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European Journal of Pharmacology 492 (2004) 1–11

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Selective inhibition of anandamide cellular uptake versus enzymatic hydrolysis—a difficult issue to handle

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Received 6 November 2003; received in revised form 4 February 2004; accepted 25 March 2004

Abstract

There is considerable debate at present as to whether the uptake of anandamide (AEA) into cells is by a facilitated transport process or by passive diffusion driven by fatty acid amide hydrolase (FAAH). The possibility that both processes occur, but to different extents depending upon the cell type used, has been difficult to investigate pharmacologically since available compounds show little selectivity between inhibition of AEA uptake and inhibition of FAAH. Recently, three compounds, UCM707 [*N*-(Fur-3-ylmethyl)arachidonamide], OMDM-1 and OMDM-2 [the 1′ -(*S*)- and 1′ -(*R*)-enantiomers of the 1′ -4-hydroxybenzoyl analogue of oleoylethanolamide], selective for the uptake process, have been described and we have used these compounds, together with AM404 [(*N*-(4-hydroxyphenyl) arachidonoyl amide)] and VDM11 [(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide]), with the initial aim of determining which mechanism of uptake predominates in C6 glioma and RBL-2H3 cells. AM404 and VDM11 were both found to decrease the uptake of 2 μM AEA into cells (IC_{50} values 6–11 μM), but they also inhibited rat brain FAAH (IC_{50} values 1–6 μM). However, when using a different FAAH assay protocol, VDM11 was a much less potent FAAH inhibitor (IC_{50}>50 μM) regardless of the cell type and animal species used. In contrast, we confirmed that UCM707, OMDM-1 and OMDM-2 were weak inhibitors of FAAH (IC_{50} values >50 μM) under all conditions used. However, their potency as inhibitors of AEA cellular accumulation appears to be largely dependent on the cell type and assay conditions used. In particular, the potency of UCM707 (IC_{50} value ≥25 μM) was considerably lower than the submicromolar potency previously reported for U937 cells. It is concluded that the cause/effect relationship between AEA uptake and hydrolysis cannot be investigated uniquely by using supposedly selective inhibitors of each process.

Keywords: Endocannabinoid; Anandamide uptake; Fatty acid amide hydrolase; C6 glioma cell

1. Introduction

Whilst it is well established that the action of the endocannabinoid anandamide (arachidonoyl ethanolamide, AEA) upon cannabinoid receptors is terminated by its cellular uptake and metabolism (Deutsch and Chin, 1993; Di Marzo et al., 1994), there is considerable controversy at present as to the nature of this uptake process. Thus, some authors have suggested that the uptake is due to a facilitated diffusion process (Hillard et al., 1997), whereas others have described it as a passive process driven by the anandamide metabolising enzyme fatty acid amide hydrolase (FAAH, Deutsch et al., 2001; Day et al., 2001) (for a current discussion of these two points of view, see Glaser et al., 2003; Hillard and Jarrahian, 2003).

One of the surprising features of the uptake studies reported in the literature is its apparent ubiquity. However, this may reflect the net effect of a contribution of the two processes, facilitated diffusion and FAAH-driven diffusion, rather than a widespread distribution of a single uptake process. Indeed, in some cell types, inhibition of FAAH produces large reductions in the observed uptake (e.g. Rakhshan et al., 2000; Day et al., 2001), whereas in others,

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doi:10.1016/j.ejphar.2004.03.048

FAAH inhibitors are without effect (e.g. Day et al., 2001; Kathuria et al., 2003; Ruiz-Llorente et al., 2004).

Regardless of the predominant pathway operative for a given cell, the use of compounds that are equally good inhibitors of anadamide uptake and its metabolism by FAAH would be expected to show rather similar AEA uptake inhibition. One such compound is AM404 (*N*-(4-hydroxyphenyl) arachidonoylamide) (Beltramo et al., 1997), which inhibits FAAH activity and the uptake of AEA into cerebellar granule cells with very similar potencies (Jarrahian et al., 2000). This compound would thus be expected to inhibit AEA accumulation to a rather similar extent regardless of the relative components contributing to the uptake. Consistent with this hypothesis, AM404 inhibits the uptake of anandamide by a wide variety of cells with in general rather similar potencies (see e.g. Beltramo et al., 1997; Hillard et al., 1997; De Petrocellis et al., 2000; Jacobsson and Fowler, 2001).

Recently, four compounds have been reported which demonstrate a clear-cut selectivity between their abilities to inhibit AEA uptake and FAAH. UCM707 (*N*-(Fur-3-ylmethyl)arachidonamide) inhibits the uptake of 100 nM AEA into U937 human lymphoma cells with an IC_{50} value of 0.8 μM, but is a rather weak inhibitor of rat brain FAAH (IC_{50} 30 μM; López-Rodríguez et al., 2003). Ortar et al. (2003) reported that the compounds OMDM-1 and OMDM-2 (the 1′-(*S*)- and 1′-(*R*)-enantiomers, respectively, of the 1′-4-hydroxybenzoyl analogue of oleoylethanolamide) inhibited the uptake of 5 μM AEA by RBL-2H3 basophilic leukaemia cells with IC_{50} values of 2.4 and 3 μM, respectively, whereas the compounds did not inhibit FAAH activity in N18TG2 cell membranes (IC_{50} values >50 μM). VDM11 ((5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide), tested in the same cells, also exhibited a similar selectivity for the uptake process (De Petrocellis et al., 2000). In theory, such compounds should be ideal in characterising the extents to which FAAH-driven diffusion and facilitated transport processes contribute to the cellular uptake of AEA within a given cell. In consequence, we have used these compounds to characterize the nature of the AEA uptake process in C6 glioma cells and RBL-2H3 basophilic leukaemia cells.

