Anandamide amidohydrolase activity, released in the medium by *Tetrahymena pyriformis*. Identification and partial characterization

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Abstract Anandamide, an endogenous cannabinoid receptor ligand, was rapidly metabolized by *Tetrahymena pyriformis* in vivo. Metabolic products were mainly phospholipids as well as neutral lipids, including small amounts of free arachidonic acid. Anandamide amidohydrolase activity was detected in the culture medium by the release of [^{3}H]arachidonic acid from [^{3}H]anandamide, in a time- and concentration-dependent manner. Kinetic experiments demonstrated that the released enzyme had an apparent K_m of 3.7 μM and V_{max} 278 pmol/min/mg protein. Amidohydrolase activity was maximal at pH 9–10, was abolished by phenylmethylsulfonyl fluoride and was Ca^{2+}- and Mg^{2+}-independent. Thus, *T. pyriformis* is capable of hydrolyzing anandamide in vivo and releasing amidohydrolase activity.

Key words: Anandamide metabolism; Fatty acid amidohydrolase; Secreted enzyme; *Tetrahymena pyriformis*

1. Introduction

Anandamide (*N*-arachidonoylethanolamide) is the most representative member of an emerging class of endogenous lipids, including amides and esters of long-chain fatty acids, termed collectively 'endocannabinoids', that have been recently suggested to have a physiological role [1]. Anandamide has been isolated from the brain lipids of various mammalian species [2,3].

Endocannabinoids are capable of binding to cannabinoid receptors and of eliciting cannabinimetic responses [4], such as neuromodulatory effects [5,6], inhibition of cancer cell growth while they also affect reproductive functions [5,7] and modulate immune responses [5].

Anandamide is released from *N*-arachidonoylphosphatidylethanolamine by the action of a phospholipase D-like enzyme [5,8]. It is inactivated by re-uptake by cells followed by hydrolysis (to arachidonic acid (AA) and ethanolamine) and/or esterification process [5,9]. The enzyme responsible for the hydrolysis (anandamide amidohydrolase, recently named fatty acid amidohydrolase, FAAH) is found in various mammalian tissues including brain liver and kidney and cell lines [10–16]. The enzyme is localized on microsomal and mitochondrial membranes [3,11,17] and is likely to be the same amidase previously shown to catalyze the hydrolysis of saturated and monounsaturated amides [18].

Anandamide amidohydrolase has been suggested to be identical to the enzyme catalyzing oleamide hydrolysis which was cloned from a cDNA library of rat liver by Cravatt et al. [19]. A recombinant enzyme overexpressed by transfection in host cells was more active with anandamide than oleamide [19]. Moreover, recently, it has been shown that the same enzyme causes the hydrolysis of another endogenous cannabinimetic compound, 2-arachidonoyl glycerol, to AA and glycerol [20].

Cannabinoids elicit biological action in invertebrates [21] which also contain anandamide as well as other endocannabinoids and its precursors [8,22–24]. For example, it has been suggested that anandamide may be an oocyte-derived cannabinimetic regulator of sea urchin fertility [23] and a physiological stimulant of NO release in invertebrate ganglia [25]. Furthermore, FAAH activity has been found in invertebrates such as Mytilus and leech [5].

Interestingly, it has been reported that THC (Δ^9-tetrahydrocannabinol) elicited an effect on movement, cellular growth and division in *Tetrahymena pyriformis* [26,27]. *Tetrahymena* species are widely used models for studies in diverse fields of biochemistry and molecular biology including lipid metabolism and enzyme secretion. It has been known that *Tetrahymena* releases large amounts of proteases, nucleases, glucosidases, lipases and phospholipases [28]. The release process takes place in both media and dilute salt solutions [29].

In the present study, we report the existence of anandamide amidohydrolase activity in the unicellular eukaryote *T. pyriformis*. Furthermore, we show that the enzyme is secreted from the cell into the medium. To our knowledge, the existence of a secreted form of the enzyme as well as the presence of anandamide amidohydrolase in simple eukaryote has not been reported.

