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Review

The movement of *N*-arachidonoylethanolamine (anandamide) across cellular membranes

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Abstract

This review presents and explores the hypothesis that *N*-arachidonoylethanolamine (AEA, also called anandamide) is transported across cellular membranes by a process that is protein-mediated. Support for this hypothesis comes from experiments demonstrating that cellular accumulation of extracellularly applied AEA is saturable, time and temperature dependent and exhibits selective inhibition by various structural analogs of AEA. The accumulation of AEA is cell specific; data is presented demonstrating that several cell types, including the bovine adrenal zona glomerulosa cell, exhibit very high capacity for AEA accumulation while others, such as the HeLa cell, have a very low capacity. The transport process has the characteristics of facilitated diffusion; it is bi-directional, not dependent on either ATP or extracellular sodium and exhibits the *trans* effect of flux coupling. Several important questions remain to be answered regarding the carrier, including its molecular structure and its role in the release and inactivation of endogenously produced AEA.

Keywords: Cannabinoid; Endocannabinoid; Transporter; Flux coupling

1. Introduction

The evidence supporting a role for the endocannabinoid *N*-arachidonoylethanolamine (AEA or anandamide) as an intercellular signaling molecule is mounting. AEA is present in measurable amounts in the brain (Devane et al., 1992; Schmid et al., 1995; Felder et al., 1996; Schmid et al., 1996), uterus (Schmid et al., 1997) and testes (Sugiura et al., 1996; Kondo et al., 1998). AEA binds and activates the CB1 cannabinoid receptor

Abbreviations: AEA, *N*-arachidonoylethanolamine; CB1, neuronal cannabinoid receptor; FAAH, fatty acid amide hydrolase; MAFP, methylarachidonylfluorophosphate; *N*-arachPE, *N*-arachidonyl phosphatidyl ethanolamine; PMSF, phenylmethylsulfonylfluoride.

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(Devane et al., 1992; Felder et al., 1993; Fride and Mechoulam, 1993) and mimics the physiological and biochemical effects of the classical cannabinoids (see Hillard and Campbell, 1997 for review). In the rodent brain, there is a reasonably good match between the regional distribution of AEA or its precursor, *N*-arachidonoylphosphatidylethanolamine (*N*-arachPE) (Bisogno et al., 1999; Yang et al., 1999) and the distribution of the CB1 cannabinoid receptor (Tsou et al., 1998a; Herkenham et al., 1990). The cellular synthesis of AEA has been demonstrated in neurons in primary culture (Di Marzo et al., 1994; Cadas et al., 1996); human umbilical vein endothelial cells (HUVECs) (Maccarrone et al., 2000); neuroblastoma cells (Di Marzo et al., 1996); macrophages and macrophage-derived cell lines (Di Marzo et al., 1996; Bisogno et al., 1997; Pestonjamasp and Burstein, 1998; Varga et al., 1998) and basophilic cells (Bisogno et al., 1997; Rakhshan et al., 2000).

Among the criteria for the classification of an intercellular signaling molecule or transmitter is evidence of a process by which its signaling is terminated. The actions of most transmitters are terminated by (1) diffusion away from its site of action; (2) metabolism to one or more inactive compounds; (3) transport to an intracellular compartment where the transmitter is sequestered from its site of action; or (4) a combination of these processes.

There is considerable evidence that AEA is metabolized to molecules that do not activate the CB1 cannabinoid receptor. AEA is a substrate for an amidohydrolase which converts AEA to arachidonic acid (which is rapidly reesterified into cellular phospholipids) and ethanolamine (Deutsch and Chin, 1993). The AEA amidohydrolase activity is identical to that of a cloned fatty acid amide hydrolase (FAAH; Cravatt et al., 1996) which also catabolizes the putative sleep inducing lipid oleamide (Cravatt et al., 1995). AEA amidohydrolase is a membrane protein whose distribution in cellular membrane fractions correlates with the distribution of markers for endoplasmic reticular membranes (Hillard et al., 1995; Ueda et al., 1995). Conversely, membrane fractions from rat brain that are enriched in plasma membranes have very low AEA amidohydrolase activity (Hillard et al., 1995). Immunohistochemical data support an intracellular localization for AEA amidohydrolase in brain (Tsou et al., 1998b). Therefore, if AEA amidohydrolase plays a role in the inactivation of AEA that has been released, then a process must be present for the movement of AEA from the extracellular space into cells that contain AEA amidohydrolase.