2. Materials and methods

2.1. Materials

Anandamide [ethanolamine-1-^{3}H] ([Et^{3}H]AEA; specific activity 60 Ci mmol^{-1}) and anandamide, [arachidonyl-5,6,8,9,11,12,14,15-^{3}H] ([Ara^{3}H]AEA; specific activity 200 Ci mmol^{-1}) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). UCM707, OMDM-1 and OMDM-2 were synthesised in the laboratories of co-authors López-Rodríguez and Di Marzo. AM404 was purchased from Tocris Cookson (Bristol, UK). VDM11 from two-sources was tested for comparative purposes: from Tocris Cookson (“VDM11(toc)”) and from the laboratory of co-author Di Marzo (“VDM11(it)”). Non-radioactive AEA was obtained from the Cayman Chemical Co., Ann Arbor, MI, USA. Fatty acid-free bovine serum albumin was obtained from Calbiochem, San Diego, CA, USA. Charcoal (acid washed with hydrochloric acid; serial number C-4386, Lot 12K0179) was obtained from Sigma (St. Louis, MO, USA). Culture medium, sera and supplements were obtained from Invitrogen, Sweden.

2.2. Cell cultures

Unless otherwise stated, uptake experiments were undertaken using C6 glioma cells (either passage range 48–52 or a mixture of passages 38 and 50 which were then used 2–7 passages later, American Type Culture Collection, MD, USA). The cells were grown in 75 cm^2 culturing flasks at 37 °C, 5% CO_2 in air at normal atmospheric pressure. The cells were cultured in Ham’s F10 medium, supplemented with 10% foetal bovine serum and 100 units ml^{-1} penicillin + 100 μg ml^{-1} streptomycin. The culture media were changed three times a week. In additional experiments, a separate batch of C6 glioma cells was obtained from the European Collection of Cell Cultures (Porton Down, UK) and used over a passage range of 11–12. RBL-2H3 cells were used over a passage range of 36–39 (Protocol #1). The RBL-2H3 cells were cultured in Eagle’s minimum essential medium, 2 mM L-glutamine supplemented with 15% foetal bovine serum and 100 units ml^{-1} penicillin + 100 μg ml^{-1} streptomycin (Protocol #1).

2.3. FAAH assay

2.3.1. Protocol #1

Frozen brains (minus cerebellum) stored at −70 °C from adult Sprague–Dawley rats were thawed and homogenized (at 4° C, glass homogenizer) in 20 mM HEPES buffer, pH 7.0, with 1 mM $MgCl_2$. The homogenates were centrifuged at 36,000 × *g* for 20 min at 4 °C, resuspended and recentrifuged. The tissue pellets were re-suspended in homogenisation buffer and incubated at 37 °C for 15 min. After a further centrifugation at 36,000 × *g* for 20 min at 4 °C, the membranes were re-suspended in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$. The protein content in the membrane preparations was determined (Harrington, 1990), with bovine serum albumin as standard, and the membranes were then stored at −70 °C until used for assay. The assay used was a modification of the standard FAAH assay of Omeir et al. (1995) whereby a charcoal extraction stage (Wilson et al., 2003) was used instead of the chloroform extraction step. Briefly, membranes, test compounds (10 μl, dissolved in ethanol), [Et^{3}H]AEA and assay buffer (10 mM Tris–HCl, 1 mM EDTA, 1% (w/v) fatty acid-free bovine serum albumin) (final assay volume of 200 μl) were incubated for 10 min at

37 °C. Two assay pH were tested, with the protein concentrations/assay being adjusted to account for the pH profile of the enzyme (Schmid et al., 1985). Thus, protein concentrations of 7.5 and 3 μg/assay were used at pH values of 6.2 and 8.4, respectively. The ethanol concentration was kept constant throughout (~5% v/v). Controls contained the same concentration of ethanol in place of the test compounds. Reactions were stopped by putting the tubes on ice and adding 80 μl charcoal mixed in 320 μl of 0.5 M HCl. The tubes were mixed, left at room temperature for ~30 min and then centrifuged at 2000 × *g* for 10 min at room temperature in a bench centrifuge to sediment the charcoal. Aliquots (200 μl) of the "supernatant" were removed and analysed for tritium content. Blanks contained buffer instead of the membrane preparation. This assay has been found to give a robust measure of FAAH activity, and to show the appropriate sensitivity to inhibition by the FAAH inhibitor palmitoyltrifluoromethylketone (IC_{50} value 37 nM; L. Boldrup, S.J. Wilson, A.J. Barbier and C.J. Fowler, unpublished data).

2.3.2. Protocol #2

The effect of compounds on the enzymatic hydrolysis of AEA was studied using a widely exploited procedure first described by Maurelli et al. (1995). Membranes were prepared after centrifugation at 10,000 × *g* of homogenates (in Tris–HCl 50 mM, pH = 7.4) of: (i) mouse neuroblastoma N18TG2 cells, (ii) rat RBL-2H3 cells; (iii) human EFM-19 and MCF-7 mammary carcinoma cells; (iv) rat brain (including cerebellum), and (v) mouse brain (including cerebellum). Membranes (30 μg/assay) were incubated with increasing concentrations of the test compounds and [Et^{14}C]AEA (5 mCi/mmol, 4 μM, 10,000 cpm) in 0.5 ml of 50 mM Tris–HCl, pH 9, for 30 min at 37°C. [^{14}C]Ethanolamine produced from [Et ^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol.).

2.4. AEA uptake assays

2.4.1. Protocol #1

The assay was essentially that of Rakhshan et al. (2000), albeit with a higher assay AEA concentration (routinely 2 μM here compared with 1 nM in the original paper). This difference was simply to allow measurements near the K_M value for AEA uptake (~10 μM for this assay and RBL-2H3 cells, Rakhshan et al., 2000; Jacobsson and Fowler, 2001). In any case, it should be pointed out that the added AEA concentrations are nominal rather than exact, since we have found significant binding of the AEA to glass and plastic during preparation of the substrate samples, unless fatty acid-free bovine serum albumin is present (Karlsson et al., in press). A second difference between Protocol #1 and the original assay of Rakhshan et al. (2000) is that they used parallel wells preincubated with 100 μM AM404 as a measure of non-specific uptake, whereas the present study used parallel wells lacking cells for each condition.

Briefly, cells were plated on 24-well plates at an initial density of 2×10^5 cells/well and incubated at 37 °C for 18 h under an atmosphere of 5% CO_2 in air at normal atmospheric pressure. Cells were then washed once with 0.5 ml warm assay buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4; gassed with 95% O_2, 5% CO_2) and pre-incubated in 350 μl buffer at 37 °C for 15 min with the test compounds or ethanol carrier. [Ara^{3}H]AEA (50 μl) was added to the wells and the cells were incubated at 37 °C. Parallel 24 well plates treated in the same way, but lacking cells, were also used.