2. Materials and methods

2.1. Materials

[^{3}H]Anandamide (200 Ci/mmol) and [^{3}H]AA (30 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (MO, USA). Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 5,5′-dithio-di(2-nitrobenzoic-acid) (DTNB), caffeic acid, acetylsalicylic acid, palmitoyl chloride and oleoyl chloride were from Sigma. 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% 9 mM Fe^{2+}–EDTA (pH 5.5). At the end of the

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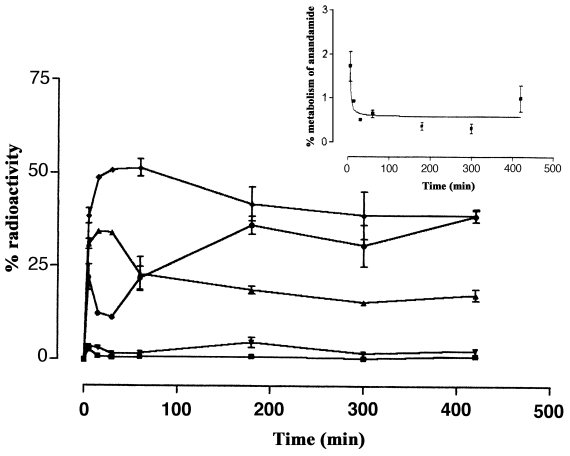

Fig. 1. Metabolic fate of [^{3}H]anandamide in intact cells. *T. pyriformis* cells were incubated with 5×10^{-10} M [^{3}H]anandamide at 25°C. At the indicated times, a 1 ml aliquot of the suspension was removed and lipids were extracted and subjected to TLC and radioactivity was measured as described in Section 2. (●) NLs, (■) anandamide, (♦) PLs, (▲) phosphatidylethanolamine, (▼) free AA. Results are expressed as mean ± S.E.M. of three to six experiments performed in duplicates. Insert: The degradation of [^{3}H]anandamide.

logarithmic phase (approximately 60 h), cells were harvested by centrifugation at $500\times g$ for 10 min, the cell pellet was washed using 5 mM Tris–HCl (or saline), pH 9, and resuspended in the same solution ($2.5–3.0\times10^6$ cells/ml).

2.3. In vivo metabolism of [^{3}H]anandamide, lipid analysis

T. pyriformis cells ($2.5–3.0\times10^6$ cells/ml) were incubated for different time intervals with [^{3}H]anandamide (5×10^{-10} M, 1 μCi/ml). Aliquots of 1.0 ml were washed twice with NaCl 0.9% (w/v) and the lipids were extracted according to the Bligh–Dyer method [30]. Lipids were then separated by thin-layer chromatography (TLC) on heat-activated silica-gel G plates using $CHCl_3/CH_3OH/NH_4OH$ (28%) 80:20:2 (v/v) and were visualized by exposure to iodine vapors. Bands corresponding to anandamide and its metabolic products (e.g. free AA, phospholipids (PL), neutral lipids (NL)) were scraped off the plate and the radioactivity was quantitated by liquid scintillation counting using 5 ml of a toluene-based scintillation fluid, in a liquid scintillation counter (Wallac 1209 Rackbeta of Pharmacia). Methanol–water phase containing soluble metabolites was also assayed for radioactivity by counting 100-μl aliquots, using 10 ml of a dioxan-based scintillation fluid.

2.4. FAAH-activity assay in the medium

Cells, harvested as described above, were resuspended in Tris–HCl, pH 9, buffer or alternatively in saline ($2.5–3.0\times10^6$ cells/ml) and remained at room temperature for 30 min under constant stirring. Cells were then removed by centrifugation ($500\times g$ for 10 min) and the supernatant was incubated with [^{3}H]anandamide (5×10^{-10} M, 1 μCi/ml) in 1 ml final volume for 10 min unless stated otherwise. The rate of cleavage of [^{3}H]anandamide was determined by measuring the radioactivity incorporated into AA and other metabolic products as described for the in vivo metabolism (Section 2.3).

