# Further evidence for the existence of a specific process for the membrane transport of anandamide

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Indirect evidence for the existence of a specific protein-mediated process for the cellular uptake of endocannabinoids has been reported, but recent results suggested that such a process, at least for AEA [*N*-arachidonoylethanolamine (anandamide)], is facilitated uniquely by its intracellular hydrolysis by FAAH (fatty acid amide hydrolase) [Glaser, Abumrad, Fatade, Kaczocha, Studholme and Deutsch (2003) Proc. Natl. Acad. Sci. U.S.A. **100**, 4269–4274]. In the present study, we show that FAAH alone cannot account for the facilitated diffusion of AEA across the cell membrane. In particular, (i) using a short incubation time (90 s) to avoid AEA hydrolysis by FAAH, AEA accumulation into rat basophilic leukaemia or C6 cells was saturable at low $\mu$M concentrations of substrate and non-saturable at higher concentrations; (ii) time-dependent and, at low $\mu$M concentrations of substrate, saturable AEA accumulation was observed also using mouse brain synaptosomes; (iii) using synaptosomes prepared from FAAH-deficient mice, saturable AEA accumulation was still observed, although with a lower efficacy; (iv) when 36 AEA and *N*-oleoylethanolamine analogues, most of which with phenyl rings in the polar head group region, were tested as inhibitors of AEA cellular uptake, strict structural and stereochemical requirements were needed to observe significant inhibition, and in no case the inhibition of FAAH overlapped with the inhibition of AEA uptake; and (v) AEA biosynthesis by cells and sensory neurons was followed by AEA release, and this latter process, which cannot be facilitated by FAAH, was still blocked by an inhibitor of AEA uptake. We suggest that at least one protein different from FAAH is required to facilitate AEA transport across the plasma membrane in a selective and bi-directional way.

Key words: anandamide, 2-arachidonoylglycerol (2-AG), cannabinoid, endocannabinoid, fatty acid amide hydrolase (FAAH), transporter.

## INTRODUCTION

Endocannabinoids are endogenous compounds binding to, and functionally activating, at least one of the two cannabinoid $CB_1$ and $CB_2$ receptors for marijuana's active principle $\Delta^9$-tetrahydrocannabinol [1,2]. Five endocannabinoids have been identified to date: AEA [*N*-arachidonoylethanolamine (anandamide)] [3], 2-AG (2-arachidonoylglycerol) [4,5], 2-arachidonoylglyceryl ether (noladin) [6,7], *O*-arachidonoylethanolamine (virodhamine) [8] and NADA (*N*-arachidonoyldopamine) [9,10]. The proposed function as neuromodulators for the endocannabinoids [11] requires for these substances the existence of specific biosynthetic and metabolic mechanisms subject to regulation during physiological and pathological conditions.

To become substrates for the intracellular enzymes catalysing their metabolism, all endocannabinoids need to be taken up by cells. A common mechanism (see [12] for a review) has been suggested to facilitate the uptake of all endocannabinoids, according to the concentration gradient across the cell membrane. This suggestion is based on several observations, including: (i) AEA, 2-AG, noladin and NADA are rapidly taken up by both neuronal and non-neuronal cells in a saturable, temperature-dependent and, at least for AEA, energy-independent manner [7,10,13–16]; (ii) 2-AG, noladin, NADA and virodhamine compete with AEA cellular uptake, and AEA competes with 2-AG, noladin and NADA uptake [7,9,10,15,16]; and (iii) AEA and 2-AG uptakes are subject to selective inhibition by some AEA analogues and to stimulation by nitric oxide [16–19].

Unlike enzymes for AEA and 2-AG hydrolysis {e.g. FAAH (fatty acid amide hydrolase) (see [20] for a review) and the monoacylglycerol lipase [21]}, no membrane transporter for endocannabinoids has been cloned yet. This fact may suggest that endocannabinoid transport across the plasma membrane is not carrier mediated and it occurs uniquely via passive diffusion in a way that depends on the rate of intracellular metabolism by FAAH, monoacylglycerol lipase or other enzymes [16,22,23]. Indeed, some inhibitors of FAAH also interfere with AEA cellular uptake [24] and vice versa [22,23], and recent crystallographic data have suggested that, although normally located on intracellular membranes, FAAH may undergo conformational changes giving it potential access to the inner layer of a plasma membrane [25]. However, numerous indirect observations support the existence of a protein-mediated mechanism for membrane transport of endocannabinoids, which would not rely entirely on intracellular metabolism: (i) many substances selectively inhibiting AEA cellular uptake without affecting FAAH activity have been reported [26–28]; (ii) some FAAH inhibitors enhance, and AEA uptake inhibitors inhibit, AEA accumulation in cells [29]; (iii) cells that do not express FAAH still rapidly take up AEA [22,30]; (iv) inhibitors of AEA cellular uptake enhance those effects of AEA that are mediated by cannabinoid receptors, but inhibit those effects that are exerted on the cytosolic side of membrane proteins, such as the stimulation of vanilloid VR1 receptors [31] or the inhibition of T-type $Ca^{2+}$ channels [32]; (v) NADA and noladin are still rapidly taken up by cells, and yet they are refractory to enzymic hydrolysis [7,10]; (vi) lipo-polysaccharides inhibit

Abbreviations used: AEA, *N*-arachidonoylethanolamine (anandamide); 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; HEK-293 cells, human embryonic kidney 293 cells; NADA, *N*-arachidonoyldopamine; RBL-2H3, rat basophilic leukaemia.

