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MINI REVIEW

Cellular accumulation of anandamide: consensus and controversy

*,[1]Cecilia J. Hillard & [1,2]Abbas Jarrahian

[1]Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A

The endocannabinoids *N*-arachidonylethanolamine (AEA or anandamide) and 2-arachidonylglycerol (2-AG) are hypothesized to function in the brain as interneuronal signaling molecules. Prevailing models of the actions of these molecules require that they traverse cellular plasma membranes twice; first, following cellular synthesis and second, prior to enzymatic hydrolysis. The transmembrane movement of AEA has been studied in multiple laboratories with a primary focus on its cellular accumulation following extracellular administration. Although there are areas of consensus among laboratories regarding AEA accumulation, several aspects are very unclear. In particular, there is a lack of consensus in the literature regarding the importance of AEA hydrolysis by fatty acid amide hydrolase in maintaining the driving force for accumulation. Furthermore, evidence for and against a transmembrane carrier protein has been published. We have reviewed the available literature and present a working model of the processes that are involved in the cellular accumulation of AEA. It is our hypothesis that transmembrane movement of AEA is regulated by concentration gradient between extracellular and intracellular free AEA. Furthermore, it is our view that a significant portion of the intracellular AEA in most cells is sequestered either by a protein or lipid compartment and that AEA sequestered in this manner does not equilibrate directly with the extracellular pool. Finally, we discuss the available data that have been presented in support of a transmembrane carrier protein for AEA. *British Journal of Pharmacology* (2003) **140,** 802–808. doi:10.1038/sj.bjp.0705468

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Abbreviations: AA, arachidonic acid; AEA, *N*-arachidonylethanolamine; 2-AG, 2-arachidonylglycerol; 4-AP, 4-aminopyridine; BSA, bovine serum albumin; CB_1, cannabinoid receptor of type 1; CNS, central nervous system; E2, beta-estradiol; FAAH, fatty acid amide hydrolase; EA, ethanolamine; HUVECs, human umbilical vein endothelial cells; MAFP, methyl arachidonyl fluorophosphonate; PMSF, phenylmethylsulfonyl fluoride; siRNA, small interfering ribonucleic acid

Introduction

The psychoactive principle of *Cannabis sativa*, Δ^9-tetrahydrocannabinol, alters CNS function because it is a partial agonist of the G protein-coupled receptor, CB_1 (Matsuda *et al*., 1990). To date, at least four brain-derived compounds have been identified that bind to the CB_1 receptor and have been given the designation 'endocannabinoids'. The CB_1 receptor agonists are *N*-arachidonylethanolamine (Devane *et al*., 1992) (anandamide or AEA), 2-arachidonylglycerol (2-AG) (Sugiura *et al*., 1995), and noladin ether (Hanus *et al*., 2001); and the CB_1 receptor antagonist virodhamine (Porter *et al*., 2002). There is considerable experimental support for the hypothesis that AEA and 2-AG are signaling molecules in the brain, the other endocannabinoids have received less attention thus far. The content of AEA and 2-AG in brain tissue and in brain-derived cells in culture is regulated by both synthesis and inactivation (Hillard, 2000; Schmid, 2000; Sugiura & Waku, 2000). The syntheses of AEA and 2-AG result from stimulus-dependent release of the lipids from phospholipid precursors through regulatable signal transduction cascades. Inactivation of both AEA and 2-AG occurs *via* hydrolysis to arachidonic acid and ethanolamine or glycerol, respectively. Both endocannabinoids are substrates *in vitro* for fatty acid amide hydrolase (FAAH) (Ueda *et al*., 2000), but studies in FAAH knockout mice suggest that FAAH is not essential for 2-AG catabolism *in vivo* (Lichtman *et al*., 2002). Piomelli and coworkers have recently characterized monoacylglycerol lipase in brain and it is likely that this enzyme plays a pivotal role in the hydrolysis of 2-AG (Dinh *et al*., 2002).