The subject of this review is the evidence that supports the hypothesis that AEA transport across cellular membranes occurs via a protein-mediated mechanism. One possible function of this transport process is to act in series with intracellular AEA amidohydrolase to inactivate extracellular AEA. Another function of the transport process may be to sequester and functionally inactivate AEA by removing it from its receptors. Finally, it is also possible that a transport process serves to release AEA from cells after its synthesis, implying a role for an AEA transport protein in the process of activation of the AEA/cannabinoid signaling system.

2. Cellular distribution of AEA transport activity

AEA transport across cellular membranes, measured almost exclusively using cellular accumulation of radiolabeled AEA, has been demonstrated using cell types derived from brain and the immune system. AEA is accumulated by both cortical (Di Marzo et al., 1994; Beltramo et al., 1997) and cerebellar granule (Hillard et al., 1997 and Fig. 1) neurons in primary culture. Cortical astrocytes in primary culture accumulate AEA (Beltramo et al., 1997) as do C6 rat glioma cells (Deutsch and Chin, 1993 and Fig. 1), endothelial cells (Maccarrone et al., 2000 and Fig. 2) and the neuroblastoma cell lines N18TG2 (Deutsch and Chin, 1993), CHP100 (Maccarrone et al., 1998), and the human astrocytoma line CCF-STTG1 (Piomelli et al., 1999). Immune cells have also been studied with regard to AEA accumulation. AEA accumulation occurs in the rat basophilic leukemia cell line, RBL-2H3 (Bisogno et al., 1997; Rakhshan et al., 2000); J774 macrophages

(Bisogno et al., 1997); and cells from the human lymphoma cell line, U937 (Maccarrone et al., 1998).

Although all of the cells mentioned above exhibit saturable accumulation of AEA, the reported kinetic parameters of K_m and V_{max} for AEA accumulation in these cell types (summarized in Table 1) demonstrate a degree of variability which may be indicative of different molecular characteristics of the transporter involved. A low affinity, high capacity form of AEA transport is seen in cerebellar granule cells in primary culture and in RBL-2H3 cells. Astrocytes in primary culture as well as two different neuroblastoma cell lines express a higher affinity but lower capacity accumulation process as do the U937 lymphoma cells and HUVECs. Primary neurons from rat cerebral cortex exhibit an intermediate affinity for AEA. The catalytic efficiency of transport in these cells (i.e. the ratio of V_{max} to K_m) exhibits a 20-fold variability, with the U937 cells having the highest efficiency and the CCF-STTG1 human astrocytoma cells having the lowest among these cells.

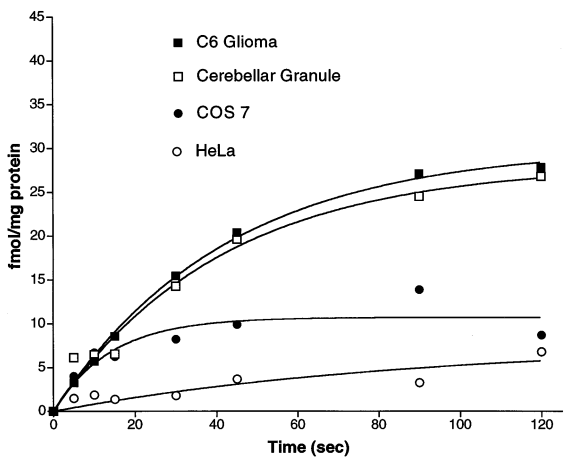

Fig. 1. AEA accumulation by various cell types. Cells were placed into culture and maintained using standard methods. The cells were used at approximately 70% confluence (or on days 7–8 in vitro for the cerebellar granule cell experiments). Cells were washed free of media in warm (37°C) KRH buffer. The buffer was replaced with fresh buffer containing 0.1 nM [^{3}H]AEA, and then removed at the times indicated. Cells were scraped into fresh buffer. Both the buffer and scraped cells were counted and the amount of AEA accumulation was expressed as fmol [^{3}H]AEA accumulated/mg protein. Nonspecific association was accounted for by subtracting data obtained in a parallel incubation at 4°C. Data are mean values of three experiments performed in triplicate. Lines drawn were determined by nonlinear regression fitting the data to a single site, association equation.

