Synthesis and Characterization of a Fluorescent Substrate for the $N$-Arachidonoylethanolamine (Anandamide) Transmembrane Carrier$^1$

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ABSTRACT

$N$-Arachidonoylethanolamine (AEA) is a proposed endogenous ligand of the central cannabinoid receptor (CB1). Previous studies indicate that AEA is translocated across membranes via a process that has the characteristics of carrier-mediated facilitated diffusion. To date, studies of this mechanism have relied on [$3H$]AEA as that has the characteristics of carrier-mediated facilitated diffusion. In the present study, we have synthesized an analog of AEA, SKM 4-45-1, that is nonfluorescent in the extracellular environment. When SKM 4-45-1 is exposed to intracellular esterases, it is de-esterified and becomes fluorescent. We have carried out studies to demonstrate that SKM 4-45-1 accumulation in cells occurs via the AEA carrier. SKM 4-45-1 is accumulated by both cerebellar granule cells and C6 glioma cells. Uptake of SKM 4-45-1 into C6 glioma is inhibited by AEA (IC$_{50}$=53.8 ± 1.8 $\mu$M), arachidonoyl-3-aminopyridine amide (IC$_{50}$=10.1 ± 1.4 $\mu$M), and arachidonoyl-4-hydroxyaniline amide (IC$_{50}$=6.1 ± 1.3 $\mu$M), all of which also inhibit [$3H$]AEA accumulation. Conversely, [$3H$]AEA accumulation by cerebellar granule cells is inhibited by SKM 4-45-1 with an IC$_{50}$ of 7.8 ± 1.3 $\mu$M. SKM 4-45-1 is neither a substrate nor inhibitor of fatty acid amide hydrolase, an enzyme that catabolizes AEA. SKM 4-45-1 does not bind the CB1 cannabinoid receptor at concentrations <10 $\mu$M. In summary, the cellular accumulation of SKM 4-45-1 occurs via the same pathway as AEA uptake and provides an alternative substrate for the study of this important cellular process.

$N$-Arachidonoylethanolamine (AEA) was isolated from porcine brain and has been postulated to be an endogenous ligand of the central cannabinoid receptor (CB1) (Devane et al., 1992). AEA administration to animals produces the physiological effects characteristic of the classical cannabinoids, including the tetrad of hypothermia, analgesia, catalepsy, and decreased locomotion (Fride and Mechoulam, 1993). Biochemical effects of AEA are similar to those of other CB1 agonists, including inhibition of adenylyl cyclase activity (Fride and Mechoulam, 1993). Biochemical effects of AEA are similar to those of other CB1 agonists, including inhibition of adenylyl cyclase activity (Fride and Mechoulam, 1993). Inhibition of voltage-operated calcium channels (Mackie et al., 1993) and inhibition of voltage-operated calcium channels (Mackie et al., 1993) by cerebellar granule cells is inhibited by SKM 4-45-1 with an IC$_{50}$ previously to be 41 ± 15 $\mu$M and 0.61 ± 0.04 nmol/min/10$^6$ cells, respectively (Hillard et al., 1997). AEA uptake also is inhibited competitively by several AEA analogs, including benzylarachidonamide (Hillard et al., 1997) arachidonoyl-3-aminopyridine amide (A3AP (Muthian et al., 1998), and arachidonoyl-4-hydroxyaniline amide (AM404) (Beltramo et al., 1997; Calignano et al., 1998) but not by arachidonic acid or N-palmitoylethanolamine (PEA) (DiMarzo et al., 1994). Cellular uptake of AEA has been demonstrated in a lymphoma cell line (Maccarone et al., 1998) and neuroblastoma cells (Deutsch and Chin, 1993; Maccarone et al., 1998). Although the biochemical evidence supports a carrier protein-mediated uptake of AEA, many questions remain regarding this process; including the identification of the carrier protein itself and whether AEA uptake is regulated by other signaling events in the cell.

Studies of the uptake carrier in the past have relied exclusively on the use of radiolabeled AEA. The aim of the present

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ABBREVIATIONS: AEA, $N$-arachidonoylethanolamine; CB1, central cannabinoid receptor; A3AP, arachidonoyl-3-aminopyridine amide; AM404, arachidonoyl-4-hydroxyaniline amide; PEA, N-palmitoylethanolamine; CGC, cerebellar granule cell; FAAH, fatty acid amide hydrolase; LCFA, long-chain fatty acid.
Materials and Methods

Preparation of Cerebellar Granule Cells (CGCs) and C6 Glioma Cells. Cell experiments were carried out with CGCs or C6 glioma cells. CGCs were cultured from 6- to 8-day-old Sprague-Dawley rat pups as described in Hillard et al. (1997). Cells were maintained in basal minimal Eagle’s medium (Life Technologies, Gaithesburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 5 mM glutamine, 25 mM KCl, 0.1 mg/ml gentamicin, and 0.01 mg/ml ampicillin. Cells were grown at 37°C in 5% CO2.