One of the important functional roles of endocannabinoid-mediated signaling in the CNS is mediation of stimulus-dependent, retrograde inhibition (Freund *et al*., 2003). In this role, the endocannabinoid is synthesized by postsynaptic neurons, released into the extracellular space, and activates CB_1 receptors on presynaptic terminals. Since the hydrolytic enzymes that terminate the actions of AEA and 2-AG are intracellular (Dinh *et al*., 2002; Hillard *et al*., 1995), the endocannabinoids must re-enter a cell to be inactivated. Therefore, it is likely that an endocannabinoid must traverse cellular plasma membranes twice; once after synthesis and once prior to hydrolysis.

Endocannabinoid transmembrane movement has been most thoroughly studied for AEA, which will be the focus of this

*Author for Correspondence; E-mail: chillard@mcw.edu
[2]Current address: Department of Pharmaceutical Sciences, Butler University College of Pharmacy and Health Sciences, 4600 Sunset Avenue, Indianapolis, IN 46208, U.S.A.
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review. Both virodhamine (Porter *et al*., 2002) and noladin ether (Fezza *et al*., 2002) act as competitive inhibitors of AEA accumulation and therefore could share the same accumulation process. The issue of 2-AG accumulation appears to be more complicated; in a human astrocytoma cell line (CCF-STTG1), a single process appears to mediate accumulation of both AEA and 2-AG (Beltramo & Piomelli, 2000), while in C6 glioma cells, one process appears to accumulate both ligands while another is involved only in AEA accumulation (Bisogno *et al*., 2001).

AEA is rapidly accumulated within cells when it is added to the extracellular media. There are several characteristics of AEA cellular accumulation that have been demonstrated in multiple laboratories and can be considered as well supported (see Giuffrida *et al*. (2001) and Hillard & Jarrahian (2000) for comprehensive reviews). To summarize, the accumulation of AEA by cells is dependent upon its concentration gradient; is saturable by solute; inhibitable by a variety of structural analogs; temperature dependent; and does not require sodium or ATP. Among the multiple laboratories using an array of cellular models, there is very good agreement among the equilibrium constants determined for AEA accumulation. In addition, there is good agreement in the inhibitor profiles that have been published by different laboratories. These accumulated findings and more recent data discussed below are consistent with a hypothesis that AEA movement across cellular plasma membranes involves binding to a saturable cellular component. However, the identity of these component(s) is one of the enigmas in the field. It has been suggested that AEA accumulation occurs *via* a true carrier protein that binds and translocates AEA from one side of the plasma membrane to the other. Alternatively, others have suggested that AEA accumulation can be explained by FAAH-mediated hydrolysis following translocation of AEA through the membrane *via* simple diffusion. We propose in this review that a third process could be operative which involves intracellular sequestration of AEA either in lipid compartments or by binding to a protein. These three processes are summarized schematically in Figure 1. It is our view that the current literature is not sufficient to allow for a conclusion regarding the importance of one or the other of these three processes in the regulation of intracellular and, more importantly, extracellular AEA concentrations. We will begin by considering the fate of AEA once it has been accumulated by a cell and whether intracellular processes drive AEA accumulation. Then, we will consider the possible mechanisms by which AEA crosses the plasma membrane of cells. Finally, we will present what is currently known about the regulation of AEA transmembrane movement.