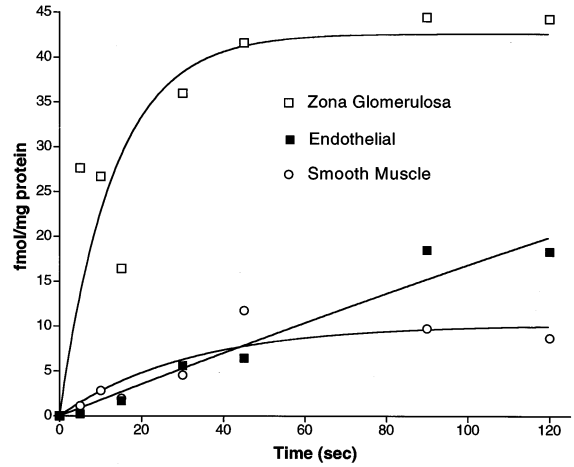

Fig. 2. AEA accumulation by various cell types. Cells were placed into culture and maintained using standard methods. The cells were used at approximately 70% confluence. Cells were washed free of media in warm (37°C) KRH buffer. The buffer was then replaced with fresh buffer containing 0.1 nM [^{3}H]AEA, and then removed at the times indicated. Cells were scraped into fresh buffer. Both the buffer and scraped cells were counted and the amount of AEA accumulation was expressed as fmol [^{3}H]AEA accumulated/mg protein. Nonspecific association was accounted for by subtracting data obtained in a parallel incubation at 4°C. Data are mean values of three experiments performed in triplicate. Lines drawn were determined by nonlinear regression fitting the data to a single site, association equation.

We have begun to explore the relative rates and T_m of AEA accumulation among a variety of cell types (Fig. 1 and Fig. 2 and Table 2). Among the cell types investigated, the zona glomerulosa cells of the bovine adrenal cortex exhibit the greatest T_m for AEA accumulation (Fig. 2). These cells contain lipid-filled organelles, which may be the storage site for AEA following its accumulation. As was shown previously (Deutsch and Chin, 1993), C6 glioma cells accumulate AEA, although the capacity for accumulation appears to be less than that of the cerebellar granule cells (Table 2). Both endothelial cells and smooth muscle cells taken from bovine coronary vessels accumulate

Table 1
Kinetic parameters of AEA accumulation

Cell type	K_m(μM)	V_{max} (nmol/min per mg protein)	V_{max}/K_m	Citation
Cerebellar granule neurons	41 ± 15	6.1 ± 0.4[a]	0.15	(Hillard et al., 1997)
Cerebral cortical neurons	1.2	0.091	0.076	(Beltramo et al., 1997)
Cerebral cortical astrocytes	0.32	0.171	0.53	(Beltramo et al., 1997)
CHP100 neuroblastoma	0.2 ± 0.02	0.03 ± 0.003	0.15	(Maccarrone et al., 1998)
CCF-STTG1 astrocytoma	0.6 ± 0.1	$0.015 \pm .0015$	0.0245	(Piomelli et al., 1999)
Human umbilical vein endothelial	0.19 ± 0.01	0.045 ± 0.003	0.24	(Maccarrone et al., 2000)
RBL-2H3	33	6[a]	0.18	(Bisogno et al., 1997)
	11.4 ± 2.3	1.8 ± 0.2[a]	0.16	(Rakhshan et al., 2000)
U937	0.13 ± 0.01	0.14 ± 0.015	1.08	(Maccarrone et al., 1998)

[a] Data were originally published as nmol/min/10^6 cells; a factor of 0.1 mg protein/10^6 cells was used to convert the data units.

AEA, although the endothelial cells appear to have a slightly greater capacity (Fig. 2). COS 7 and HeLa cells exhibit only a small amount of AEA accumulation (Fig. 1); in particular, HeLa cells may prove useful as uptake null cells for transfection studies.

Taken together, data from multiple laboratories consistently demonstrate saturable accumulation of AEA by neurons, astroglial cells and cells of the immune system. The K_m values for AEA vary between 0.13 and 41 μM, which may indicate different subtypes of AEA carrier although methodological differences among laboratories cannot be discounted. In addition to brain-derived cells and circulating immune cells, our preliminary data suggest that cells of the cardiovascular system and the zona glomerulosa cells also accumulate AEA. Not all cells accumulate AEA to the same degree which can be taken as evidence for cell-specific, accumulation process(es). As we argue below, the available data suggests that the transport process is protein-mediated and that protein expression or lack thereof is likely to be a major determinant of AEA accumulation by the various cell types.