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FAAH expression without affecting AEA cellular uptake [33]; in contrast, nitric oxide, peroxynitrite and superoxide anions stimulate AEA cellular re-uptake [19], and acute or chronic ethanol inhibit this process [34], in both cases with no effect on FAAH activity; and (vii) in some cells, even the presence of a very active FAAH is not sufficient to observe temperature-dependent uptake of AEA [35]. Furthermore, as elegantly discussed by Hillard and Jarrahian [36], intracellular-binding proteins or organelle-mediated processes may also be responsible for the sequestration from the extracellular medium, and accumulation of endocannabinoids into cells, without necessarily requiring their enzymic hydrolysis.

Although only the cloning and molecular characterization of the putative protein(s) responsible for endocannabinoid transport across the cell membrane will set the final word on whether or not this process is mediated by a plasma membrane carrier, experimental strategies are available to confirm further or rule out the possibility that AEA uptake is uniquely mediated by FAAH. In the present study, we have employed these strategies, including: (i) the use of assay conditions, where FAAH activity is minimal, and of two types of cells; (ii) the use of synaptosomes from the brain of FAAH-deficient mice [37], where no interference of this enzyme with the uptake process is expected to occur; (iii) the testing of the effect of a large series of AEA analogues with closely related chemical structures on AEA cellular uptake, and hydrolysis by the same cell type; and (iv) the study of the efflux from cells of *de novo* biosynthesized AEA, a process which cannot be facilitated by FAAH and was suggested to be mediated by the same protein responsible for AEA uptake [14].

## MATERIALS AND METHODS

### Synthesis of compounds

Amides OMDM-1 to OMDM-4 and OMDM-7 to OMDM-36 were synthesized by treatment of the appropriate amine with oleic or arachidonic acid using 1-hydroxybenzotriazole/*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodi-imide hydrochloride as the carboxylate activator. Acyl hydrazines OMDM-5 and OMDM-6 were prepared as described previously [27]. VDM11 [*N*-arachidonoyl-(2-methyl-4-hydroxy)phenylamine] was also synthesized as described before [38].

### AEA uptake experiments in intact cells

RBL-2H3 (rat basophilic leukaemia) and C6 cells, cultured according to the manufacturer's instruction, were plated on to 6-well culture plates at a density of $(1–2) \times 10^6$ cells/well. Confluent cells, washed free of serum, were incubated for either 3 or 90 s at 37 °C in 500 $\mu$l of medium (minimal essential medium and RPMI 1640 respectively) containing increasing concentrations of [$^{14}$C]AEA (2.5–125 $\mu$M, radiolabelled on the ethanolamine moiety) and, for a pilot experiment, 0.4 % fatty acid-free BSA. After the incubation, the media were separated from the cells, which were washed three times with 3 ml of medium containing 0.4 % BSA, and then scraped off the dishes. Both incubation media and cells were extracted with chloroform/methanol (2:1, v/v), and radioactivities of both the aqueous and organic phase were counted in a $\beta$-scintillation counter. The radioactivity found in the organic phase from the cell extracts was taken as a measure of AEA accumulated into cells. The radioactivity found in the aqueous phase from either the cell extracts or the media was taken as a measure of AEA hydrolysis during the incubation. Specific AEA uptake was determined by subtracting the radioactivity in the organic phase of the cell extracts after 5 s incubation from that measured after 90 s incubation, as described previously [23]. In a different experiment performed with RBL-2H3 cells, cells were incubated for 5 min with correspondingly increasing concentrations of OMDM-2 (5–250 $\mu$M, 2:1 ratio with [$^{14}$C]AEA concentrations) before incubation with [$^{14}$C]AEA. In another series of experiments, RBL-2H3 cells were incubated with 10 $\mu$M [$^{14}$C]AEA in the presence of increasing concentrations (1–25 $\mu$M) of 36 different AEA and *N*-oleoylethanolamine analogues. The inhibitory effect on the uptake was expressed as the $K_i$ calculated from the $IC_{50}$ values of [$^{14}$C]AEA uptake inhibition (after subtraction of non-specific uptake) using the Cheng–Prusoff equation.

### AEA uptake experiments in mouse synaptosomes

To prepare brain synaptosomes, male FAAH$^{+/+}$ and FAAH$^{-/-}$ mice were decapitated and the entire brain (minus cerebellum) of each mouse was quickly removed and gently disrupted by Ultra-Turrax (11 000 rev./min for 1 min) in 0.32 M sucrose containing 0.1 mM EDTA buffered with 5 mM Hepes (pH 7.4), thus following the previously reported method [40]. Then brain homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C to remove tissue debris as precipitate ($P_1$). The supernatants were then centrifuged again at 12 000 ***g*** for 20 min at 4 °C to yield the crude synaptosomal pellet ($P_2$). This pellet, containing the synaptosomes, was resuspended in 136 mM NaCl, 5 mM KCl, 0.16 mM $CaCl_2$, 0.1 mM EGTA, 1.3 mM $MgCl_2$, 10 mM glucose and 10 mM Tris/HCl buffer (pH 7.4), at protein concentration of 7 mg/ml. Synaptosomes (300 $\mu$g/sample) were incubated at 37 or 4 °C with [$^{14}$C]AEA. Incubation was blocked by gentle centrifugation (at 2000 ***g*** for 2 min at 4 °C) to yield supernatants corresponding to external phase and pellet ($P_3$) corresponding to incubated synaptosomes. External phases were extracted with chloroform/methanol (2:1, v/v) and radioactivities of both the aqueous and organic phases were counted with a $\beta$-scintillation counter. The $P_3$ pellet was washed three times with 1 ml of PBS containing 1 % BSA, re-suspended in 250 $\mu$l of PBS and finally extracted with chloroform/methanol (2:1, v/v); again, radioactivities of both the aqueous and organic phases were counted with the $\beta$-scintillation counter. The radioactivity in the organic phase was taken as a measure of [$^{14}$C]AEA accumulation. Specific [$^{14}$C]AEA accumulation into synaptosomes with different incubation times (1, 3, 5, 7.5 and 10 min) or with different concentrations of [$^{14}$C]AEA (2.5–125 $\mu$M) was obtained by subtracting the uptake at 4 °C from the uptake at 37 °C. Radioactivity in the aqueous phases was again taken as a measure of [$^{14}$C]AEA enzymic hydrolysis.