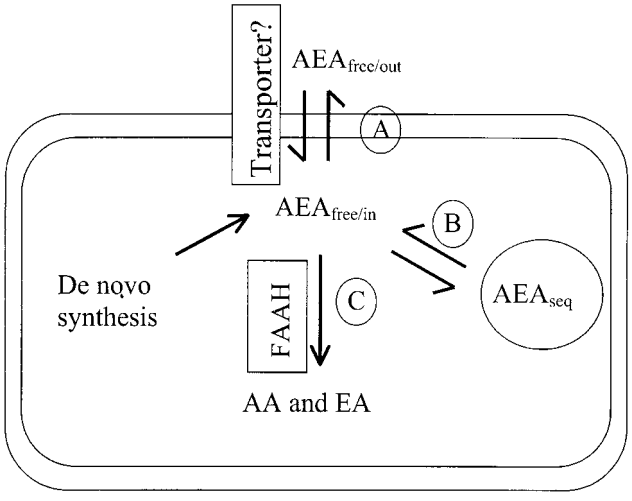

Figure 1 Model of AEA accumulation in cells. Free AEA concentrations are equal on both sides of the plasma membrane because AEA either freely diffuses across plasma membranes (a) or is translocated by a transporter in a process of facilitated diffusion. Once inside the cell, it is likely that a significant portion of the internalized AEA is sequestered (AEA_{seq}) and this sequestered pool is in equilibrium with $AEA_{free/in}$. The amount of $AEA_{free/in}$ is also influenced by *de novo* synthesis of AEA (b) and FAAH-mediated hydrolysis (c). AA denotes arachidonic acid; EA denotes ethanolamine.

Transmembrane movement of AEA is driven by the concentration gradient

When radiolabeled AEA is added to the extracellular media of cells (Deutsch & Chin, 1993), brain slices (Beltramo *et al*., 2000), or synaptosomes (Maccarrone *et al*., 2001), the radioisotope is accumulated within the intracellular compartment. The accumulation is not dependent upon ATP or sodium ion and is not inhibited by ouabain (Hillard *et al*., 1997). While AEA accumulation is temperature sensitive, the process exhibits a Q_{10} value less than 2 (Hillard *et al*., 1997; Maccarrone *et al*., 2000a), which is consistent with diffusion rather than an active transport process. Taken together, the available data support the conclusion that AEA accumulation is driven by its transmembrane concentration gradient.

There is some evidence in the literature that AEA can move in both directions across the plasma membrane and that the direction of movement is dependent upon its concentration gradient across the membrane. AEA efflux has been demonstrated to occur after preloading in cerebellar granule cells (Hillard *et al*., 1997), striatal neurons *in situ* (Gerdeman *et al*., 2002), and human umbilical vein endothelial cells (HUVECs) (Maccarrone *et al*., 2002). While static efflux following preloading is consistent with bidirectional movement, more extensive studies are required to differentiate between true efflux and solute exchange. Studies in our laboratory demonstrate that conditions shown to induce AEA synthesis in neurons reduce the accumulation of AEA added to the outside of the cells (Table 1). The effects of four agents that either depolarize neurons or increase intracellular calcium directly were studied: ionomycin, a calcium ionophore; KCl, which will clamp the membrane potential at a depolarized state; veratridine, a sodium channel activator; and 4-aminopyridine (4-AP), a potassium channel blocker. Ionomycin, 4-AP, and KCl all produced significant inhibition of AEA accumulation while depolarization with veratridine did not. The three active compounds were all shown by Di Marzo *et al*. (1994) to increase neuronal AEA content. These findings are consistent with bidirectional movement of AEA; when neurons synthesize AEA, intracellular AEA is increased and the driving force for the accumulation of AEA is reduced. However, an alternative explanation is that newly synthesized AEA is released and reduces the accumulation of radiolabeled AEA by competition.

Table 1 The accumulation of [^{3}H]AEA is reduced in cerebellar granule cells by treatments known to increase AEA synthesis

Treatment	*AEA accumulation (% of control ± s.e.m.)*
Ionomycin (300 nM)	44 ± 2
4-Aminopyridine (1 mM)	59 ± 10
KCl (35 mM)	80 ± 3
Veratridine (60 μM)	91 ± 6

Uptake was measured as described in Hillard *et al.* (1997). Rat cerebellar granule cells were preincubated with the compounds indicated for 5 min; [^{3}H]AEA accumulation was determined after 2 min incubation with radiolabeled AEA as the ratio of dpm in the cells/(cells + media). Each value is the mean of three separate experiments, except ionomycin which is the mean of 4. The control accumulation during 2 min was 0.23 ± 0.02.