In addition to differences in the kinetics of AEA accumulation, the various cell types investigated also differ in the expression of FAAH and cannabinoid receptors. For example, the cerebellar granule cells express high levels of the CB1 cannabinoid receptor (Pacheco et al., 1993; Hillard et al., 1999) and have very low FAAH activity (Hillard et al., 1997). These characteristics along with the presence of a low affinity, high capacity accumulation process suggest that the cerebellar granule cell is primarily a target cell designed to allow maximal AEA access to the CB1 receptor. Conversely, the U937 cells express hardly detectable levels of mRNA for either CB1 or CB2 receptors (Galiegue et al., 1995) and very high levels of FAAH protein and activity (Maccarrone et al., 1998) and exhibit high affinity,

Table 2
AEA accumulation by various cell types[a]

Cell type	Half-life (s)	T_{max} (fmol/mg protein)
Cerebellar granule	72 (53–112)	45 ± 3
C6 glioma	29 (26–33)	30 ± 1
Bovine zona glomerulosa	20 (11–125)	55 ± 6
Bovine coronary endothelial	56 (35–140)	22 ± 2
Bovine coronary smooth muscle	56 (30–552)	16 ± 3
COS 7	14 (7–74)	12 ± 1
HeLa	53 (31–189)	7 ± 1

[a] Cells were placed into culture and maintained using standard methods. The cells were used at approximately 70% confluence (or on days 7–8 in vitro in the cerebellar granule cell experiments). Assays were carried out as described in the legends to Fig. 1 and Fig. 2. Kinetic parameters were obtained from the best fit of the data combined from three separate experiments to the first order association equation using least squares, nonlinear regression analyses (GraphPad Prism). Data for the half-life are reported with the 95% confidence intervals, data for T_{max} are reported with standard error values.

moderate capacity AEA accumulation (Maccarrone et al., 1998). These characteristics suggest that the U937 cell (or its progenitor) plays a role in the rapid and efficient removal, degradation and recycling of AEA and is itself not a target of AEA.

3. Mechanism of AEA cellular transport

As was discussed above, several investigators have demonstrated that the cellular accumulation of AEA is saturable. This is one of several criteria of a protein carrier mediated transport process. Another key criteria of carrier-mediated processes is dependence on incubation temperature. The temperature dependence of AEA accumulation has been demonstrated in neurons (Di Marzo et al., 1994; Hillard et al., 1997), HUVECs (Maccarrone et al., 2000), RBL-2H3 cells (Bisogno et al., 1997; Rakhshan et al., 2000), human neuroblastoma cells (Maccarrone et al., 1998) and U937 lymphoma cells (Maccarrone et al., 1998).

The transport process for AEA in cerebellar granule cells has the characteristics of facilitated diffusion (Hillard et al., 1997). AEA accumulation is not dependent upon cellular ATP synthesis; AEA transport is not decreased significantly by the inhibition of neuronal metabolism by cyanide or iodoacetate (Hillard et al., 1997) or by the treatment of neuroblastoma cells with the mitochondrial inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Maccarrone et al., 1998). Analyses of the temperature dependence of the initial rates of AEA transport into cerebellar granule cells (Hillard et al., 1997), neuroblastoma cells (Maccarrone et al., 1998) and HUVECs (Maccarrone et al., 2000) give Q_{10} values of 1.4–1.6. These findings also suggest that AEA accumulation is driven by diffusion and is not an active, energy requiring process. Similar results have been reported recently for AEA accumulation by RBL-2H3 cells (Rakhshan et al., 2000).

AEA accumulation is not sodium dependent; neither substitution of sodium in the buffer with choline nor preincubation of cerebellar granule cells with ouabain, an inhibitor of Na^+/K^+ ATPase activity, affects AEA accumulation (Hillard et al., 1997). A similar lack of sodium dependence has also been reported in cortical astrocytes (Beltramo et al., 1997) and RBL-2H3 cells (Rakhshan et al., 2000). The lack of dependence of the AEA accumulation process on intracellular ATP synthesis or an intact sodium gradient makes this transport process fundamentally different from the catecholamine and amino acid transport processes.