In some experiments, the cell medium was centrifuged at $100\,000\times g$ for 1 h and tested for amidohydrolase activity.

The influence of metal ions and inhibitors on the hydrolytic activity was investigated after preincubation of the cell medium with the appropriate concentrations of the reagent solution at 25°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. [31].

The integrity of the cells was tested by assay of lactate dehydrogenase (LDH) [32] in the medium and observation under the microscope.

2.5. 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 TLC using $CHCl_3/CH_3OH/NH_4OH$ (28%) 80:20:2 (v/v/v) as the solvent system ($R_f = 0.7$) [2].

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

The structure of *N*-palmitoylethanolamine was verified with electrospray mass spectrometry using a Finning Quadruple MAT SSQ700. There was one main peak at *m/z* 300.3 which corresponds to the $[M+H]^+$.

3. Results

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

[^{3}H]Anandamide was taken up and rapidly metabolized by *T. pyriformis* W cells. The main metabolic products were PLs commigrating with phosphatidylethanolamine on TLC and other more polar PL, while a significant amount of radioactivity was incorporated into NL ($R_f = 0.9–1$). After 5 min, 95% of [^{3}H]anandamide was metabolized since only 5% of the total radioactivity remained in anandamide and the radioactivity of PL and NL was 70% and 20% respectively (Fig. 1). At the same time, the percentage of radioactivity corresponding to AA was very low (< 6%) (Fig. 1). These results clearly show that anandamide was rapidly hydrolyzed to [^{3}H]AA and probably ethanolamine by the action of an amidohydrolase-like activity, and AA and/or its metabolite(s) was then incorporated into various lipids. Preincubation of the cells with PMSF (2 mM) for 15 min resulted to the inhibition of anandamide metabolism at a great extent since 83.6 ± 5.0% of the total radioactivity remained in anandamide after 5 min. Only a small amount of radioactivity was found in polar lipids and free fatty acid (2.6 ± 0.7% up to 6 h), indicating that [^{3}H]anandamide was not hydrolyzed to AA by an amidohydrolase in the presence of PMSF (Fig. 1). Interestingly, a significant amount of radioactivity remained incorporated into NL (17.7 ± 5.2% in 5 min) even in the presence of PMSF, but their identity has not been elucidated.

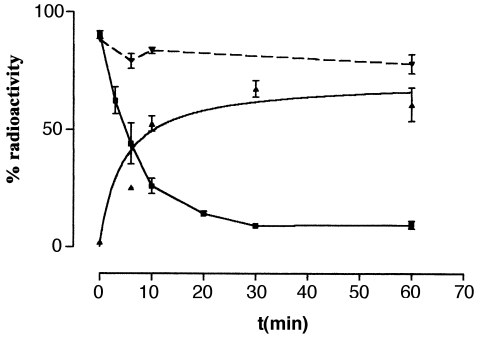

Fig. 2. Anandamide amidohydrolase activity in the medium of *T. pyriformis* cells. The medium (100 μg/ml protein) was incubated at 30°C in 0.05 M Tris–HCl buffer, pH 9, containing [^{3}H]anandamide (5×10^{-10} M). At the indicated times, aliquots of the incubation mixture were collected and lipids were extracted, separated on TLC and their radioactivity was measured as described in Section 2. (▲) Free AA, (■) anandamide, (▼) PMSF. Data represent means ± S.E.M. of four to eight experiments performed in duplicate.