AEA is sequestered by cells

In apparent contradiction to the hypothesis that AEA accumulation is dependent upon its transmembrane gradient, several laboratories using different cellular models have estimated that the concentration of radiolabeled AEA within cells at equilibrium is far greater than its concentration in the extracellular media. For example, we find that the concentration of AEA within cerebellar granule neurons approaches 200 nM and is, therefore, 1000-fold greater than its concentration in the extracellular media (Hillard & Jarrahian, 2000). Similarly, Rakhshan *et al.* (2000) estimate that the intracellular concentration of intact AEA exceeds 100 nM in RBL-2H3 cells incubated with 5 nM AEA. N18 neuroblastoma, C6 glioma and Hep2 cells also concentrate AEA and reach intracellular concentrations of 500–1700 nM (Deutsch *et al.*, 2001). Interestingly, the intracellular concentrations of AEA following equilibration with extracellular AEA do not vary by more than 15-fold. In sum, these data indicate that AEA concentrations inside and outside of the cell are not equal to each other at equilibrium. Since there is no evidence that AEA is accumulated against its concentration gradient by an active process, these data led to the conclusion that only a small fraction of the total intracellular, intact AEA is free and in equilibrium with extracellular AEA. The majority of accumulated AEA cannot be immediately exchanged with the extracellular pool; we refer to this AEA as sequestered or bound (Figure 1).

One can suggest two general mechanisms by which AEA could be sequestered. First, the lipid character of AEA suggests that it could associate with membranous compartments. In support of this mechanism, Barker and colleagues have presented a preliminary report that subcellular fractions containing lipid rafts and caveolae are enriched in tritium following exposure of RBL-2H3 cells to tritiated AEA (McFarland *et al.*, 2003). A second possibility it that AEA is bound by an intracellular protein. There are several intracellular proteins that function to shuttle fatty acids, cholesterol and other hydrophobic molecules among intracellular compartments (Stremmel *et al.*, 2001). In theory, binding of AEA to an intracellular protein would exhibit saturability and competition from molecules that also bound to the protein. In addition, AEA association with an intracellular binding protein would also be consistent with an accumulation of AEA beyond its concentration in the extracellular environment.

If AEA binds in a reversible manner to an intracellular site that is a protein with limited capacity and the ability to discriminate among potential ligands, then this sequestration process could underlie AEA accumulation by cells. In other words, the characteristics of AEA uptake described above (particularly saturability and inhibition by similar molecules) could be due to AEA binding to an intracellular protein and not a transmembrane carrier. Furthermore, if this intracellular protein were ubiquitous, it would explain why so many diverse cell types accumulate AEA with similar equilibrium constants. Clearly, more experiments are needed to clarify the mechanism(s) involved in the intracellular sequestration of AEA that has been observed.

Role of FAAH-mediated catabolism in the cellular accumulation of AEA

As discussed above, it is likely that AEA movement from the outside to the inside of the cell is driven by the difference in concentration between *free* AEA concentrations on each side of the membrane. Many cells that have been used to study the accumulation of AEA express the AEA hydrolyzing enzyme, FAAH. FAAH is an intracellular enzyme (Ueda *et al.*, 2000) and its hydrolysis of intracellular AEA could maintain intracellular concentrations of free AEA at a low level and thereby drive the movement of AEA from the outside to the inside of the cell (Figure 1). Indeed, recent studies in N18 neuroblastoma cells demonstrate that at incubation times of 5 min, the substrate dependence of FAAH and of AEA uptake are very similar. This led to the suggestion by these authors that the kinetics of FAAH-mediated hydrolysis of AEA accounts for the saturability of AEA uptake at long incubation times (Glaser *et al.*, 2003).

