Title

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Journal

Lipids in Health and Disease, 1(1)

ISSN

1476-511X

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Publication Date

2002

DOI

10.1186/1476-511x-1-1

Peer reviewed

Lipids in Health and Disease

Short paper

Anandamide uptake by synaptosomes from human, mouse and rat brain: inhibition by glutamine and glutamate

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Published: 3 September 2002

Lipids in Health and Disease 2002, **1**:1

Received: 1 August 2002
Accepted: 3 September 2002

This article is available from: http://www.Lipidworld.com/content/1/1/1

Abstract

Anandamide (*N*-arachidonoylethanolamine, AEA) belongs to an emerging class of endogenous lipids, called "endocannabinoids". A specific AEA membrane transporter (AMT) allows the import of this lipid and its degradation by the intracellular enzyme AEA hydrolase. Here, we show that synaptosomes from human, mouse and rat brain might be an ideal *ex vivo* system for the study of: i) the accumulation of AEA in brain, and ii) the pharmacological properties of AMT inhibitors. Using this *ex vivo* system, we demonstrate for the first time that glutamine and glutamate act as non-competitive inhibitors of AEA uptake by human, mouse and rat brain AMT.

Findings

Endocannabinoids are an emerging class of lipid mediators, which activate cannabinoid receptors and regulate sleep induction, interact with GABAergic, serotonergic and dopaminergic neurotransmission, and are also formed during glutamate-induced neurotoxicity [1,2]. The biological activity of anandamide (*N*-arachidonoylethanolamine, AEA), a major endocannabinoid, is controlled by cellular uptake through a specific AEA membrane transporter (AMT) [3], followed by intracellular degradation by anandamide hydrolase (fatty acid amide hydrolase, FAAH) [4]. The critical role of AMT in controlling AEA action, and the potential use of AMT inhibitors in the treatment of human pathologies such as multiple sclerosis, Parkinson's disease and Huntington's disease [1,5,6], prompted us to investigate whether brain synaptosomes might be used as a new *ex vivo* system, able to accumulate AEA through a selective transporter with the features of AMT.

Human brain specimens were obtained from twelve different male patients (aged 73–77), undergoing surgical operation to remove meningioma tumors from the pterional area. Brain tissues, removed and donated by Dr. G. De Caro (Neurosurgery Division, University of Rome "Tor Vergata", Sant'Eugenio Hospital, Rome, Italy), were kept on ice until processed (within 30 min). In each case the perilesional white matter (150 mg of fresh tissue) surrounding the tumor area (300 mg) was removed and used as healthy control [7]. Clearance of the local Ethnics Committee was obtained to use human brain biopsies. Swiss mice weighing 20–25 g were from Charles River (Calco, Italy), and Wistar rats weighing 250–280 g were from Morini (San Paolo D'Anza, Italy). All animal procedures met the guidelines of the U.S. National Institutes of Health, detailed in the *Guide for the Care and Use of Laboratory Animals*, and the European Community directives regulating animal research. The experimental procedures were also approved by the local Animal Care Committee.

Table 1: Kinetic Constants of the AEA Membrane Transporter (AMT) in Brain Synaptosomes and Inhibition by Glutamine and Glutamate

Kinetic constant	Human Meningioma	Mouse Brain	Rat Brain
K_m (nM)	793 ± 128	660 ± 80	483 ± 52
V_{max} (pmol/min per mg protein)	219 ± 19	370 ± 23	357 ± 18
K_i of L-Glutamine (nM)[a]	1100 ± 90	970 ± 80	850 ± 80
K_i of D-Glutamine (nM)[a]	525 ± 50	480 ± 50	430 ± 45
K_i of L-Glutamate (nM)[a]	4000 ± 370	3580 ± 300	3300 ± 310
K_i of D-Glutamate (nM)[a]	1230 ± 110	1100 ± 90	850 ± 80

[a]Non-competitive inhibitor of AMT in all cases.