For facilitated diffusion mediated processes, both the direction and the amount of solute movement are dependent upon the solute concentration gradient across the membrane. This suggests that AEA movement across cellular membranes should be bi-directional. Several experiments were carried out to test this prediction. In the first set of experiments, we demonstrated that time and temperature-dependent AEA efflux occurs when cerebellar granule cells were preloaded with radiolabeled AEA (Hillard et al., 1997). In a second set of experiments, we have demonstrated that the transcellular movement of AEA exhibits the *trans* effect of flux coupling. According to this concept, a protein carrier that moves solute in both directions across a membrane barrier must be capable of binding solute on both sides of the membrane. A high concentration of solute on one side of the membrane favors the accumulation of carrier molecules with their solute binding sites facing the opposite side of the membrane. As a consequence, a cell preloaded with solute will accumulate solute against its concentration gradient. We have demonstrated that the transport of AEA by cerebellar granule cells exhibits this characteristic. In this experiment, cells were preloaded with 100 μM AEA for 30 min. After washing, [^{3}H]AEA was added to the extracellular media and removed at the times indicated (Fig. 3). In spite of a large concentration gradient that should favor AEA movement out of the cell, a transient inward movement of radiolabeled AEA occurs. This data is consistent with a carrier that can bind solute at either side of the membrane and whose movement from one side of the membrane to the other is regulated by solute binding.

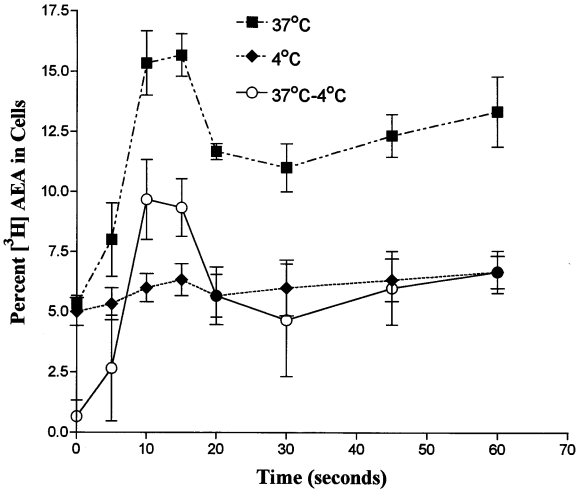

Fig. 3. The *trans* effect of the flux-coupling phenomena occurs with AEA transport. Cerebellar granule cells were preloaded with 100 μM AEA for 30 min at 4 or 37°C as noted. The buffer was replaced with fresh buffer containing 10 nCi [^{3}H]AEA, and then removed at the indicated time intervals. Cells were scraped into fresh buffer. Both the buffer and scraped cells were counted and the percent accumulation was calculated as (dpm in cells)/(dpm in cells + dpm in buffer)*100. Data are mean values of three experiments performed in triplicate.

Inhibitor studies have been used to provide evidence that AEA transport is protein mediated. The nonspecific alkylating agent phenylmethylsulfonylfluoride (PMSF) partially inhibits AEA accumulation in leukocytes (Bisogno et al., 1997), neuroblastoma cells (Maccarrone et al., 1998) and RBL-3H3 cells (Rakhshan et al., 2000). In neuroblastoma cells, PMSF acts as a noncompetitive inhibitor and these authors suggest that it may act by alkylating a critical cysteine residue in the carrier protein (Maccarrone et al., 1998). Phloretin, a nonspecific inhibitor of several membrane transport processes, inhibits AEA transport with noncompetitive kinetics in cerebellar granule cells (Hillard et al., 1997). Phloretin also inhibits AEA uptake into RBL-2H3 cells with an IC_{50} of 80 μM (Di Marzo et al., 1998a) but has been reported to have no effect on AEA transport in cortical astrocytes at a concentration of 50 μM (Beltramo et al., 1997). Bromcresol green, an inhibitor of PGE2 uptake, inhibits AEA accumulation by astrocytes and neurons with IC_{50} values of 12 and 4 μM, respectively (Beltramo et al., 1997) but has no effect on AEA accumulation by RBL-2H3 cells. *N*-ethylmaleimide (NEM; 100 μM) has been reported to inhibit AEA transport (Bisogno et al., 1997; Maccarrone et al., 1998). However, this data may be artifactual as we have found that this concentration of NEM also alkylates AEA itself and that the adduct is not accumulated by cells (unpublished data).

Several inhibitors that have been reported to be without effect on the cellular accumulation of AEA include: the FAAH inhibitors (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one and linoleyl trifluoromethyl ketone (Beltramo et al., 1997); inhibitors of phospholipid uptake verapamil and quinidine (Beltramo et al., 1997); and diisothiocyanostilbene, an inhibitor of the cellular accumulation of fatty acids (Hillard et al., 1997).