The choice of incubation time for first order processes like AEA accumulation is somewhat complex: a sufficient time needs to pass to allow measurable accumulation over background (often a greater problem at higher assay concentrations since uptake is saturable, whereas the background value will increase linearly with substrate concentration), whereas as short an incubation time as possible should be used to ensure that the initial velocity is measured. Inevitably, the chosen incubation time is a tradeoff between these two conflicting criteria. It is reasonable to assume that the time to saturation will decrease as the substrate concentration is decreased (see Ruiz-Llorente et al., 2004, for time-dependency curves for PC-3 prostate epithelial cells). With respect to C6 cells, this appears to be the case according to the literature, since at an assay AEA concentration of 0.1 nM, linearity of uptake is clearly lost by about 60 s (Hillard and Jarrahian, 2000), at 100 nM between 3 and 6 min (depending upon whether or not FAAH was inhibited, Deutsch et al., 2001), and at 4 μM between 8 and 10 min (Bisogno et al., 2001). In consequence, in the present study, incubation times of 4 and 8 min were used for the experiments using 100 nM and 2 μM AEA, respectively.

After incubation, the plates were then placed on ice and the wells were rinsed three times with ice-cold buffer containing 1% bovine serum albumin. The buffer was then removed, 0.2 M NaOH (500 μl/well) was added, and the wells were incubated at 75 °C for 15 min. Aliquots (300 μl) of the NaOH were transferred to scintillation vials and assayed for tritium content by liquid scintillation spectroscopy with quench correction. Test substances and AEA were diluted in ethanol and buffer and the ethanol concentration was constant between the different wells. In general, the pre-incubation phase contained 10 μl ethanol in a volume of 350 μl, i.e. an ethanol concentration of 2.9%. This was reduced slightly during the incubation phase (to 2.8% [11.3 μl in a volume of 400 μl]. Although this concentration is high, experiments using RBL-2H3 cells have demonstrated that in our hands this concentration of ethanol does not affect the uptake of [^{3}H]AEA at all, and that concentrations higher than 5% are required

before a drop in uptake is seen (Jacobsson and Fowler, 2001).

2.4.2. Protocol #2

The assay described in Bisogno et al. (1997), and later modified by Di Marzo et al. (2001), was used. Briefly, the effect of compounds on the uptake of [Et^{14}C]AEA by intact, confluent rat basophilic leukemia (RBL-2H3, passage 20–40) cells in 6-well dishes (cell density 8×10^5 cells/well) was studied by using 4 μM (10,000 cpm) of [^{14}C]AEA. Cells were incubated with [^{14}C]AEA in serum-free, bovine serum albumin-free Dulbecco's minimal essential medium for 5 min at 37 °C, in the presence or absence of increasing concentrations of the inhibitors in methanol (0.5% final concentration). Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3/CH_3OH$ 2:1 (by vol.), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells. Previous studies (Bisogno et al., 1997) had shown that, after a 5-min incubation, the amount of [^{14}C]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [^{14}C]AEA in the cell extract. Non-specific binding of [^{14}C]AEA to cells and plastic dishes was determined incubating cells at 4 °C and was never higher than 35%. For both FAAH and uptake assays, Protocol #1 experiments were undertaken in Sweden and Protocol #2 experiments in Italy.

2.5. Analysis of data

FAAH activities were expressed as % of control and used for further analysis as described below. For the uptake experiments, when using Protocol #1, the observed "uptake" in the absence of cells (but in the presence of the test compound) was subtracted from the uptake in the presence of cells, and the values then expressed as % of the corresponding controls. "Uptake" (in this case NaOH releasable adsorption) of the AEA in the wells alone at each inhibitor concentration was also expressed as % of control. When using Protocol #2, the uptake at 4 °C was subtracted from the uptake at 37 °C, and the net uptake observed in the presence of inhibitors then expressed as % of the total net uptake in the presence of vehicle. The pI_{50} ($-\log_{10}[IC_{50}]$ values) and hence IC_{50} values for the inhibitable portion of the FAAH activity or uptake were determined using the built-in equation "sigmoid dose–response (variable slope)" of the GraphPad Prism computer programme (GraphPad Software, San Diego, CA, USA) as described previously (Jonsson et al., 2001). The data set was constrained so that the "top" value (i.e. uninhibited) was set to 100 and the "bottom" value (reflecting the minimum remaining activity) determined. This allows for the determination of the total observable inhibition and the pI_{50} value of the inhibitable fraction. When the 95% confidence limits of the bottom value straddled zero, the data were reanalysed with the bottom value set to 0 to avoid bias due to artifactually low bottom values.

3. Results

3.1. Inhibition of FAAH by AM404, VDM11, UCM707, OMDM-1 and OMDM-2

The inhibition of the hydrolysis of 2 μM AEA by rat brain membranes by the five compounds, obtained when using Protocol #1, is shown in Fig. 1 and summarised in Table 1. In all cases, the maximum inhibition calculated by the curve fitting programme was not significantly different from 100%, although, of course, this level of inhibition was not always reached at the highest concentration tested. AM404 and VDM11 (from both sources) were found to exert a pronounced inhibition of FAAH with little pH dependency in their inhibition. In contrast, UCM707, OMDM-1 and OMDM-2 only weakly inhibited FAAH at