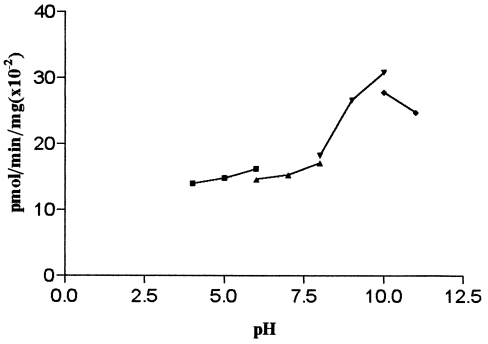

Fig. 3. pH-dependence of the hydrolysis of anandamide. The medium (100 μg/ml) was incubated with [^{3}H]anandamide (5×10^{-10} M) at the indicated pH for 10 min. The buffers (50 mM) used were: citrate acid/sodium citrate at pH 4.0, 5.0, 6.0 (■), KH_2PO_4 at pH 6.0, 7.0, 8.0 (▲), Tris–HCl at pH 8.0, 9.0, 10.0 (▼), and $NaHCO_3$ at pH 10.0, 11.0 (◆). Lipids were extracted, separated on TLC and radioactivity was measured. Other details in Fig. 2. Results from one representative experiment of four experiments performed in duplicate.

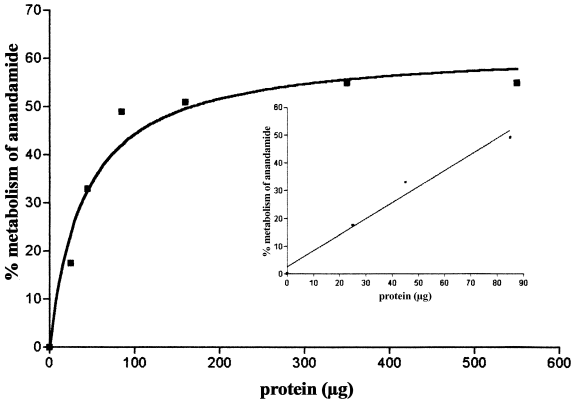

Fig. 4. Anandamide amidohydrolase activity as a function of protein concentration. [^{3}H]Anandamide (5×10^{-10} M) was incubated with different protein amounts of *T. pyriformis* medium for 10 min. Results from one of three experiments performed in duplicate. Insert: Metabolism of anandamide from 0 to 90 μg protein.

3.2. Release of amidohydrolase activity in the culture medium

The rapid hydrolysis of [^{3}H]anandamide led us to test the hypothesis that anandamide was attacked by the enzyme extracellularly. Thus, *T. pyriformis* cells were harvested and transfered into inorganic medium. After 30 min of incubation under constant stirring, cells were removed by centrifugation and the supernatant was tested for amidohydrolase activity. Almost 80% of anandamide was metabolized to AA (60% of the total radioactivity) (Fig. 2), while 10–15% was found in the water–methanol phase of the Bligh–Dyer extraction after 10 min (not shown). The metabolic fate of [^{3}H]anandamide in the supernatant indicates that *T. pyriformis* cells are capable of releasing enzyme(s) in the medium which is responsible for anandamide hydrolysis. PMSF inhibited almost thoroughly the hydrolysis showing the presence of amidohydrolase activity (Table 1; Fig. 2). Anandamide hydrolysis was not affected by the addition of EGTA, EDTA or Mg^{2+}, showing that the activity was not dependent on Ca^{2+} or Mg^{2+} (Table 1). It was also unaffected by the presence of DTT or DTNB, suggesting that sulfhydryl groups do not play important role in the activity. [^{3}H]Anandamide metabolism remained the same after preincubation with cafeic acid, indomethacin and acetylsalicylic acid at the indicated concentrations (Table 1), suggesting the absence of lipoxygenase (LOX) and cycloxygenase (COX) activity. The hydrolysis of anandamide was dependent on the temperature with optimum between 20 and 30°C (not shown) and also on pH, exhibiting a pH optimum at 9–10 (Fig. 3).

The rate of anandamide hydrolysis increased depending on the protein amount. The extent of the metabolism was a linear function of protein concentration between 0 and 85 μg/ml, while at higher concentrations, a plateau was reached (Fig. 4). It also varied with the substrate concentration. The apparent K_m of hydrolysis was 3.7 μM and the V_{max} in the medium of *T. pyriformis* cells was 278 pmol/min/mg protein (Fig. 5).

To test the substrate specificity, we synthesized *N*-palmitoylethanolamine and *N*-oleoylethanolamine and found that at pH 9 the supernatant of *T. pyriformis* could also hydrolyze *N*-palmitoylethanolamine and *N*-oleoylethanolamine (Table 1).