There is accumulating evidence that AEA accumulation is regulated by nitric oxide. Using CHP100 cells, U937 cells and HUVECs, Maccarrone and coworkers have demonstrated that nitric oxide donors, such as sodium nitroprusside (SNP), increase the V_{max} for the accumulation of AEA (Maccarrone et al., 1998, 2000). The enhancement is particularly striking in CHP100 neuroblastoma cells where treatment of the cells with 5 mM SNP results in a 22-fold increase in V_{max}. The authors suggest that NO binds to a critical cysteine in the carrier molecule to enhance activity. This is a very interesting potential point of interaction between the AEA/cannabinoid system and NO, which have both been implicated in long term potentiation in the hippocampus (Nowicky et al., 1987; Schuman and Madison, 1991; Terranova et al., 1995) and in long term depression in the cerebellum (Crepel and Jaillard, 1990; Levenes et al., 1998).

4. What is the driving force for AEA accumulation?

One intriguing feature of AEA accumulation by cerebellar granule cells is that accumulation of AEA reaches a steady state, but that the steady states does not occur when the concentrations of

AEA are equal on both sides of the cell membrane. The data in Fig. 4 illustrate this point; the accumulation of [^{3}H]AEA and [^{14}C]urea were measured simultaneously in cerebellar granule cells. Urea is freely diffusable through cellular membranes and is not concentrated within cells so its distribution within and without the cell represents a steady state. This equilibrium occurs when approximately 0.5% of the total urea added is within the cells. The calculated total volume of the cells is 25 nl. In contrast, the accumulation of AEA within the cells reaches equilibrium when nearly 30% of the added AEA is cell-associated. These data suggest that AEA within the cell is either metabolized which maintains the concentration gradient or it is removed to a compartment that is not free to equilibrate with the extracellular AEA pool. In cerebellar granule cells, it is likely that the second explanation is more important since these cells exhibit very little FAAH activity (Hillard et al., 1997). The same conclusion was reached by Piomelli and coworkers using primary astrocytes (Beltramo et al., 1997). The identity of this sequestration process or processes is not known.

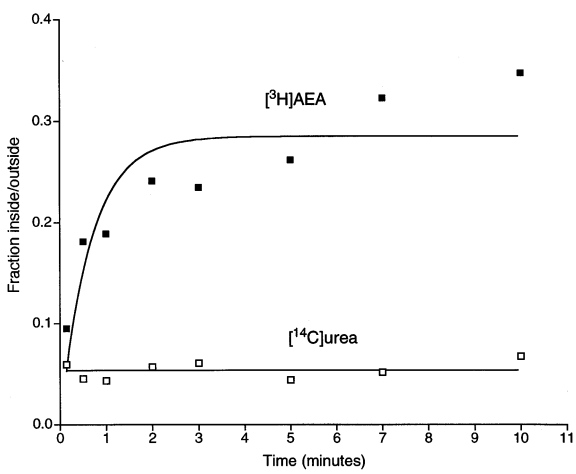

Fig. 4. AEA accumulation by cerebellar granule cells exceeds the distribution of urea. Cells were washed free of media in warm (37°C) KRH buffer. The buffer was then replaced with fresh buffer containing both 40 pM [^{3}H]AEA and 32 μM [^{14}C]urea and then removed at the indicated time intervals. Cells were scraped into fresh buffer. Both the buffer and scraped cells were counted and the fraction inside/outside was calculated as (dpm in cells)/(dpm in cells + dpm in buffer). Nonspecific association was accounted for by subtracting data obtained in a parallel incubation at 4°C. Data are mean values of three experiments performed in triplicate.

As was discussed above, it is likely in other cell types that the accumulation process and FAAH-mediated hydrolysis of AEA act in series to inactivate AEA. Deutsch and colleagues have recently reported that the FAAH inhibitor methylarachidonoyl fluorophosphate (MAFP) inhibits AEA accumulation by neuroblastoma cells (Deutsch et al., 1999). Furthermore, Maccarrone and colleagues have reported that the accumulation of AEA by CHP100 and U937 cells exhibits very similar affinities for AEA but the U937 cells take up AEA with a greater velocity (See Table 1). One explanation for this difference may be that the U937 cells have a much higher FAAH activity, which may drive AEA accumulation. Data obtained in RBL-2H3 cells, which have significant FAAH activity, suggest that FAAH-mediated hydrolysis may play a role in the accumulation of AEA, but that it is not completely responsible for the concentration of AEA in these cells (Rakhshan et al., 2000).