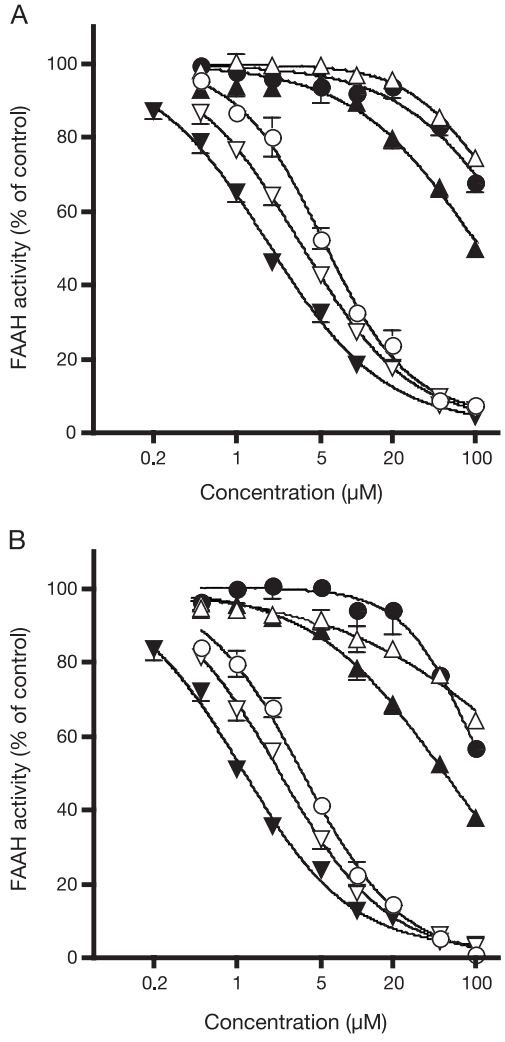

Fig. 1. Inhibition of 2 μM AEA hydrolysis by rat brain membranes at an assay pH of 6.2 (Panel A) and 8.4 (Panel B) by: VDM-11(toc) (▼), VDM-11(it) (▽), AM404 (○), OMDM-2 (▲), OMDM-1 (△) and UCM707 (●). "toc" and "it" refer to the sources of VDM-11 (TocrisCookson and co-author Di Marzo's laboratory, respectively). Shown are means ± S.E.M. (when not enclosed by the symbol), $n = 3$. All experiments were conducted using Protocol #1. Note that the abscissae are log scales.

Table 1
Summary of FAAH and AEA uptake inhibitory properties of the compounds tested in the present study

Compound	pI_{50} [IC_{50}, μM[a]] for inhibition of FAAH (Protocol #1)		Inhibition of AEA uptake (C6 cells)[b] (Protocol #1)		Inhibition of AEA uptake (RBL2H3 cells) (Protocol #2)	
	pH 6.2	pH 8.2	Max. inhibition[c]	pI_{50} [IC_{50}, μM[a]]	Max. inhibition[c]	pI_{50} [IC_{50}, μM[a]]
AM404	5.23 ± 0.02 [5.9]	5.45 ± 0.02 [3.6]	100	4.96 ± 0.02 [11]	100	4.99 ± 0.03[d] [10]
VDM11 (toc)	5.70 ± 0.02 [2.0]	5.93 ± 0.02 [1.2]	100	5.22 ± 0.02 [6.1]	–	–
VDM11 (it)	5.43 ± 0.02 [3.7]	5.64 ± 0.02 [2.3]	100	5.14 ± 0.06 [7.2]	100	4.95 ± 0.05[d] [11]
UCM707	3.59 ± 0.09 [>100]	3.92 ± 0.05 [>100]	53 ± 22	4.39 ± 0.32 [41]	100	4.60 ± 0.09 [25]
OMDM-1	3.60 ± 0.06 [>100]	3.41 ± 0.10 [>100]	100	4.49 ± 0.23 [>20]	100	5.58 ± 0.09[e] [2.6]
OMDM-2	3.97 ± 0.06 [>100]	4.26 ± 0.03 [54]	54 ± 9	4.78 ± 0.12 [17]	100	5.49 ± 0.07[e] [3.2]

Data are means ± S.E.M. of analyses from three experiments, except for UCM707 where the inhibition of uptake (C6 cells) were from eight experiments, and for OMDM-2 where $n = 4–5$. The AEA concentration was 2 μM for the FAAH and Protocol #1 uptake experiments.

[a] IC_{50} values are taken from the mean pI50 values. However, when the pI_{50} value was lower than the maximum concentration tested, the IC50 value is given as >this concentration.

[b] All data points calculated from the uptake (measured using Protocol #1) in the presence of the cells minus the corresponding value in their absence.

[c] When the maximum inhibition was not significantly different from 100% (i.e. when the calculated "bottom" value straddled zero) the value of 100% was taken and used in the analysis (see Materials and methods). Naturally, the maximum inhibition value used has considerable implications for the calculated pI_{50} and hence IC_{50} values. If, for example, the inhibition of AEA uptake by OMDM-2 using protocol #1 had been calculated assuming a maximum inhibition of 100%, a pI_{50} value of 4.30 ± 0.05, corresponding to an IC_{50} value of 50 μM, would have been obtained. The corresponding values for UCM707 using this protocol would be 3.89 ± 0.09 (>100 μM).

[d] These data have been published previously (De Petrocellis et al., 2000).

[e] These data have been published previously (Ortar et al., 2003).

either pH. When using Protocol #2 and FAAH preparations from various sources, VDM11, OMDM-1 and OMDM-2 inhibited the hydrolysis of 4 μM AEA only at high (>25 μM) concentrations (Table 2).

3.2. Inhibition of AEA uptake into C6 cells by AM404, VDM11, UCM707, OMDM-1 and OMDM-2

The inhibition of the uptake of 2 μM AEA into C6 cells by the compounds, obtained when using Protocol #1, is shown in Fig. 2 and summarised in Table 1. AM404 and VDM11 (from both sources) showed the expected potencies, with IC_{50} values in the range of 6–11 μM. In contrast, UCM707, OMDM-1 and OMDM-2 only weakly inhibited the accumulation of AEA.

In a second set of experiments using Protocol #1, the ability of UCM707 to inhibit AEA uptake was compared using different AEA concentrations (2 μM vs. 100 nM) and using C6 cells obtained on different occasions, and with very different passage numbers (11–12 vs. >40). Concentration ranges of UCM707 of 5–100 μM were used (although in the case of the high passage cells and the low AEA concentration, the data at an UCM707 concentration of 2 μM was not used due to a very large data spread). In all cases the maximum observed inhibition was not significantly different from 100%. The pI_{50} values (with IC_{50} values in parantheses) were: 2 μM AEA, low passage 4.14 ± 0.05 (72 μM); high passage 3.55 ± 0.20 (>100 μM); 100 nM AEA, low passage 4.29 ± 0.13 (51 μM); high passage 4.34 ± 0.07 (46 μM).