### AEA hydrolysis experiments

Membranes from RBL-2H3 cells were incubated in the presence of test compounds and 10 $\mu$M [$^{14}$C]AEA for 30 min at 37 °C in 50 mM Tris/HCl (pH 9.5). The incubation was terminated by the addition of chloroform/methanol (1:1, v/v). The aqueous phase, containing [$^{14}$C]ethanolamine produced by [$^{14}$C]AEA hydrolysis, was measured by $\beta$-scintillation counting. Results are expressed as the $K_i$ calculated from the $IC_{50}$values of [$^{14}$C]AEA hydrolysis inhibition using the Cheng–Prusoff equation.

### Measurement of anandamide in stimulated HEK-293 cells (human embryonic kidney 293 cells) and dorsal root ganglia neurons

Confluent HEK-293 cells were washed with PBS without $Mg^{2+}$/$Ca^{2+}$ and incubated with 1 $\mu$M thapsigargin with or without 5 min preincubation with 4 $\mu$M VDM11 for the indicated time intervals in Hanks balanced salt solution (+ 2.5 mM EGTA, pH 7.4)

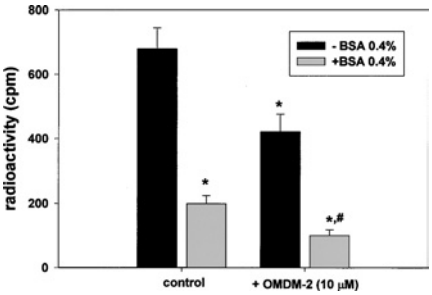


**Figure 1 Net uptake of [$^{14}$C]AEA (5 $\mu$M) from RBL-2H3 cells (after 45 s incubations, subtracted of the uptake after 5 s incubations)**

The effect of BSA (0.4 %), or the specific anandamide uptake inhibitor OMDM-2 (10 $\mu$M), or both, is shown. Results are means $\pm$ S.D. for at least $n = 3$ separate experiments. $^{*}P < 0.05$ versus control and $^{\#}P < 0.05$ versus BSA; calculated by ANOVA followed by the Bonferroni's test.

without $CaCl_2$, and at room temperature (25 °C). Incubation was terminated by removing the buffer. Dorsal root ganglia neurons from neonatal rats, prepared as described previously [10], were similarly incubated, but in the additional presence of 1 $\mu$M methylarachidonoylfluorophosphonate to inhibit FAAH. Lipids were extracted from the cells by the Bligh and Dyer procedure, whereas anandamide released in the incubation buffer was separated by solid-phase extraction. HPLC coupled with atmospheric pressure chemical ionization MS, using $d^8$-AEA (10 pmol) as internal standard, was applied to quantify anandamide as described in [7].

## RESULTS

### Effect of BSA on AEA cellular accumulation

In the first series of experiments, we decided to use an experimental procedure similar to that used by Glaser et al. [23] (i.e. short incubation time, non-specific accumulation calculated by means of 5 s incubations and 0.4 % BSA in the incubation medium), and to analyse the uptake of 5 $\mu$M [$^{14}$C]AEA by RBL-2H3 cells. Under these conditions, the presence of 0.4 % BSA in the incubation medium had a stronger inhibitory effect on AEA accumulation when compared with the previously described selective inhibitor of AEA uptake, OMDM-2 [27] (Figure 1). A similar result was obtained when using C6 cells (results not shown). Therefore the use of BSA was avoided in the following experiments.

### Saturability of AEA accumulation in cells

Glaser et al. [23] reported recently that AEA accumulation in cells is not a saturable process when the FAAH-catalysed hydrolysis of the endocannabinoid is minimized by using short incubation times. They proposed that the saturability of this process with increasing concentrations of AEA, used as evidence in favour of the existence of a membrane transporter, is simply a reflection of FAAH saturation [23]. Using RBL-2H3 cells and a method very similar to that used by these authors (including the same method for the calculation of non-specific uptake), we were not able to detect any [$^{14}$C]ethanolamine in either the incubation media or cells, indicating that no [$^{14}$C]AEA was hydrolysed under these conditions (results not shown). As shown in Figure 2(A), we confirmed that AEA accumulation is not a saturable process when high concentrations (up to 120 $\mu$M) of [$^{14}$C]AEA were used. However, if concentrations of