5. Structure activity relationships

Another key criterion for defining carrier-mediated transport is the demonstration of selectivity of the carrier binding site for structural analogs of the solute. Substrate selectivity for the accumulation process has been demonstrated in several cellular models and a series of AEA structural analogs have been synthesized that inhibit AEA accumulation.

The first set of analogs that have been investigated can be classified as other biologically active eicosanoids. None of these analogs compete with AEA for cellular accumulation. The analogs that have been investigated include PGE_2 and leukotriene B_4 (Beltramo et al., 1997; Maccarrone et al., 1998); leukotriene C_4 and thromboxane B_2 (Beltramo et al., 1997); and 5-HETE, 12-HETE, 15-HETE, 5,6 EET, and 8,9 EET (Piomelli et al., 1999). Although arachidonic acid does not affect AEA accumulation in neurons (Beltramo et al., 1997; Maccarrone et al., 1998), it does inhibit

AEA accumulation by RBL-2H3 cells (Rakhshan et al., 2000).

The second set of analogs includes ethanolamides of fatty acids other than arachidonic acid. The *N*-ethanolamine derivatives of long chain, saturated fatty acids, including palmitoyl (Beltramo et al., 1997; Bisogno et al., 1997; Hillard et al., 1997); stearoyl (Di Marzo et al., 1994; Piomelli et al., 1999) and arachidoyl (Di Marzo et al., 1994) do not compete for AEA accumulation. However, ethanolamides of unsaturated fatty acids of 18 carbons or more will compete for AEA accumulation (Hillard et al., 1997; Piomelli et al., 1999). The best inhibitor among this group of compounds is *N*-oleoylethanolamine, which has been shown in two laboratories to have a higher affinity for the carrier than AEA itself (Hillard et al., 1997; Piomelli et al., 1999).

Ethanolamides of arachidonic or stearic acid that have been modified along the backbone by the addition of hydroxyl groups or epoxides do not compete well for AEA accumulation (Hillard et al., 1997; Maccarrone et al., 1998). Similarly, an AEA analog with a *trans* double bond along the backbone does not bind well to the carrier (Piomelli et al., 1999). Similarly, Melck and coworkers demonstrated that the *trans* isomer of olvanil bound to the carrier with at least 5-fold lower affinity than olvanil itself which has a single double bond in the *cis* position (Melck et al., 1999). These studies suggest that the backbone length and position of the double bonds is less critical than the conformation of the acyl chain. The addition of hydroxyls and *trans* double bonds will cause kinks in the backbone that impose steric hindrance to binding.

The final group of analogs that have been tested as inhibitors of AEA transport are amides and esters of arachidonic acid with various head group substitutions for the ethanolamine of AEA (Beltramo et al., 1997; Hillard et al., 1997; Melck et al., 1999; Piomelli et al., 1999; Jarrahian et al., 2000; Muthian et al., 2000). While readers are referred to the original articles for the details of these analogs, some generalizations can be made from these studies. First, the AEA binding site can tolerate very bulky additions to the head group region, provided they are hydrophobic. Second, the carrier does not require a secondary amine in the carboxamido group. Third, the presence of an electron-donating group at the 2′ position can stabilize binding to the carrier but this interaction is very sensitive to the orientation and position of this group. Fourth, aromatic substitutions in the head group region stabilize binding to the carrier, possibly because of the introduction of aromatic stacking interactions.

These studies have resulted in the identification of three very promising lead compounds that inhibit the accumulation of AEA by the carrier. The first is 4-hydroxyphenylarachidonoylamide (also called AM404; Beltramo et al., 1997) and the second is 3-pyridinylarachidonoylamide (Jarrahian et al., 2000). Both of these analogs inhibit the binding of AEA to the carrier and AM404 has been shown to be accumulated by cells (Piomelli et al., 1999). Both analogs also inhibit the hydrolysis of AEA by AEA amidohydrolase so they cannot be used to demonstrate the relative roles of the transporter and AEA amidohydrolase in the inactivation of endogenous or exogenous AEA. The third lead compound is α-linolenoyl-vanillyl-amide (Melck et al., 1999; Maccarrone et al., 2000). This analog also binds to the AEA carrier in the low micromolar range but has the added advantage that it does not inhibit FAAH until concentrations are above 50 μM (Melck et al., 1999).