If FAAH is an important regulator of intracellular free AEA, one would predict that the expression of FAAH in a previously FAAH-null cell would enhance accumulation of radioisotope when the cells are incubated with radiolabeled AEA. Deutsch *et al.* (2001) have shown that transfection of Hep2 cells, which have no measurable endogenous FAAH activity, with FAAH resulted in a doubling of radioisotope accumulation compared to the wild-type cell. Similarly, Barker and colleagues have demonstrated that transfection of FAAH-null HeLa cells with FAAH results in a two-fold increase in radioisotope accumulation (Day *et al.*, 2001). The enhancement in AEA uptake required the hydrolytic activity of FAAH; a catalytically inactive FAAH mutant was ineffective and a FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) blocked the effect of FAAH transfection with an IC_{50} value of 7.6 μm (Day *et al.*, 2001). These studies demonstrate that the introduction of FAAH to a cell can result in an enhancement of AEA uptake from the extracellular media and are in agreement with the hypothesis that the transmembrane concentration gradient of AEA is the driving force for its uptake into cells.

While the introduction of FAAH into a cell demonstrates that the uptake of AEA *can* be regulated by FAAH, it does not speak to the question of the requirement for FAAH-mediated hydrolysis in AEA accumulation. In fact, several studies demonstrate that FAAH-mediated AEA hydrolysis is *not*

required for the transmembrane movement or cellular accumulation of AEA. For example, FAAH-null cells (Day *et al*., 2001; Glaser *et al*., 2003) and cells with very low FAAH activity, including cerebellar granule neurons (Hillard *et al*., 1997) and mast cells (Maccarrone *et al*., 2000c), exhibit robust and saturable accumulation of AEA. In those cells expressing FAAH, its role in AEA accumulation has been studied using several FAAH inhibitors. The nonselective FAAH inhibitor PMSF has been shown to reduce AEA accumulation in human neuroblastoma (CHP100), lymphoma (U937), J774 macrophages, RBL-2H3 basophils, and FAAH-transfected HeLa cells, but does not affect accumulation in FAAH null HeLa cells (Bisogno *et al*., 1997; Maccarrone *et al*., 1998; Day *et al*., 2001). The inhibition produced by PMSF never exceeds 50–60% suggesting that FAAH is not sufficient for AEA accumulation to occur. A second inhibitor of FAAH, methyl arachidonyl fluorophosphonate (MAFP), also produces a partial (40–50%) reduction of AEA accumulation in several cell lines (Rakhshan *et al*., 2000; Day *et al*., 2001; Deutsch *et al*., 2001). However, the concentrations of MAFP required are 100–1000-fold higher than needed to inhibit FAAH (Deutsch *et al*., 1997). In addition, similar concentrations of MAFP also inhibit AEA accumulation in cell lines that do not express FAAH (Day *et al*, 2001; Glaser *et al*., 2003), suggesting that MAFP can inhibit AEA accumulation *via* a non-FAAH mechanism. Finally, FAAH inhibitors do not reduce the accumulation of AEA in astrocytes (Beltramo *et al*., 1997) or cortical neurons (Kathuria *et al*., 2003) in spite of the fact that these cells have FAAH activity.

In summary, the addition of FAAH to a cell can enhance the uptake of AEA, but its complete inhibition results in, at best, partial inhibition of AEA uptake and, in some cellular models, has no significant effect on uptake. Therefore, we conclude that FAAH-mediated hydrolysis alone is not sufficient to explain the uptake of AEA by cells. The variability in the effects of FAAH inhibition on the accumulation of AEA suggests that the contribution of FAAH to the regulation of free, intracellular AEA is cell-specific. In some cells, AEA uptake and FAAH-mediated hydrolysis are more tightly coupled than in other cells. In our model, we have included FAAH-mediated hydrolysis as one mechanism by which the free intracellular AEA concentration can be regulated (Figure 1).

Is there a protein carrier that is responsible for AEA movement across membranes?

