmembrane protein, localized mainly in microsomes and/or mitochondria [17,33–41]. From our results, we can conclude that the enzyme found in *T. pyriformis* is also bound to the cell membranes. The specific activity found in the microsomal fraction was almost as high as in the plasma membrane, but the protein amount in the latter fraction was very low.

Our attempts to solubilize the enzyme using various detergents, in order to purify it, were not successful. We found that commonly used detergents like TritonX-100, Tween 20, NP-40, taurodeoxycholate and CHAPS, at concentrations between 0.01% and 1%, inhibited FAAH activity (data not shown). The reasons of such an inhibition have not been investigated. Similar inhibition of the activity has been reported for rat liver *N*-acylethanolamine amidohydrolase by Triton X-100, sodium cholate and sodium dodecyl sulfate [42,43].

Finally, subcellular fractions with high enzymatic activity namely membranes, were subjected to SDS-PAGE and Western blot analysis, using an anti-FAAH antibody [31]. The results (Fig. 5) showed the presence of a strongly reactive band of approximately 66 kDa in the non-microsomal membrane fraction, and a second one of approximately 45 kDa localized mainly in the 100,000 × *g* supernatant (cytosol). Using the same antibody Maccarrone and coworkers have identified FAAH in mouse uterus [31] and in human brain [35]. It has been reported that mammalian FAAH is a 63 kDa protein [19,20] or a 67 kDa protein [31,35]. It is worth mentioning that in invertebrates, a 46 kDa protein band apparently with FAAH activity has been found with an optimum pH 7 [21]. The presence of a 45 kDa in *T. pyriformis* is very interesting, because a significant FAAH activity at pH 7 is present as well (Fig. 4). Overall, our results from immunoblot analysis (Fig. 5) suggest the presence of at least two isoforms of FAAH in *Tetrahymena*.

The physiological substrates of the enzyme in *Tetrahymena* have not yet been identified and are under investigation in our laboratory. The physiological significance of these findings is not yet known and will be addressed in an independent study. Nevertheless, our results support the notion that *Tetrahymena* is a good model for metabolic studies on endocannabinoids, and could be also used as a convenient experimental model for the study of drugs targeted towards FAAH [44,45].

In conclusion, results reported here demonstrate for the first time the presence of a FAAH with characteristics similar

to the mammalian enzyme in a unicellular eukaryote, the protozoon *Tetrahymena*, suggesting the importance of the enzyme throughout evolution. The enzyme could inactivate endogenous signaling lipids which might have manifold roles in *T. pyriformis*, as suggested already in invertebrates [23] and mammals [1].

Acknowledgments

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