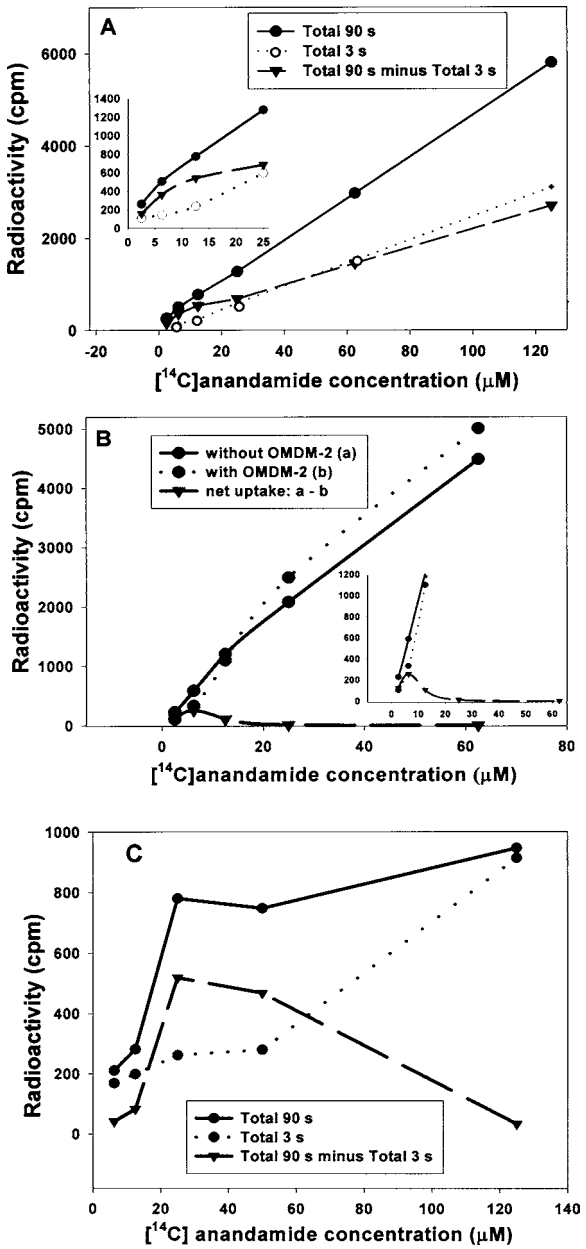


**Figure 2 Uptake of [$^{14}$C]anandamide by RBL-2H3 and C6 glioma cells**

Saturation of net [$^{14}$C]anandamide accumulation in RBL-2H3 cells after 90 s incubations, using (**A**) the uptake at 5 s or (**B**) the co-incubation with increasing concentrations (5–250 $\mu$M) of OMDM-2, to calculate non-specific uptake (in both cases the radioactivity still found to be associated with cells was subtracted from that measured after 90 s incubations and in the absence of inhibitor). (**A**) Inset: enlargement of the graph; (**B**) inset: Y-axis enlargement of the graph. Results are means for at least $n = 3$ separate experiments. Error bars are not shown for the sake of clarity, and were never higher than 5 % of the means. (**C**) Saturation of net [$^{14}$C]anandamide accumulation in C6 cells.

substrate were considered more in the physiological range (2.5–10 $\mu$M), a strong trend towards saturation [estimated $K_m = 10 \pm 2$ $\mu$M, $B_{max} = 3.5 \pm 1.1$ nmol $\cdot$ min$^{-1}$ $\cdot$ (mg of protein)$^{-1}$] of this process can be observed, irrespective of the type of method used to calculate non-specific uptake (Figures 2A and 2B). Furthermore, when using C6 glioma cells, and again a very short incubation time (90 s), a clear-cut saturation of AEA accumulation was observed [estimated $K_m = 16 \pm 2$ $\mu$M, $B_{max} = 2.8 \pm 1.0$ nmol $\cdot$ min$^{-1}$ $\cdot$ (mg of protein)$^{-1}$] (Figure 2C).

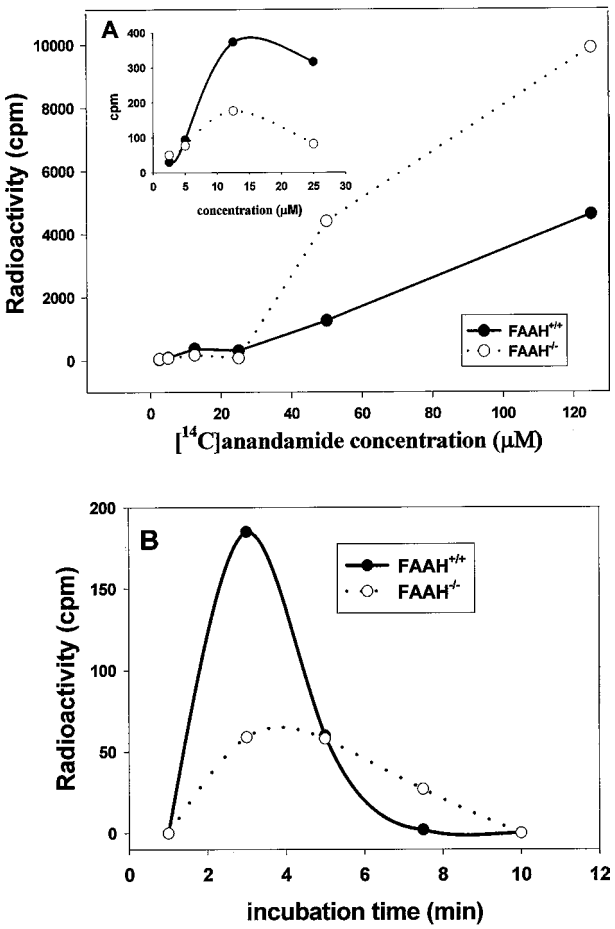


**Figure 3 Accumulation of [$^{14}$C]anandamide in mouse brain synaptosomes**

(**A**) Saturation of net [$^{14}$C]anandamide accumulation into mouse brain synaptosomes after 90 s incubations. (**B**) Time dependence of net [$^{14}$C]anandamide (4 $\mu$M) accumulation. Net uptake was calculated by using the uptake at 4 °C as non-specific binding (in this case the radioactivity still found to be associated with synaptosomes was subtracted from that measured after 90 s incubations). (**A**) Inset: enlargement of the graph. Results are means for at least $n = 3$ separate experiments. Error bars are not shown for the sake of clarity and were never higher than 5 % of the means.