In a recent study, Deutsch and coworkers reported that AEA accumulation by N18TG2 neuroblastoma and C6 glioma cell lines is saturable with substrate at long (i. e. 5 min) but not very short (i.e. 25 s) incubation times (Glaser *et al*., 2003). Based on these data and the assumption that a carrier-driven process would saturate at short incubation times, the investigators suggest that AEA accumulation does not require a carrier. They hypothesize that cellular entry occurs *via* a nonsaturable process such as simple diffusion and that accumulation is driven by an intracellular process that is saturable, such as FAAH-mediated hydrolysis. Unfortunately, cell lines rather than primary cultures of neurons were utilized for these studies so they do not address accumulation in the cells that have been demonstrated to exhibit the *trans* effects of flux coupling (see below). In addition, bovine serum albumin (BSA; 0.4%) was included in the incubation media during the uptake period, which is not done in other laboratories (Beltramo *et al*., 1997; Bisogno *et al*., 1997; Hillard *et al*., 1997; Maccarrone *et al*., 1998; Rakhshan *et al*., 2000). In fact, Di Marzo *et al*. (1994) demonstrated in a very early study that 0.5% BSA completely inhibited the uptake of AEA by cortical neurons likely because BSA has a high capacity for binding to molecules with fatty acyl chains. Therefore, it is likely that the assay conditions and cells chosen for this study (Glaser *et al*., 2003) have precluded AEA entry into cells *via* a carrier, if one exists, and favor AEA accumulation *via* other processes.

That being said, the study of Deutsch and colleagues does support the hypothesis that AEA can enter cells *via* noncarrier-mediated process(es). Several other observations are consistent with this hypothesis. First, many diverse cell types accumulate AEA so the accumulation function does not exhibit the cellular selectivity of expression one would expect for a specific carrier. Second, protein inactivating agents and membrane-impermeable proteases do not affect AEA accumulation in RBL-2H3 cells, suggesting either that a plasma membrane protein is not involved or that the protein is embedded in the hydrophobic core of the plasma membrane and therefore inaccessible to these reagents (Rakhshan *et al*., 2000). Third, AEA should be sufficiently lipophilic to partition from the aqueous phase into the membrane bilayer without the aid of a protein carrier.

On the other hand, there are studies that cannot be easily explained by noncarrier mechanisms. First, two laboratories (Gerdeman *et al*., 2002; Maccarrone *et al*., 2002) have demonstrated that AEA efflux is inhibited by AM404, an inhibitor of AEA accumulation and presumptive inhibitor of the AEA carrier (Beltramo *et al*., 1997). If AM404 was functioning as either an inhibitor of FAAH-mediated AEA hydrolysis or as a competitor for AEA intracellular sequestration, it would increase AEA efflux rather than inhibit. These data are consistent with a site of action of AM404 on a membrane carrier and thereby also support the presence of a carrier.

One hallmark of carrier-dependent movement of solute across a membrane is the *trans* effect of flux coupling. The *trans* effect is based upon two characteristics of bidirectional carriers: they have a single binding site for solute that can face either side of the membrane and the rate of movement of the binding site from one side of the membrane to the other is accelerated by solute binding. Therefore, high concentrations of solute on the *cis* side of the membrane favors the accumulation of carrier molecules with their binding sites facing the *trans* side of the membrane. Therefore, solute will move against its concentration gradient utilizing the accumulated carriers. We have demonstrated that this phenomenon occurs for AEA in cerebellar granule neurons (Hillard & Jarrahian, 2000) and another group has reported that the phenomenon occurs in endothelial cells but did not show the data (Maccarrone *et al*., 2002). The *trans* effect of flux coupling is not consistent with AEA accumulation in these cells by any process other than a bidirectional carrier.