The absence of LDH activity in the medium (not shown) indicated the integrity of the cells, suggesting that the hydrolytic activity was not due to membrane vesicles that may have leaked out of the cells.

In addition, in some experiments the hydrolytic activity of the supernatant obtained after centrifugation of the conditioned medium at $100\,000\times g$ for 1 h was also tested. The results showed that $\sim73\pm20\%$ ($n=6$) of the total activity was recovered in the supernatant solution.

Table 1
Effects of various compounds on FAAH activity

Inhibitor	Activity pmol/min/mg protein	% Inhibition of anandamide degradation
–	0.3 ± 0.04	–
PMSF (0.2 mM)	0.05 ± 0.02	83.9 ± 4.0
N-Palmitoylethanolamine (5×10^{-10} M)	0.015 ± 0.004	95 ± 7.0
N-Oleoylethanolanine (5×10^{-9} M)	0.010 ± 0.002	96.7 ± 2.0
EGTA (2 mM)	0.35 ± 0.01	–
EDTA (2 mM)	0.3 ± 0.01	–
DTT (0.33 mM)	0.25 ± 0.00	16.7 ± 0.0
DTNB (0.1 mM)	0.29 ± 0.00	3.3 ± 0.0
Cafeic acid (0.3 mM)	0.29 ± 0.01	3.3 ± 0.0
Acetylsalicylic acid (0.1 mM)	0.32 ± 0.03	–
Indomethacin (10^{-3} mM)	0.3 ± 0.01	–
Mg^{2+} (1 mM)	0.3 ± 0.00	–

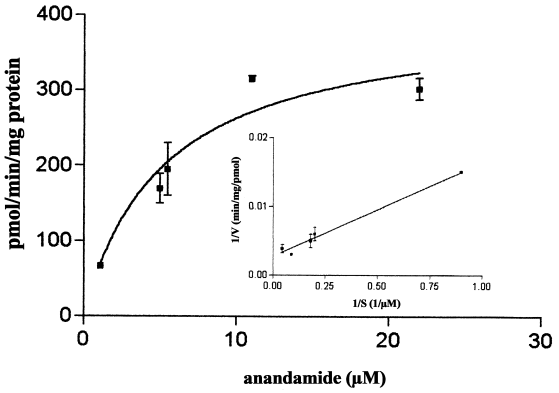

Fig. 5. Anandamide amidohydrolase activity as a function of substrate concentration. *T. pyriformis* supernatant (100 μg/ml) was incubated with different concentrations of [^{3}H]anandamide at pH 9 for 10 min. Results are from one of three experiments performed in duplicate. Insert: Lineweaver–Burk.

4. Discussion

In this study we report that a unicellular organism, the protozoon *T. pyriformis*, is able to incorporate and rapidly metabolize [^{3}H]anandamide. The main metabolic products were [^{3}H]PL and [^{3}H]NL (Fig. 1). The production of [^{3}H]PL was totally inhibited by the addition of PMSF (a known serine protease inhibitor), suggesting the presence of FAAH activity in intact *T. pyriformis* cells. It has been shown that FAAH is a serine hydrolase [33,34]. FAAH activity results in the degradation of [^{3}H]anandamide to ethanolamine and [^{3}H]AA, which is probably rapidly incorporated into PLs and non-polar lipids since the levels of free [^{3}H]AA were very low. The production of [^{3}H]NL in significant amounts even in the presence of PMSF could be attributed to the direct metabolism of anandamide by other enzymes, rather than through AA, since it was only partially inhibited in the presence of PMSF.

It should be noted that AA was not considered a constituent of PL or NL of *T. pyriformis* [35], but it has been recently shown that lipids of *T. pyriformis* may contain AA [36]. The possibility that [^{3}H]AA is converted to other fatty acid(s) (e.g. linoleic acid which is a major constituent of *T. pyriformis* PLs [35]) and then incorporated into PL could not be excluded.