### AEA accumulation into synaptosomes from the brain of FAAH$^{+/+}$ and FAAH$^{-/-}$ mice

When using brain synaptosomes from FAAH$^{+/+}$ mice to study the uptake process of [$^{14}$C]AEA, we found again an apparent lack of saturation at high substrate concentrations. Also in this case, a closer look at more physiological AEA concentrations (1–20 $\mu$M) revealed a saturable accumulation [estimated $K_m = 7 \pm 1$ $\mu$M, $B_{max} = 0.11 \pm 0.02$ nmol $\cdot$ min$^{-1}$ $\cdot$ (mg of protein)$^{-1}$, $t_{1/2} \sim 1.7$ min] (Figures 3A and 3B). We could not detect any [$^{14}$C]ethanolamine in either the incubation media or synaptosomes, which indicates that no [$^{14}$C]AEA was hydrolysed under these conditions. However, for longer ($\geqslant 5$ min) incubation times, up to 90 % of the radioactivity was found to be associated with the aqueous phases (results not shown). Importantly, when the synaptosomes were prepared from the brain of the FAAH$^{-/-}$ littermates, a significantly less efficacious, but still saturable accumulation of [$^{14}$C]AEA was observed [estimated $K_m = 5 \pm 1$ $\mu$M, $B_{max} = 0.05 \pm 0.01$ nmol $\cdot$ min$^{-1}$ $\cdot$ (mg of protein)$^{-1}$, $t_{1/2} \sim 1.8$ min] (Figures 3A and 3B). This finding indicates that, although FAAH significantly contributes to force the cellular uptake of AEA, this process can still take place in a protein-mediated manner in the absence of the enzyme.

### Effect of several different analogues of AEA on AEA uptake and hydrolysis in RBL-2H3 cells

The effect on [$^{14}$C]AEA cellular uptake and enzymic hydrolysis of 36 aromatic or constrained AEA and *N*-oleoylethanolamine analogues (Figure 4), structurally related to previously reported uptake inhibitors, is shown in Table 1. Little structural differences, such as stereochemistry, the presence of an additional hydroxy group and/or of a methylene spacer, and the position of the aromatic ring on the ethanolamine moiety, produced significant differences in potencies (cf. OMDM-21 with OMDM-22; OMDM-29 with OMDM-30; OMDM-31 with OMDM-32; OMDM-30 with OMDM-21/-22; OMDM-13 with OMDM-21; and OMDM-14 with OMDM-22). Indeed, some of the compounds efficaciously inhibited the uptake process with $K_i$ values in the low range (2.4–13 $\mu$M), whereas others were inactive at the highest (25 $\mu$M) concentration tested. None of the 36 derivatives significantly inhibited FAAH from RBL-2H3 cells up to a concentration of 50 $\mu$M. These results indicate that the chemical determinants regulating the influence of these compounds on the uptake process are very stringent and differ from those regulating the interaction with FAAH. As a corollary to this finding, it can be concluded that the inhibition of AEA cellular uptake by some of these compounds is not due to inhibition of FAAH. The detailed structure–activity relationship study of the effect of the OMDM compounds on AEA uptake will be published elsewhere.

### AEA biosynthesized *de novo* is released from cells via a process that can be inhibited by a selective AEA uptake inhibitor

One of the reasons why AEA uptake was suggested to be uniquely mediated by FAAH is that the three uptake inhibitors, i.e. AM404, arvanil and olvanil, were shown to be incapable of inhibiting AEA accumulation by cells under conditions where AEA hydrolysis cannot occur [23]. Therefore it was suggested that these compounds could inhibit AEA uptake by inhibiting FAAH. In fact, AM404, arvanil and olvanil were found to inhibit FAAH at the same concentrations that were required to inhibit the uptake process, although not on the enzyme prepared from the same cells used to study the uptake [23]. In Figure 5(a), we show that HEK-293 cells stimulated with thapsigargin produce AEA in the absence of extracellular calcium. This effect is probably due to the mobilization of intracellular calcium and subsequent *de novo* biosynthesis of AEA, as suggested by the concomitant formation of an AEA biosynthetic precursor, *N*-arachidonoyl-phosphatidyl-ethanolamine (results not shown). When the endocannabinoid was measured in both the cells and the extracellular medium, we could demonstrate an intracellular peak of AEA, which was immediately followed by its release into the extracellular medium (Figure 5b). However, if cells were preincubated with 4 $\mu$M VDM11, an inhibitor of AEA uptake in these and other cells [31,38], the amount of AEA found in the extracellular medium after a 30 min stimulation was significantly decreased (Figure 5c), and the AEA associated with cells was correspondingly increased from $28 \pm 5$ to $63 \pm 5$ % ($n = 3$, $P < 0.05$), without changing the total amounts of AEA. Similar results were obtained when cultured dorsal root ganglia neurons from neonatal rats were stimulated with thapsigargin in the presence or absence of VDM11, and also in the presence of the FAAH inhibitor methylarachidonoylfluorophosphonate. In this case, VDM11 increased the intracellular levels of AEA from $0.062 \pm 0.002$ to $0.085 \pm 0.004$ pmol/dish ($P < 0.05$, $n = 3$), without significantly changing the total amounts of AEA