In all experiments using Protocol #1, parallel culture plates were run with the same assay conditions and inhibitor

Table 2
Summary of the IC_{50} for the inhibition by VDM-11 of FAAH from various membrane preparations, determined using Protocol #2

FAAH preparation	IC_{50} values for VDM-11
Rat brain	N.M. (39% inhibition at 50 μM)
Rat RBL-2H3 cells	48.0 ± 3.1 μM (54% inhibition at 50 μM)
Mouse brain	47.0 ± 2.6 μM (55% inhibition at 50 μM)
Mouse N18TG2 cells	N.M. (43% inhibition at 50 μM)[a]
Human MCF-7 cells	N.M. (16% inhibition at 50 μM)

Data are means ± S.E.M. of analyses from three experiments. N.M., not measured since less than 50% inhibition was attained at the highest concentration used.

[a] Using this protocol and this preparation, OMDM-1 and OMDM-2 were previously found not to inhibit FAAH (IC_{50}>50 μM) (Ortar et al., 2003).

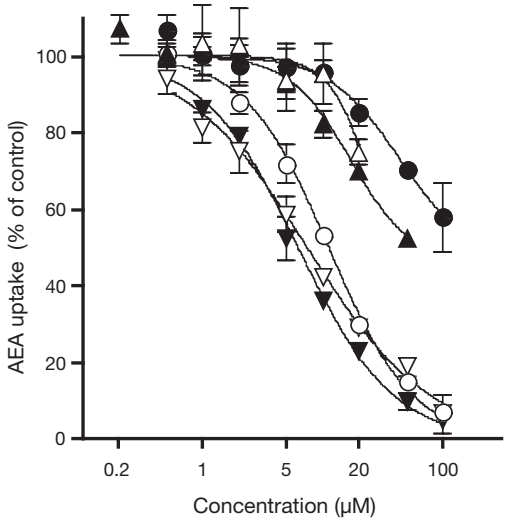

Fig. 2. Inhibition of the uptake of 2 μM [^{3}H]AEA into C6 glioma cells by VDM-11(toc) (▼), VDM-11(it) (▽), AM404 (○), OMDM-2 (▲), OMDM-1 (△) and UCM707 (●). Shown are means ± S.E.M. (when not enclosed by the symbol), $n = 3–8$. All experiments were conducted using Protocol #1. Note that the abscissa is a log scale.

concentrations, but in the absence of cells, to control for AEA binding to the wells. As expected, apparent "uptake" (due to adsorption and release into the NaOH of AEA from the wells) was found, approximating 10% of the total binding in the presence of cells. Although negligible as compared to the uptake by cells, and possibly overestimated due to the absence of a cell layer in the well, this apparent "uptake" for the wells alone could also be inhibited by some of the uptake inhibitors, particularly with AM404 and VDM11. Thus, pI_{50} values for the maximal attained inhibition (which in all cases was >60%) were: AM404, 5.50 ± 0.06; VDM11 (toc), 6.00 ± 0.13; VDM11 (it), 5.81 ± 0.32; UCM707, 3.68 ± 0.23; OMDM-1, 4.97 ± 0.32; OMDM-2, 5.59 ± 0.07. The data are shown for VDM(it) and OMDM-2 as examples in Fig. 3, and the data for VDM(toc) and AM404 are presented elsewhere (Karlsson et al., in press submitted).

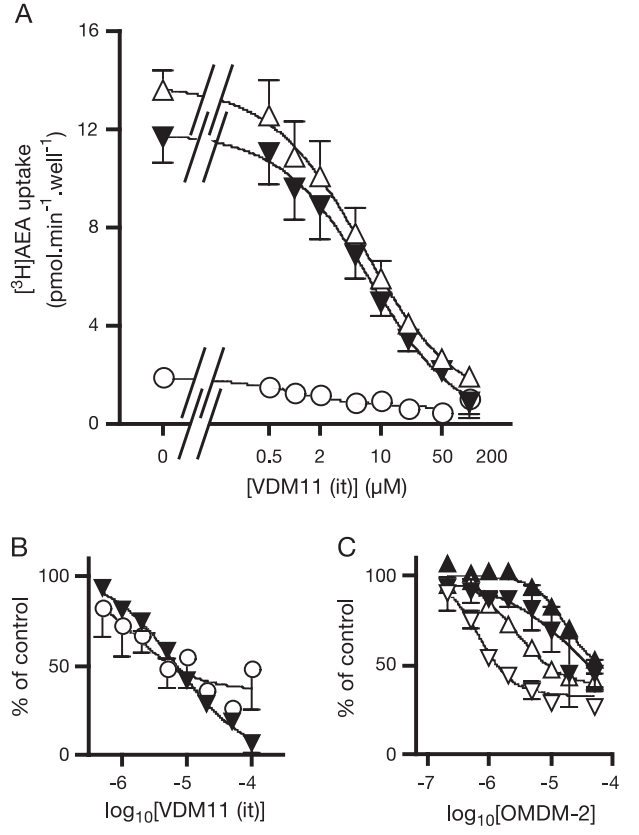

Fig. 3. Inhibition of AEA uptake into C6 glioma cells by VDM-11 (it) and OMDM-2. In Panel A, the absolute uptake values (pmol min^{-1} $well^{-1}$) are shown for total uptake (△), "cell-specific" uptake (▼, i.e. total uptake minus "uptake" for the wells alone) and "uptake" for the wells alone (○). Note that the absolute values assume an assay [^{3}H]AEA concentration of 2 μM, which is an overestimation (see Results). In Panel B, the corresponding values for the cell-specific and well uptake are shown as % of their own controls. In Panel C, data are shown for "cell specific uptake" at AEA assay concentrations of 2 μM (▲) and 100 nM (▼), and "uptake" for the wells alone at AEA assay concentrations of 2 μM (△) and 100 nM (▽). Data are means ± S.E.M. (when not enclosed by the symbol), $n = 3–5$. All experiments were conducted using Protocol #1. Note that the abscissae are log scales.