The rapid metabolism observed in intact cells (Fig. 1, insert) led us to hypothesize that the hydrolysis might start extracellularly. It is well established that *Tetrahymena* species secrete large amounts of hydrolytic enzymes. The enzyme secretion has been shown to take place in growth media as well as in starvation medium and the major types of released enzymes are acid phosphatases, proteases, nucleases, glucosidases, lipases and phospholipases [29,37]. In the present work we demonstrated that, indeed, *T. pyriformis* starvation medium contained FAAH activity (Fig. 2) and we have characterized the enzyme.

The hydrolysis products (chloroform-rich phase of Bligh–Dyer extraction) were mainly [^{3}H]AA as expected (Fig. 2), while a relatively small percentage of radioactivity ($<15\%$, not shown) was found in the methanol–water phase. The latter could be due to small amounts of [^{3}H]anandamide and/or [^{3}H]AA produced by the enzyme since extraction of [^{3}H]anandamide or [^{3}H]AA in the absence of cells or medium resulted in approximately 10% of radioactivity in the water–methanol phase (not shown). However, we cannot rule out the possibility that soluble products of oxidative degradation of [^{3}H]anandamide and/or [^{3}H]AA were formed to some extent, the identity of which has not been established yet. Thus, we can conclude that a FAAH activity released from *T. pyriformis* converts [^{3}H]anandamide to [^{3}H]AA and ethanolamine as it has been shown for the enzyme derived from a variety of mammalian cells [3,10–16].

Furthermore, the hydrolytic activity of *T. pyriformis* medium was PMSF sensitive (Fig. 2; Table 1), suggesting that the enzyme is a serine hydrolase as it is well established for FAAH (or amidohydrolase) derived from mammalian cells and/or tissues [3]. DTT and DTNB had no effect on the enzymatic activity, suggesting that the sulfhydryl groups do not play an important role in the mechanism of action of the enzyme and was not Mg^{2+}- or Ca^{2+}-dependent since the activity was not affected by the presence of chelators (EGTA or EDTA) (Table 1). It has been reported that FAAH activity in mammalian cells is Mg^{2+}- or Ca^{2}-independent [11,38].

It has been shown that anandamide could be a substrate for both LOX and COX in mammalian cells [39–41]. Our results show that caffeic acid and acetylsalicylic acid or indomethacin did not affect the activity, suggesting the absence of LOX- and COX-like activity (Table 1).

The hydrolytic activity was abolished by boiling, and kinetic studies showed that the enzyme followed Michaelis–Menten kinetics, yielding an apparent K_m of 3.7 μM and a V_{max} equal to 278 pmol/min/mg protein. As it has been reported from similar kinetic studies on the FAAH from various cell types and tissues, there is a wide range of K_m and V_{max} values (0.3–400 μM and 0.015–5.6 nmol/min/mg protein respectively [3,10,11,14,24,42,43]). The kinetic values of our enzyme are similar to those reported for rat and human brain enzymes (3.4 μM [11] and 2 μM respectively [44]).

The unsaturated and monounsaturated analogues of anandamide (*N*-palmitoyl and *N*-oleoylethanolamine) completely inhibited [^{3}H]anandamide metabolism by *T. pyriformis* medium, suggesting that the enzyme is not specific for anandamide, as it has been reported for rat and porcine brain microsomes [3,10].

Finally, it should be mentioned that LDH activity was absent from the medium and that most of the total hydrolytic activity found in the medium remained in solution after centrifugation at $100\,000 \times g$, suggesting that the activity was not due to membrane vescicles that may have leaked out of the cells.

Nevertheless, the mechanism by which the enzyme is secreted as well as its subcellular origin were not addressed in this work and further studies are required. *Tetrahymena* has at least two secretory pathways that are well characterized: a constitutive release mechanism and another responsible for the release of hydrolytic enzymes apparently from lysosomes [45]. Interestingly, *Tetrahymena* also possesses a regulated mucocyst-mediated pathway in which a dense core vesicle (mucocyst) is stably docked at the plasma membrane, at the site of exocytosis. The use of mutants that are available [46] will allow us to clarify the origin of the secreted FAAH-like activity.

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