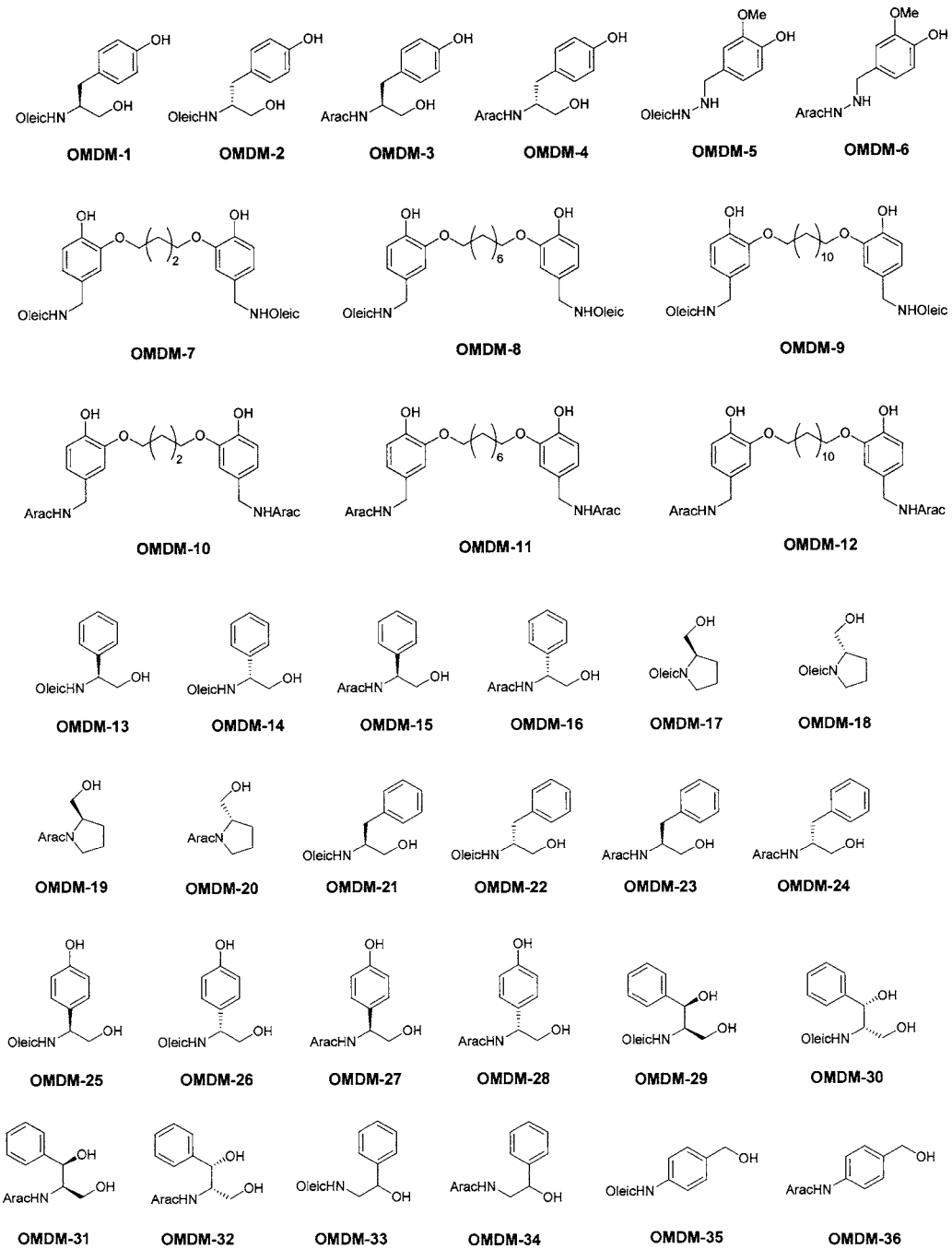


**Figure 4 Chemical structures of OMDM-1 to OMDM-36**

Oleic, acyl chain of oleic acid; Arac, acyl chain of arachidonic acid.

produced by thapsigargin [$0.091 \pm 0.006$ versus $0.101 \pm 0.006$ pmol/dish ($n = 3$, $P = 0.29$)]. These findings strongly suggest that (i) VDM11, unlike the compounds tested by Glaser et al. [23], does not inhibit AEA uptake by inhibiting FAAH; (ii) AEA release is probably facilitated by the same selective and pharmacologically inhibitable mechanism proposed for AEA cellular uptake; and (iii) the latter process does not rely uniquely on FAAH. In fact, in the absence of a membrane transporter, FAAH would be expected to minimize AEA release (by reducing the intracellular concentration of AEA, and, hence, the extent of the gradient across the membrane), and its hypothetical inhibition by VDM11 should have led to enhancement, rather than reduction, of extracellular levels of AEA in HEK-293 cells. Furthermore, in dorsal root ganglion neurons, and with FAAH activity inhibited, VDM11 still counteracted the release of AEA, again with no effect on overall AEA biosynthesis.

## DISCUSSION

In the present study, we reported new results suggesting the existence of a specific, saturable, pharmacologically inhibitable and bi-directional mechanism facilitating the transport across the plasma membrane of AEA, and presumably also of other endocannabinoids. This mechanism might be regulated by, but

**Table 1 Effect of OMDM compounds on [$^{14}$C]AEA uptake by RBL-2H3 cells**

Results are means ± S.E.M. for $n = 3$ experiments and are expressed as the $K_i$ for the inhibition. None of the compounds were found to exert an appreciable inhibitory activity on [$^{14}$C]AEA hydrolysis by RBL-2H3 cell membranes ($IC_{50} \geqslant 50$ $\mu$M, results not shown).