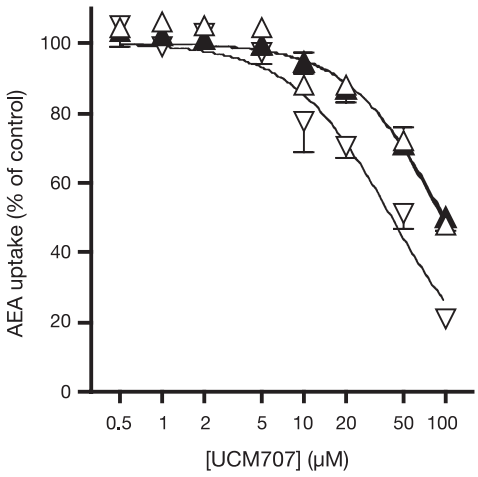

Fig. 4. Inhibition of the uptake of 2 μM AEA into RBL-2H3 cells. Conditions were: ▽, Standard assay, UCM707 dissolved in DMSO; △, standard assay, UCM707 dissolved in ethanol; ▲, 0.15% fatty acid free bovine serum albumin present throughout the assay, UCM707 dissolved in ethanol. Data are means ± S.E.M. (when not enclosed by the symbol), $n = 3$. The data are shown as % of control, which, in the case of the experiments for UCM707, dissolved in ethanol are as shown in Fig. 5. All experiments were conducted using Protocol #1. Note that the abscissa is a log scale.

3.3. Inhibition of AEA uptake into RBL-2H3 cells by UCM707

The ability of UCM707 to inhibit 2 μM AEA uptake was investigated in RBL-2H3 cells under four different conditions: (a) Protocol #1, UCM707 dissolved in dimethylsulfoxide (DMSO); (b) Protocol #1, UCM707 dissolved in ethanol; (c) 0.15% fatty acid-free bovine serum albumin present throughout the assay, carried out with Protocol #1, UCM707 dissolved in ethanol; and (d) Protocol #2, UCM707 dissolved in ethanol. The experiments using DMSO as solvent were run separately, the other two experiments using Protocol #1 were run concomitantly. The data are shown in Figs. 4 and 5 for the Protocol #1 experiments. Dissolution of UCM707 in DMSO produced an improvement in the potency of UCM707 (pI_{50} value 4.38 ± 0.04 [IC_{50} value 42 μM]) compared with the potency of this compound when dissolved in ethanol (pI_{50} value 4.02 ± 0.05 [IC_{50} value 95 μM]). When using Protocol #2 in RBL-2H3 cells, UCM707 inhibited AEA uptake with a pI_{50} value of 4.60 ± 0.09 [IC_{50} value 25 μM] Table 1).

As pointed out in Materials and methods, the assay AEA concentrations are nominal rather than exact, since there is significant binding of the AEA to glass and plastic during preparation of the substrate samples, unless fatty acid-free bovine serum albumin is present (Karlsson et al., in press). This was confirmed in the present study in an experiment where assays were conducted either in the absence or presence throughout of fatty acid free bovine serum albumin (0.15%). Assuming a concentration of 2 μM (i.e. no appreciable binding to the tubes during preparation of the

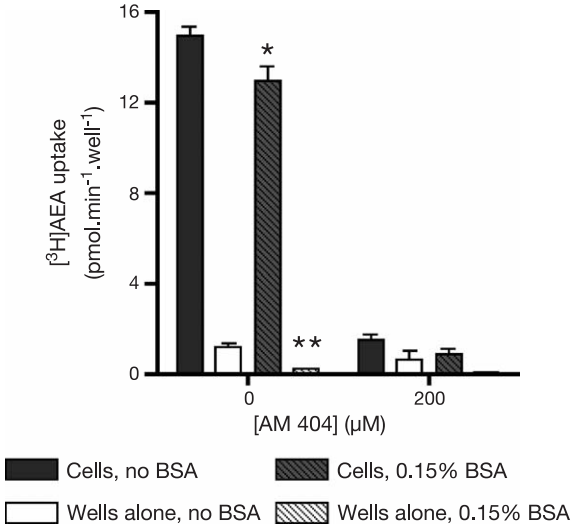

Fig. 5. Effect of inclusion throughout the assay of 0.15% fatty acid-free bovine serum albumin upon the uptake of AEA into RBL-2H3 cells. Data are means ± S.E.M., $n=3$ of the total uptake either for RBL-2H3 cells or the wells alone following preincubation with either vehicle (ethanol) or 200 μM AM 404. Data are means ± S.E.M., $n=3$. *$P<0.05$, **$P<0.01$ vs. the corresponding value for assays conducted in the absence of fatty acid-free BSA (Protocol #1).

radioactive solutions) for the added [^{3}H]AEA stock solutions in the presence of fatty acid-free bovine serum albumin, the recovered radioactivity for the samples prepared concomitantly but in the absence of fatty acid-free bovine serum albumin indicated an actual concentration of 1.2 ± 0.2 μM. The presence of fatty acid free bovine serum albumin in the assay throughout reduced considerably the level of tritium "uptake" for the wells alone (from 8.0 ± 1.1% of the total uptake for cells + wells in its absence to 1.9 ± 0.1% in its presence) (see Fig. 5). In contrast, the presence of 0.15% fatty acid-free bovine serum albumin throughout the assay had only a small (albeit significant) effect upon the observed AEA uptake (Fig. 5) and did not affect the observed inhibitory potency of UCM707 (pI_{50} value 4.00 ± 0.03 [IC_{50} value 100 μM]) (Fig. 4). The ability of both UCM707 and AM404 to inhibit the adsorbtion of AEA to the wells was also preserved in the presence of 0.15% fatty acid-free bovine serum albumin: the "uptake" for wells alone in the presence of 50 μM UCM707, 100 μM UCM707 and 200 μM AM404 was 58 ± 4%, 43 ± 3% and 32 ± 7%, respectively.