Therefore, it is our current interpretation of the available data that AEA accumulation by cells occurs *via* several mechanisms. The best evidence for a plasma membrane protein carrier is found in neurons and endothelial cells; which is consistent with a role for these cells in the synthesis and/or inactivation of AEA as a signaling molecule. The

accumulation of AEA by other cells could occur by a process that does not require a transmembrane carrier but perhaps a membrane-associated binding protein as has been suggested for the intracellular accumulation of fatty acids (Stremmel *et al*., 2001). This process involves delivery of the lipid solute to the cell as complexes with albumin or other extracellular proteins, dissociation from albumin and binding to the plasma membrane protein, movement from the outer to inner leaflet of the membrane bilayer by simple diffusion or flip-flop, and association with an intracellular binding protein at the cytosolic surface of the plasma membrane. Alternatively, since AEA is uncharged and hydrophobic it likely can traverse membrane bilayers by simple diffusion, although the kinetics of its translocation across bilayers is not available in the literature.

Selectivity of AEA cellular accumulation inhibitors

Many compounds have been synthesized and screened for their ability to inhibit AEA accumulation in cells (Beltramo *et al*., 1997; Di Marzo *et al*., 1998; 2002; Piomelli *et al*., 1999; De Petrocellis *et al*., 2000; Jarrahian *et al*., 2000; Muthian *et al*., 2000; Lopez-Rodriguez *et al*., 2003a, b). The effects of many of these compounds on FAAH activity, CB receptor binding, and vanilloid receptor binding have also been determined. Overall, it has been difficult to create arachidonate analogs that are reasonably potent inhibitors of AEA accumulation that do not also bind FAAH and/or the vanilloid receptor at similar concentrations (Di Marzo *et al*., 1998; 2002; De Petrocellis *et al*., 2000; Jarrahian *et al*., 2000). However, the available inhibitor data do not support the conclusion of Glaser *et al*. (2003) that all uptake inhibitors are also FAAH inhibitors. For example, Lopez-Rodriguez *et al*. (2003b) have synthesized a novel series of arachidonates and find that there is no obvious correlation between inhibition of AEA accumulation and inhibition of FAAH in this series. One analog in particular, UCM707, is a potent inhibitor of AEA accumulation with low affinities for both the vanilloid VR1 receptor and FAAH. Other examples of inhibitors of accumulation that do not inhibit FAAH include O-2109 (Di Marzo *et al*., 2002) and the pyrene carboxylic ester of arachidonic acid (Jarrahian *et al*., 2000). Conversely, there are several inhibitors of FAAH that do not inhibit AEA accumulation (discussed above). Interestingly, these compounds are not arachidonate derivatives; by analogy useful AEA uptake inhibitors could come from nonarachidonate chemical classes.

One AEA uptake inhibitor, AM404, has been used to study the role of AEA uptake in the inactivation of exogenously delivered AEA (Beltramo *et al*., 1997; Calignano *et al*., 1997) and to infer a role for endogenous AEA in physiological effects (Gonzalez *et al*., 1999; Beltramo *et al*., 2000). However, AM404 is not selective for uptake inhibition; it is a substrate for FAAH and therefore can inhibit AEA hydrolysis *in vitro* (Jarrahian *et al*., 2000) and is a full agonist of the VR1 receptor in the same concentration range (Zygmunt *et al*., 2000). *In vivo*, AM404 results in an increase in circulating levels of AEA but not palmitoylethanolamide, which is also a substrate for FAAH (Giuffrida *et al*., 2000) and produces behavioral effects that can be inhibited by the CB1 receptor antagonist SR141716 (Beltramo *et al*., 2000a). While these studies suggest that AM404 acts as an inhibitor of AEA accumulation *in vivo* without a significant effect on FAAH or the VR1 receptor, its lack of specificity *in vitro* points to a need for further development of inhibitors of AEA uptake.

There are at least three protein entities that could theoretically be involved in the accumulation of AEA by cells: a membrane carrier; an intracellular binding protein for AEA; and FAAH. The only one of these entities that has been identified molecularly is FAAH (Cravatt *et al*., 1996). The other two may or may not exist in the cells under study and a compound that is found to inhibit AEA accumulation could be acting on any one. Therefore, the site of action of compounds that inhibit AEA accumulation will not be completely clear until the proteins involved in AEA accumulation are better understood and, ideally, molecularly identified.