| Compound | $K_i$ ($\mu$M) |
|---|---|
| OMDM-1 | 2.4 ± 0.7 |
| OMDM-2 | 3.0 ± 0.6 |
| OMDM-3 | 16.6 ± 0.9 |
| OMDM-4 | 17.7 ± 1.5 |
| OMDM-5 | 4.8 ± 0.5 |
| OMDM-6 | 7.0 ± 0.6 |
| OMDM-7 | > 25 |
| OMDM-8 | > 25 |
| OMDM-9 | > 25 |
| OMDM-10 | > 25 |
| OMDM-11 | > 25 |
| OMDM-12 | > 25 |
| OMDM-13 | 12.9 ± 3.1 |
| OMDM-14 | 16.6 ± 2.1 |
| OMDM-15 | 19.4 ± 1.2 |
| OMDM-16 | > 25 |
| OMDM-17 | > 25 |
| OMDM-18 | > 25 |
| OMDM-19 | > 25 |
| OMDM-20 | > 25 |
| OMDM-21 | > 25 |
| OMDM-22 | 11.1 ± 2.0 |
| OMDM-23 | > 25 |
| OMDM-24 | > 25 |
| OMDM-25 | > 25 |
| OMDM-26 | > 25 |
| OMDM-27 | 17.5 ± 2.3 |
| OMDM-28 | 21.2 ± 4.1 |
| OMDM-29 | > 25 |
| OMDM-30 | 6.4 ± 0.5 |
| OMDM-31 | 15.7 ± 3.7 |
| OMDM-32 | > 25 |
| OMDM-33 | > 25 |
| OMDM-34 | > 25 |
| OMDM-35 | > 25 |
| OMDM-36 | > 25 |

is entirely unrelated to, FAAH. Apart from the previous results summarized in the Introduction section, this concept is based on the following original findings described in this study: (i) the accumulation of AEA into cells can be saturated by concentrations of the endocannabinoid within the physiological range, i.e. between 1 and 10 $\mu$M, also when using experimental conditions (i.e. a very short incubation time) in which FAAH-catalysed AEA hydrolysis is minimal, if at all present, provided that care is taken to avoid BSA in the incubation medium; (ii) time dependent and saturable accumulation of AEA into mouse brain synaptosomes can be observed even when FAAH is not expressed (i.e. when synaptosomes are prepared from FAAH$^{-/-}$ mice); (iii) AEA uptake from the extracellular medium by RBL-2H3 cells can be decreased by several novel substances that have no inhibitory action on FAAH; (iv) the mechanism mediating AEA cellular uptake can be inhibited by several compounds in a manner that depends on the presence of very stringent chemical prerequisites in the molecule; and (v) if the presence of an active FAAH facilitates AEA uptake, this cannot hold true also for the release from cells of AEA biosynthesized *de novo* – we have shown in the present study that AEA release is inhibited by a selective inhibitor of AEA uptake. Given the stringent chemical requirements needed to inhibit this process, this strongly suggests that the same protein mediates AEA uptake by, and release from, cells.

Our finding that BSA in the incubation medium prevents AEA accumulation in cells was to be expected from the results of the first studies on AEA biosynthesis [13], where it was found that without BSA in the medium, release of the endocannabinoid into the extracellular medium cannot be observed due to its rapid re-uptake by cells. Accordingly, AEA was found recently to bind to BSA with an affinity even higher than that of the uptake process [39]. On the other hand, our finding that accumulation of AEA in RBL-2H3 cells or mouse brain synaptosomes is not saturable only at very high concentrations can be explained partially by the fact that this compound easily binds to the plastic ware used for the uptake assays (see also below). In fact, AEA can adsorb to the plastic in a way that can be prevented by uptake inhibitors, to be subsequently released in a time- and temperature-dependent manner [40]. This process may have been particularly relevant to our assay conditions, as we could not introduce BSA, normally

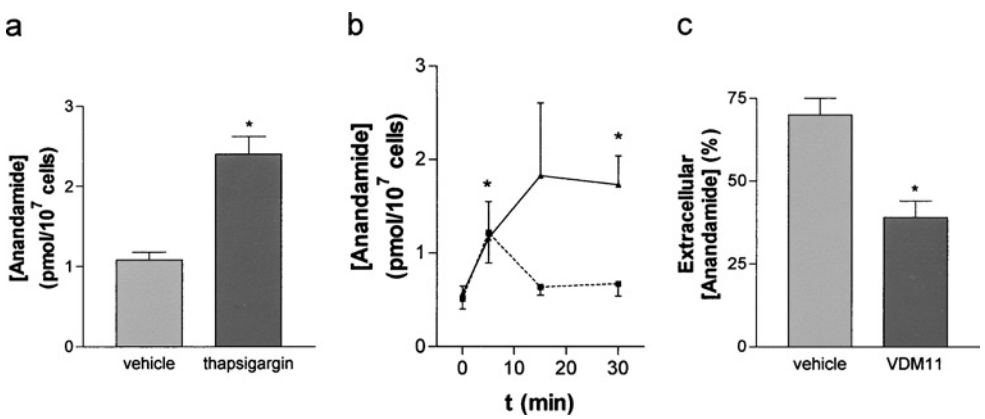


**Figure 5 An anandamide uptake inhibitor blocks the release of anandamide biosynthesized *de novo***

(**a**) Stimulation of HEK-293 cells with thapsigargin (1 $\mu$M for 30 min) in the absence of extracellular calcium leads to *de novo* formation of anandamide, presumably using intracellular calcium; (**b**) time response for the stimulation of cells with thapsigargin, showing that anandamide is first formed inside the cell (----, cell-associated anandamide) and then exits the cell (—, medium-associated anandamide); (**c**) 5 min preincubation of cells with 4 $\mu$M VDM11 significantly decreases the amount of extracellular anandamide (as percentage of total anandamide produced; see **a** and **b**) after 30 min incubation with 1 $\mu$M thapsigargin. Results are means ± S.E.M. for at least $n = 3$ determinations. Anandamide levels were measured by isotope-dilution liquid chromatography-MS (see the Materials and methods section and [7]).