4. Discussion

In the present study, a comparison has been made between the abilities of five uptake inhibitors to inhibit FAAH and to prevent the cellular uptake of AEA by C6 and RBL-2H3 cells. Although data has been published concerning the potencies of these compounds towards both FAAH and AEA uptake in different cellular systems, it is extremely difficult to compare potencies obtained in different laboratories for such lipophilic compounds. Thus, for example, IC_{50} values for AM404 as an inhibitor of FAAH reported in the literature range from 3.4 μM (Jarrahian et al., 2000; Jonsson et al., 2001) to 10.2 μM (De Petrocellis et al., 2000). An even larger range was found in the present study for VDM-11 (compare the data shown for FAAH in Table 1 (assays undertaken in Sweden) with those in Table 2 (assays undertaken in Italy)). The assay variation is not surprising, given the nature of the compounds involved-indeed, the K_M of AEA as a substrate for FAAH varies widely between laboratories, values ranging from 0.8 to 180 μM being reported in the literature (see Fowler et al., 2001). Nevertheless, the present study underlines the importance of comparing potencies obtained in the same laboratory and under the same conditions. The present study in addition has provided information on dependency on the pH of the effects of VDM-11 and AM404 on FAAH. In contrast to non-steroidal antiinflammatory agents such as ibuprofen and indomethacin which inhibit FAAH in a highly pH dependent manner (Holt et al., 2001; Fowler et al., 2003), but consistent with other substrate analogues (Vandevoorde et al., 2003), the present compounds show little pH dependency.

With respect to the abilities of the compounds to inhibit AEA uptake, several important conclusions can be made from the present study. First, when using exactly the same assay conditions, the choice of cells might be of great importance for the observed potency of a certain uptake inhibitor. The present study here with UCM707 shows this rather well, since, regardless of the detailed assay conditions used, the potency in C6 and RBL-2H3 cells is >30 fold lower than that reported for its effects upon U937 human lymphoma cells (López-Rodríguez et al., 2003). In theory, this difference may reflect the degree to which FAAH "drives" the AEA uptake in the cells studied: for a cell line where the FAAH component of AEA uptake predominates, a low potency of UCM707 would be expected. However, a recent study has reported that UCM707 is not that potent an inhibitor of AEA uptake into PC-3 prostate epithelial cells (Ruiz-Llorente et al., 2004). Interestingly, the uptake of AEA into PC-3 cells is not that sensitive to FAAH inhibitors (Ruiz-Llorente et al., 2004), which would suggest that the expression of FAAH is not the only factor involved in determining the uptake inhibitory potency of a compound. The extreme example of this is the rabbit platelet, where the accumulation of AEA is neither temperature-, VDM11- or FAAH inhibitor-sensitive (Fasia et al., 2003).

Although the choice of different cell types may produce dramatic changes in the potency of a given compound, other experimental parameters, such as the type of vehicle, the way the substrate is radiolabelled and the cell passage number may also be important and may cause significantly different K_i values. Regarding cell passage number, for cerebellar granule neurons in primary culture, for example, the rate of uptake of AEA increases from 2 to 8 days in vitro

(Basavarajappa et al., 2003). Also, for cell lines, it is well established that cell morphology and biochemistry can change with passage number and culture medium (see Fowler and Brännstrom, 1990; Gubits et al., 1992 with respect to C6 cells), and passage-dependent changes in FAAH expression could thus be an important factor in the observed uptake. This does not, however, appear to be the case, at least for the cells studied here, since the RBL-2H3 cells were of similar passage number for the #1 and #2 uptake protocols (although of course, there may be passage-independent phenotypic differences between ostensibly identical cell lines used in different laboratories). For the C6 cells studied here, both early (14–22) and late (41–60) passage cells show a similar reduction in observed uptake following treatment with an FAAH inhibitor (Holt and Fowler, 2003; Karlsson et al., in press). In this respect, Day et al. (2001), using the assay upon which Protocol #1 is based (i.e. with an AEA concentration of 1 nM and an incubation time of 5 min), reported that AM404 could inhibit the AEA uptake remaining after preincubation with FAAH inhibitors to the level seen in RBL-2H3 cells with intact FAAH, which would also argue for both FAAH-dependent and independent uptake mechanisms in the cells. However, it should be pointed out as a caveat that at the concentration used (100 μM), AM404 may have non-specific effects upon membrane function (as demonstrated for AEA and arachidonic acid by Bloom et al. (1997); non-specific effects of AM404 upon calcium influx have also been reported in several cell lines by Chen et al. (2001)).

A second observation is that, again when using exactly the same assay conditions but different concentrations of radiolabelled AEA substrate, the potency observed with a certain inhibitor can also change, and increases with lower concentrations of the substrate used. Thus, the IC_{50} value for UCM707 when tested in C6 cells almost halved when decreasing the concentration of AEA from 2 μM to 100 nM. This is completely consistent with a competitive mode of inhibition and assuming that the K_M value for uptake is ~ 10 μM (such as we have seen for RBL-2H3 cells, using this assay, Jacobsson and Fowler, 2001). A similar observation has been made using these cells for AM404 (Bisogno et al., 2001) and is also a reflection of the competitive nature of the inhibition of AEA uptake by this compound (Rakhshan et al., 2000). Nevertheless, these changes are minor compared with the large potency differences for UCM707 seen here and in PC-3 cells (Ruiz-Llorente et al., 2004) and in U937 cells (López-Rodríguez et al., 2003).

A third observation of our study concerns the use in the incubation medium of fatty acid-free bovine serum albumin, which effectively binds AEA (K_D value = 55 nM, assuming one binding site, Bojesen and Hansen, 2003), thus preventing its binding to the plastic ware. However, a concentration of 0.5% of bovine serum albumin has been shown completely to prevent the accumulation of AEA into cortical neuronal cultures (Di Marzo et al., 1994). In RBL-2H3 cells, even when using very short incubation times (90 s) and a higher concentration of AEA (4 μM), 0.4% bovine serum albumin leads to a strong inhibition of AEA uptake, inhibition that could be, however, still enhanced in the presence of OMDM-2 (Ligresti et al., in press). Yet, in the present study, the uptake into RBL-2H3 cells was hardly affected in the presence of 0.15% fatty acid-free bovine serum albumin, which would suggest that the bovine serum albumin sensitivity of AEA uptake is dependent on the assay concentration of this protein. Consistent with this conclusion, the interaction of a lower concentration of AEA (1 μM) with the intracellular site on vanilloid TRPV1 receptors, which requires AEA uptake by intact TRPV1-expressing cells, was erased at bovine serum albumin concentrations higher than 0.2%, but could still be observed at lower concentrations (De Petrocellis et al., 2001). As a corollary to this conclusion, and as shown in Fig. 5, it can be suggested that the right concentration of bovine serum albumin, preventing AEA binding to plastic without preventing uptake, should be found prior to performing the uptake experiments. The fact that the sensitivity of AEA uptake by C6 cells to inhibition to UCM707 is not decreased when the assays are conducted in the presence of fatty acid-free bovine serum albumin can be interpreted in two ways: either the uptake seen in the presence of bovine serum albumin is entirely non-specific in nature (i.e. the specificity of uptake is defined as that sensitive to bovine serum albumin; Hillard and Jarrahian, 2003) and thereby not particularly sensitive to UCM707; or, alternatively, that AEA dissociates rapidly from the bovine serum albumin and can therefore be accumulated by the transport mechanism (Bojesen and Hansen, 2003), which in C6 cells is not particularly sensitive to UCM707.