Regulation of cellular AEA accumulation

Discussed above are the data that AEA accumulation can be regulated by processes that alter its transmembrane concentration gradient. In addition, data are beginning to emerge that suggest the transmembrane movement of AEA is regulated by changes in the cellular environment and/or cellular signal transduction. Recent work suggests that the transmembrane movement of AEA in cerebellar granule cells is affected by acute and chronic exposure of the cells to ethanol (Basavarajappa *et al*., 2003). Interestingly, chronic ethanol exposure resulted in an increase in AEA synthesis, an increase in the proportion of the synthesized AEA released into the media, and a decrease in AEA uptake. FAAH activity was not affected in cell lysates but hydrolysis of AEA in cells was reduced, which is consistent with reduced AEA uptake.

A series of studies by Maccarrone and colleagues suggest that nitric oxide and/or peroxynitrite significantly increase the accumulation of AEA in human endothelial cells (Maccarrone *et al*., 2000a), human mast cells (Maccarrone *et al*., 2000b), a human lymphoma cell line U937 (Maccarrone *et al*., 1998), C6 glioma (Bisogno *et al*., 2001), and rat synaptosomes (Maccarrone *et al*., 2001). While the mechanism for this effect is not clear, the effects of NO donors can be abolished by both extracellular superoxide dismutase and intracellular glutathione (Maccarrone *et al*., 2000a). Since AEA accumulation is reduced in the same system by PMSF, the authors suggest that the site of action is a cysteine residue (Maccarrone *et al*., 1998).

The exposure of endothelial cells to nanomolar concentrations of beta-estradiol (E2) results in a complex array of changes in AEA handling by these cells (Maccarrone *et al*., 2002). In particular, a short-term exposure of HUVECs to E2 results in a six-fold increase in AEA release and a four-fold increase in AEA uptake. A variety of pharmacologic interventions affected both processes in an identical manner, which led these investigators to conclude that the same carrier was responsible for both functions (i.e. AEA release and uptake). These data are very interesting and have important implications for the cardiovascular effects of endocannabinoids and estrogen.

The regulation of AEA accumulation has also been studied in whole animals. Gubellini *et al*. (2002) have demonstrated that the apparent V_{max} for AEA accumulation and FAAH activity was reduced in a parallel manner in synaptosomes

isolated from the striata of rats lesioned with 6-hydroxydopamine. The mechanism for this effect is not clear; however, we have found that dopaminergic terminals contain a large amount of FAAH, so it is possible that the loss of AEA accumulation and FAAH reflects that selective loss of dopaminergic terminals and that AEA accumulation is normal in the terminals that remain.

Summary

In summary, a model of AEA transcellular accumulation is shown in Figure 1. We hypothesize that AEA moves across cellular membranes until equilibrium between free concentrations is reached between the outside and inside of the cell. Based upon the available data, we hypothesize that AEA can freely diffuse across membranes in all cells and that some cells also express a carrier protein that facilitates this movement. We also hypothesize that AEA is sequestered within cells by as yet unknown mechanisms so that the free concentration of intracellular AEA is held at a low level. FAAH can hydrolyze free AEA and its contribution to the regulation of free AEA concentrations, and, hence, the uptake of AEA, varies by cell type. When AEA is synthesized *de novo*, the intracellular free AEA concentration increases, which favors AEA movement from inside to outside and can be seen experimentally as a decrease in the influx of AEA (Table 1).

There is a critical need for the molecular identification of the proteins that are involved in the movement of AEA across membranes and among cellular compartments. Once this has occurred, many of the ambiguities and controversies regarding AEA cellular accumulation and release will be clarified. Based upon the data available, it is our hypothesis that multiple mechanisms could serve to accumulate AEA. Arachidonic acid is a rare and highly conserved lipid and it is likely that all mammalian cells will use 'nonselective' processes to scavenge it from molecules like AEA and 2-AG. On the other hand, it is plausible that neurons and endothelial cells, which utilize endocannabinoids as signaling molecules, would have a specific process for the movement of these molecules across membranes.

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