used to prevent AEA binding to plastic, in our uptake assays. However, AEA binding to the plastic should have prevented us from observing saturation of the uptake process also when using C6 cells, which instead appeared to sequester the endocannabinoid in a saturable manner even at concentrations higher than physiological ones. Furthermore, Glaser et al. [23] did use BSA in their assay medium, and yet they could not saturate AEA accumulation in neuroblastoma or astrocytoma cells. Indeed, the absence of BSA in the incubation medium used by these authors might explain why they could only observe a non-saturable cellular accumulation of AEA, i.e. possibly the same process that we have observed in the present study at high AEA concentrations. Clearly, from the results presented in the present study and previously, it appears that the extent to which AEA cellular accumulation can be saturated by low concentrations of the substrate depends very much on experimental variables as well as on the type of cell used.

The saturable and time- and temperature-dependent accumulation of AEA into brain synaptosomes was described for the first time by Maccarrone et al. [41] in 2001. This model has an advantage over intact cell systems since it allows studying endocannabinoid uptake in a synapse-like environment. These authors, however, used a longer incubation time than that required to minimize FAAH activity. This variable might cause interference from FAAH on AEA accumulated into synaptosomes [23]. Therefore we have used the method described by these authors, but have greatly shortened the incubation time (90 s). Also, in this case, we found an apparent non-saturable accumulation of AEA, which, however, at a closer look, was in fact saturable at low concentrations of the compound. More importantly, we observed saturable and time-dependent AEA accumulation also into synaptosomes prepared from the brain of mice with genetically inactivated FAAH. In this case, AEA uptake appeared to be less efficacious, thus suggesting that, although not necessary, an active FAAH, by maintaining a gradient of AEA concentrations across the cell membrane, is still an important factor to determine the kinetics of cellular uptake of AEA [22,42]. Interestingly, despite the lack of any FAAH activity, the amounts of intact AEA accumulated into the synaptosomes still disappeared at long incubation times, although more slowly than with synaptosomes from $FAAH^{+/+}$ mice, thus, suggesting the existence of other metabolic pathways for AEA in mouse synaptosomes. However, this process, which will certainly require further detailed investigations, appears to take place only after AEA accumulation reaches a plateau, and, therefore, is unlikely to drive AEA uptake. On the other hand, the non-saturable process observed in synaptosomes at very high, non-physiological, concentrations of AEA was apparently higher in $FAAH^{-/-}$ when compared with $FAAH^{+/+}$ mice. Since the amount of total proteins and the type of other experimental variables used were exactly the same in both the cases, only the higher abundance, in synaptosomes from $FAAH^{-/-}$ mice, of one or more proteins to which AEA binds non-specifically may explain this difference. Adaptive alterations in protein profiling in transgenic mice deficient in the expression of certain genes has been reported (see [43] for an example), but a thorough comparative analysis of differential protein expressions in $FAAH^{-/-}$ and $FAAH^{+/+}$ mice by using the microRNA array technology has not been reported yet.

The enantioselectivity of AEA cellular uptake inhibitors had been already observed in a previous study [44]. In the present study, by assessing the effect of the largest series of chemically related AEA and *N*-oleoylethanolamine polar 'head' analogues ever tested, we confirmed that enantioselectivity as well as other subtle chemical features are crucial to endow a molecule with the capability of inhibiting AEA uptake, thus confirming the hypothesis that this process involves at least one protein. Our results, showing that none of the uptake inhibitors was capable of inhibiting FAAH preparations from the same cells, strongly argue against this protein being FAAH. However, whether this protein (i) resides in the plasma membrane and behaves as a carrier protein [44]; (ii) is located in some intracellular compartment, as suggested by more recent preliminary studies [45]; or (iii) acts as a shuttle between the extracellular milieu and FAAH on intracellular membranes (see [36] for a review), cannot be established conclusively from the present or previous results. Nevertheless, our finding that VDM11, a selective uptake inhibitor [38], also blocks the release of AEA without affecting the total amounts biosynthesized *de novo* inside the cell (i) conclusively confirms that the previously reported effect of inhibitors of AEA uptake, which, when injected inside the cell, block retrograde neuromodulatory actions mediated by cannabinoid $CB_1$ receptors [46,47], is due to blockade of endocannabinoid release; and (ii) together with the FAAH-independent uptake of AEA by brain synaptosomes, corroborates the original hypothesis that both AEA uptake and AEA release are facilitated by the same 'AEA membrane transporter', which works in two directions and according to the gradient of AEA concentrations across the cell membrane [13,14]. Therefore these findings not only rule out the exclusive role of FAAH in AEA transport across the membrane, since the enzyme would only facilitate the uptake but not the release of AEA, but also point to the plasma membrane as the possible cellular compartment where the protein(s) responsible for AEA uptake should be looked for in future studies.

In conclusion, we have presented several lines of evidence indicating that FAAH cannot be the sole protein responsible for the AEA cellular uptake. None of the single experimental strategies used here, if considered alone, would provide further information on this process. However, taken together, our findings suggest that a saturable, selective, pharmacologically inhibitable and bi-directional mechanism is responsible for AEA transport in or out the cell, depending on the gradient of its concentration across the membrane itself. Our findings support future efforts aimed at isolating and cloning a membrane transporter for endocannabinoids, as well as developing more potent inhibitors of AEA transport to be used as both pharmacological tools to investigate endocannabinoid function and therapeutic drugs.

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