Most of the discussion above has been concerned with the role of FAAH in determining AEA uptake rather then the nature of putative facilitated diffusion processes proposed initially by Di Marzo et al. (1994). Hillard and Jarrahian (2003) have suggested the possibility that the extra-, intra-cellular AEA gradient may be driven by intracellular sequestration of AEA, and there is some recent data, implicating lipid rafts as a site for uptake. Thus, Sarker and Maruyama (2003) reported that treatment of PC12 cells with methyl-β-cyclodextrin, which depletes membranes of cholesterol, abolished AEA-generated superoxide production, proapoptotic signalling and ultimately cell death, and suggested that an anandamide carrier was present in cholesterol-dependent lipid rafts. Biswas et al. (2003) have also reported that a reduction of membrane cholesterol following treatment with either methyl-β-cyclodextrin or mevastatin reversed the deleterious effects of AEA upon hepatocyte cell viability. These authors also showed that cholesterol binds to AEA-coated, but not control, polymyxin beads. Finally, McFarland et al. (2003) reported in a recent abstract that treatment of RBL-2H3 cells with [^{3}H]AEA leads to an accumulation of tritium label in lipid rafts, and that depletion of cellular cholesterol following treatment with nystatin

and progesterone reduced AEA transport by RBL-2H3 cells. In this respect, it is interesting to note that methyl-ß-cyclodextrin afforded protection against AEA-induced apoptosis in C6 glioma cells (Sarker and Maruyama, 2003), which would argue for the presence of a lipid raft-directed accumulation of AEA in these cells. The finding that low (1 μM) concentrations of AM404 and VDM11 can protect against the negative effects of prolonged treatment with AEA upon the proliferation of C6 cells (Jonsson et al., 2003) may also indicate an active uptake mechanism, given that the antiproliferative effects of AEA require the intracellular activation of vanilloid receptors in this model (Jacobsson et al., 2001). If these results are mooted to suggest that the presence, or lack thereof, of an uptake mechanism in C6 cells is secondary to the assay used, it would predict that cholesterol depletion should not affect the uptake of AEA using the Protocol #1 assay here. Such a prediction is presently under investigation.

Another, to our knowledge, unexplored possibility is that a site of action of the uptake inhibitors may be intracellular rather than on the plasma membrane. If, for the sake of argument, it is assumed that AEA is both synthesised and degraded intracellularly, it would need to be transported to the cell surface before it could be released from the cell. Similarly, extracellular AEA would need to be internalised before it can reach FAAH. Inhibition of this intracellular transport would affect the intra-: extracellular AEA gradient and hence the observed accumulation of extracellular AEA. In the rat heart, the pivotal enzyme for AEA synthesis, *N*-acyl phosphatidylethanolamine (NAPE)-phospholipase D is mainly associated in microsomal and mitochondrial fractions (Schmid et al., 1983), and has now been identified the as a member of the zinc metallohydrolase family of the ß-lactamase fold (Okamoto et al., 2004). Similarly, FAAH has been found in subcellular fractionation studies to be associated with microsomal and mitochondrial fractions and, where appropriate, synaptosomal and myelin fractions (see e.g. Schmid et al., 1985; Hillard et al., 1995; Maurelli et al., 1995). Most importantly, by using an immunohistochemical method, Glaser et al. (2003) found that FAAH is immunolocalized perinuclearly in neuroblastoma cells. Of course, this does not rule out metabolic actions taking place on the cell surface (experiments with exogenous phospholipase from *S. chromofuscus* have suggested that some of the *N*-acyl phosphatidylethanolamine can be metabolised to *N*-acylethanolamines at the level of the cell membrane (Cadas et al., 1996)), but it would provide an argument as to why a transport system is required for such lipophilic compounds. There is clearly further work required before the nature of the FAAH-independent AEA uptake mechanism can be elucidated. Our data have shown that the cause/effect relationship between AEA uptake and hydrolysis cannot be investigated uniquely by using supposedly selective inhibitors of each process unless an assay is developed allowing the monitoring of both processes independently from each other in the same cell type. The ultimate proof of transport mechanisms, of course, will be the identification of the molecular target(s) involved.

A final note concerning the observation that the adsorption of AEA to the wells per se can be prevented by the uptake inhibitors is worthy of comment. AEA is a very "sticky" molecule, and does not only adhere to wells, but can be released from the wells upon medium change in a time- and temperature-dependent manner (Karlsson et al., in press). The degree to which AEA binds to the wells appears to be highly dependent upon factors such as the batch of the culture wells, and in our hands the degree of adsorption can vary from 5% to 10% (present study) to as high as 50% (Jonsson et al., 2001). The finding that this adsorption can be prevented by AEA uptake inhibitors underlines the importance of controlling for this parameter at all inhibitor concentrations tested. However, it is unlikely that this is a strong confounding factor in studies on the potency of uptake inhibitors, since OMDM-1 analogues with minor differences in their chemical structures exert dramatically different effects on AEA uptake by RBL-2H3 cells (Ligresti et al., in press), which cannot be accounted for by possible different capabilities to displace AEA from plastic.

Acknowledgements

The technical expertise of Britt Jacobsson in undertaking the Protocol #1 FAAH and #1 uptake experiments is greatly appreciated. V.D.M. and A.L. thank Dr. Maria Grazia Cascio for her valuable assistance. C.J.F. and G.T. would like to thank the Swedish Research Council (Grant no. 12158, medicine), Konung Gustav V's and Drottning Victorias Foundation, Gun and Bertil Stohne's Foundation, and the Research Funds of the Medical Faculty, Umeå University for financial support. M.L.R. would like to thank MCYT (BQU2001-1459) for financial support.

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