Another group of compounds, based upon the structure of capsaicin [*N*-(3-methoxy-4-hydroxy)-benzyl-8-methyl-6-*trans*-nonenamide], have been found to interact with the AEA carrier (Di Marzo et al., 1998a). Capsaicin is an agonist of the vanilloid receptor (VR1), a receptor present on sensory fibers that is hypothesized to transmit the sensation of heat (Caterina et al., 1997). Among the analogs of capsaicin that have been synthesized are derivatives in which the branched acyl chain has been replaced with straight chain fatty acids. One of these, olvanil (Δ^9-*cis* 18:1) is particularly interesting since it has similar pharmacological effects to capsaicin. In light of the structural similarity between olvanil and AM404, two groups have determined the effects of this compound on AEA accumulation by cells and find

similar degrees of inhibition (Di Marzo et al., 1998a; Beltramo and Piomelli, 1999; Melck et al., 1999). Di Marzo and coworkers have synthesized the arachidonate analog of olvanil (called arvanil) and report that it has a higher affinity for the AEA carrier than olvanil; in fact the IC_{50} of arvanil for is very similar to that of AM404 (Melck et al., 1999). These data support the conclusion drawn from other inhibitor studies that the substitution of the ethanolamine head group with an aromatic ring stabilizes binding to the carrier. The pharmacology of arvanil is complex; it mimics both cannabinoid and vanilloid receptor agonists (Melck et al., 1999).

The second putative endocannabinoid, 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995) competes with [^{3}H]AEA for transport into cerebellar granule cells with an IC_{50} of 13.3 μM (Jarrahian et al., 2000), into astrocytoma cells with an IC_{50} of 18.5 μM (Piomelli et al., 1999) and into RBL-2H3 cells with an IC_{50} of 27 μM (Rakhshan et al., 2000). However, data from macrophages (Di Marzo et al., 1999) and RBL-2H3 (Di Marzo et al., 1998b) cells suggest that while both 2-AG and AEA are accumulated by these cells, they do not compete with each other. Furthermore, it is not clear that the accumulation of 2-AG by macrophages is carrier mediated (Di Marzo et al., 1999); however, the accumulation of radiolabeled 2-AG by astrocytoma cells is time-dependent (Piomelli et al., 1999). Further studies must be done to investigate the role of a protein transporter in the inactivation of 2-AG.

6. Role of the carrier in the inactivation of AEA in vivo

Activation of the CB1 receptor results in inhibition of adenylyl cyclase activity (Howlett and Fleming, 1984). Like other CB1 receptor agonists, AEA inhibits forskolin-activated accumulation of cAMP in cortical neurons (Beltramo et al., 1997). This effect of AEA is potentiated by AM404 and bromcresol green, two inhibitors of AEA accumulation. This effect is likely due to inhibition of AEA accumulation rather than inhibition of AEA breakdown by AEA amidohydrolase because a positional isomer of AM404, 3-hydroxyphenylarachidonamide (AM403), does not potentiate AEA actions in this model. 3-Hydroxyphenylarachidonamide is very likely to be an inhibitor of AEA amidohydrolase since both the 2-hydroxy and 4-hydroxy isomers are potent inhibitors (Jarrahian et al., 2000).

In two studies carried out in animals, co-administration of AM404 with AEA was reported to potentiate the analgesic effects of AEA measured using the mouse hot plate test (Beltramo et al., 1997) and the hypotensive effects of AEA in anesthetized guinea pigs (Calignano et al., 1997). However, the uptake inactive isomer AM403 was not investigated in these studies. Therefore, it is possible that inhibition of AEA amidohydrolase by AM404 contributes to the potentiation of AEA effects seen in these studies.

7. Summary

Taken together, the evidence from multiple laboratories supports the hypothesis that AEA transport across cellular membranes occurs and likely involves a protein carrier molecule. The flux coupling data presented herein, in particular, suggest that the carrier can bind AEA or other solute molecules on either side of the membrane. Although inhibitor and other biochemical data support the concept that AEA transport across membranes is protein mediated, definitive evidence awaits its molecular characterization. In this regard, carriers for other lipid signaling molecules, including the prostaglandins (Kanai et al., 1995) have been cloned and may provide clues regarding the identity of the AEA transporter(s).

One major significance of these findings is that a selective mechanism exists for the removal of AEA from extraneuronal space which provides indirect support for the role of AEA as a signaling molecule. Secondly, specific and selective inhibitors of the carrier should prolong the action of AEA at the CB1 receptor and, thus, will be important pharmacological tools with which to study the endogenous cannabinoid system.

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