To prepare synaptosomes from human, mouse or rat brain, fresh tissues were resuspended in ice-cold 0.32 M sucrose, 5 mM Tris.HCl buffer (pH 7.4) and were gently disrupted by 10 up-and-down strokes in a Teflon-glass homogenizer (weight/volume ratio = 1:10). The homogenate was centrifuged at 1000 × *g* for 5 min, at 4°C, the supernatant was then centrifuged again at 17000 × *g* for 15 min, at 4°C. The final pellet 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, 10 mM Tris.HCl buffer (pH 7.4), at a protein concentration of 1 mg/ml [8]. The activity of AMT and its kinetic properties were determined as described [9]. Synaptosomes (100 μl/test) were incubated for different time intervals, at 37°C or 4°C to discriminate carrier-mediated from non-carrier-mediated transport of AEA through cell membranes, with 200 nM [^{3}H]AEA (223 Ci/mmol; NEN Dupont de Nemours, Köln, Germany). Alternatively, they were incubated for 15 min with different concentrations of [^{3}H]AEA, in the range 0–1000 nM (also in this case, the uptake at 4°C was subtracted from that at 37°C). The kinetic constants of AMT, *i.e.* apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), and the inhibition constant (K_i), were calculated by non-linear regression analysis of the experimental points, using a Prism 3 program (GraphPAD Software for Science, San Diego, CA). Q_{10} value was calculated as the ratio of AEA uptake at 30°C and 20°C [9]. The effect of various compounds (AEA, arachidonic acid, ethanolamine, D/L-glutamine, D/L-glutamate, phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid, *N*-ethylmaleimide, sodium nitroprusside (SNP), all from Sigma Chemical Co., St. Louis, MO; 2-arachidonoylglycerol (2-AG) and *N*-(4-hydroxyphenyl)-arachidonoylamide (AM404), from Research Biochemicals International, Natick, MA; VDM11, from Tocris-Cookson, Bristol, UK; leukotriene B_4 and prostaglandin E_2, from Cayman Chemical Company, Ann Arbor, MI; 3-morpholinosydnonimine (SIN-1), from Alexis Corporation, Läufelfingen, Switzerland) on AMT activity was determined by adding directly each substance to the assay buffer, at the indicated concentrations, and incubating for 15 min at 37°C [9]. Data are reported as mean (± S.D.) of at least four independent determinations, each performed in duplicate. Statistical analysis was performed by the non-parametric Mann-Whitney test, elaborating experimental data through the InStat 3 program (GraphPAD Software for Science).

Synaptosomes prepared from human healthy brain (HB) or human meningioma (HM) were found to take up [^{3}H]AEA with a saturable process, showing apparent K_m and V_{max} values of 802 ± 150 nM and 374 ± 38 pmol/min per mg protein, for HB, and 793 ± 128 nM and 219 ± 19 pmol/min per mg protein, for HM. Accumulation of 200 nM [^{3}H]AEA was linear up to a synaptosome concentration of 500 μg protein, and was undetectable in synaptosomes pre-boiled for 5 min. It was also time- ($t_{1/2}$ ~ 3 and ~5 min for HB and HM, respectively) and temperature- (Q_{10} ~ 1.5 in both cases) dependent. Uptake of [^{3}H]AEA was dose-dependently inhibited by AM404 or VDM11, selective AMT inhibitors [5,10]. Linear regression analysis (r^2 = 0.93) of the inhibition data yielded IC_{50} (concentration necessary to produce half-maximal inhibition) values of ~7 μM for both compounds in both tissues. This IC_{50} value is in the same range calculated in cell suspensions [5,10]. The uptake of [^{3}H]AEA was further characterized in synaptosomes prepared from HM, which was available in larger amounts than the healthy tissue. It was found that accumulation of 200 nM [^{3}H]AEA by HM synaptosomes was fully inhibited by 1 μM "cold" AEA, whereas arachidonic acid and ethanolamine, the hydrolysis products generated from AEA by FAAH, were ineffective at concentrations up to 10 μM. Moreover, the uptake of [^{3}H]AEA was not affected by leukotriene B_4 or prostaglandin E_2 (at 1 μM), was inhibited (~50%) by alkylating agents like PMSF, iodoacetic acid and *N*-ethylmaleimide (each used at 100 μM), and was enhanced (~180–220%) by nitric oxide-donor SNP (at 5 mM) and peroxynitrite-donor SIN-1 (at 1 mM), in much the same way as reported for neuronal and non-neuronal cells in culture [3,7,9]. Remarkably, 1 μM 2-AG inhibited almost completely the up-

take of 200 nM [^{3}H]AEA by AMT in meningioma synaptosomes, again in line with previous observations on cultured cells [3,9]. Taken together, these data suggest that both healthy and tumoral brain accumulate [^{3}H]AEA by means of a selective transporter, most probably expressed in lower amounts in the tumoral than in the healthy tissue. Also mouse brain (MB) and rat brain (RB) synaptosomes were found to take up [^{3}H]AEA with a saturable process, showing apparent K_m and V_{max} values comparable to those of AMT in human meningioma (Table 1). Interestingly, for the first time glutamine and glutamate were found to inhibit AMT in HM, MB and RB synaptosomes in a dose-dependent manner, the L-enantiomers of both compounds being less effective than the corresponding D-forms. Kinetic analysis of the inhibition of AMT by D/L-glutamine or D/L-glutamate showed that these compounds acted as non-competitive inhibitors of AMT, with K_i values in the nM-μM range and a potency D-glutamine > L-glutamine > D-glutamate > L-glutamate (Table 1). While it might be obvious that the amide was more potent than the corresponding free acid in inhibiting the amide transporter AMT, it remains unclear why the D-enantiomers were more potent than the corresponding L-forms. As yet, the lack of structural information on AMT [3,5,9] does not allow a molecular interpretation of the properties of this transporter. However, these unprecedented findings might be relevant for the role of AEA in glutamate-induced neurotoxicity [1,2], because they suggest a cross-talk between these two neurotransmitters. Indeed, here it is shown that glutamate inhibits AEA transport through AMT, whereas it has been already reported that AEA inhibits synaptic release of glutamate [reviewed in ref. [11]]. This interplay between AEA and glutamate might be relevant also in controlling excitotoxic insults [12].

In summary, this study describes how to prepare synaptosomes suitable to investigate presence and kinetic properties of the anandamide membrane transporter in human, mouse and rat brain. This system allows to extend *ex vivo* the model of AEA transport already described *in vitro* with cell suspensions, and has the potential to provide insights on the regulation and function of AMT in the context of mature brain tissue, where control mechanisms lost in immortalized cells might be retained. In this line, the finding that AMT in synaptosomes is enhanced by nitric oxide-donor SNP, and even more by peroxynitrite-donor SIN-1, speaks in favour of a physiological meaning for the nitric oxide-mediated link between AEA receptor and AEA degradation, previously demonstrated *in vitro*[9]. Moreover, the inhibition of AMT in human, mouse and rat brain synaptosomes by glutamine and glutamate seems relevant to better understand the role of AEA in glutamate-induced neurotoxity. In conclusion, brain synaptosomes appear to be a good tool for studies aimed at discovering new inhibitors of AMT of potential therapeutic value.

Authors' contributions

N. Battista and M. Bari participated equally in animal care, synaptosome preparation and uptake assays. A. Finazzi-Agrò participated in the study design and coordination. M. Maccarrone conceived and coordinated the study.

Acknowledgements

We are grateful to Dr. G. De Caro for kindly donating human brain specimens (Neurosurgery Division, University of Rome "Tor Vergata", Sant'Eugenio Hospital, Rome, Italy) and to Dr. M. Di Rienzo for skilfull assistance. This study was supported by Consiglio Nazionale delle Ricerche, Progetto strategico "Neuroscienze".

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