SKM 4-45-1 Synthesis. SKM 4-45-1 was synthesized from AEA (Cayman Chemicals, Ann Arbor, MI) and fluorescein-5-carbonyl azide diacetate (Molecular Probes, Eugene, OR). AEA in ethanol was (Cayman Chemicals, Ann Arbor, MI) and fluorescein-5-carbonyl azide diacetate was introduced into the vial containing dried under nitrogen and resuspended in anhydrous toluene. Excess azide diacetate has fluorescence only after esterase activation to form stable urethanes (Takadate et al., 1984). Shown in Fig. 1 is the structure of SKM 4-45-1, the resulting product of AEA reaction with fluorescein-5-carbonyl azide diacetate.

Esterase Modification and Fluorescence. Fluorescein diacetates have fluorescence only after esterase activation to form stable urethanes (Takadate et al., 1984). Shown in Fig. 1 is the structure of SKM 4-45-1, the resulting product of AEA reaction with fluorescein-5-carbonyl azide diacetate.

Results

Synthesis. SKM 4-45-1 was synthesized from AEA and fluorescein-5-carbonyl azide diacetate under anhydrous conditions at 80°C. The heat activation of carbonyl azide converts it to an isocyanate, which readily reacts with alcohols to form stable urethanes (Takadate et al., 1984). Shown in Fig. 1 is the structure of SKM 4-45-1, the resulting product of AEA reaction with fluorescein-5-carbonyl azide diacetate.

Esterase Modification and Fluorescence. Fluorescein diacetates have fluorescence only after esterase activation (Rothman and Papernaster, 1966; Jones and Senft, 1985). Therefore, we tested the fluorescence activation of SKM 4-45-1 by C6 glioma lysates. SKM 4-45-1 in buffer alone did not show any fluorescence (Fig. 2). However, when SKM
4-45-1 (100 nM) was incubated with cell lysates, fluorescence intensity increased in proportion to the volume of cell lysate added. In all incubations, the fluorescence intensity was time-dependent.

**Cellular Uptake of SKM 4-45-1.** SKM 4-45-1 is taken up into CGCs (Fig. 3). Shown is a photograph of an experiment where CGCs were incubated with 100 nM SKM 4-45-1 for 20 min. SKM 4-45-1 uptake occurs in CGCs as confirmed by fluorescence. Parallel experiments were carried out in the presence of either 100 μM AEA or PEA. As expected, AEA inhibited SKM 4-45-1 uptake, whereas PEA had no effect.

**SKM 4-45-1 Accumulation Occurs Via AEA Uptake Carrier.** AEA is accumulated by CGCs by an uptake mechanism that has the characteristics of facilitated diffusion (Hillard et al., 1997). Several approaches were used to determine whether SKM 4-45-1 was accumulated via the AEA transmembrane carrier.

First, SKM 4-45-1 inhibits [3H]AEA accumulation in CGCs with an IC<sub>50</sub> of 7.8 ± 1.3 μM (Fig. 4). This compares favorably with other inhibitors of AEA accumulation in CGCs, including AM404 (IC<sub>50</sub> = 3.4 ± 1.2 μM) and A3AP (IC<sub>50</sub> = 4.8 ± 1.1 μM) (Muthian et al., 1998).

AEA inhibits the accumulation of SKM 4-45-1 (100 nM) by C6 glioma cells with an IC<sub>50</sub> value of 53.8 ± 1.8 μM (Fig. 5). A3AP and AM404 also inhibit SKM 4-45-1 accumulation (100 nM) into C6 glioma cells with IC<sub>50</sub> values of 10.1 ± 1.4 and 6.1 ± 1.3 μM, respectively (Fig. 5). A comparison of the IC<sub>50</sub> values of the inhibitors used against 40 pM [3H]AEA and 100 nM SKM 4-45-1 accumulation is shown in Table 1. Collectively, these data suggest that SKM 4-45-1 accumulation occurs via the AEA transmembrane carrier, SKM 4-45-1 inhibits [3H]AEA accumulation in CGCs, and its uptake is sensitive to inhibitors of AEA uptake.

**CB1 Binding and FAAH Activity.** AEA binds to the CB1 cannabinoid receptor in the brain with high affinity (Devane et al., 1992) and it is catabolized by FAAH (Deutsch and Chin, 1993; Cravatt et al., 1996). The possibility that SKM 4-45-1 is also a ligand for the CB1 receptor and either a competitor or substrate for FAAH was explored. At concentrations <3 μM, SKM 4-45-1 has no effect on the binding of [3H]CP55940, a high-affinity cannabinoid ligand, to rat brain membranes (Fig. 6). In contrast, AEA displaced [3H]CP55940 binding with a K<sub>i</sub> of 144 nM.

FAAH activity was assessed in rat brain membranes with
[14C]AEA in the presence of AEA or SKM 4-45-1 (Fig. 7). As expected, AEA inhibited the catabolism of [14C]AEA with an IC50 value of 300 nM. However, SKM 4-45-1 had no effect on the catabolism of [14C]AEA at or below 10 μM. These data indicate that SKM 4-45-1 is neither a competitor nor a substrate for FAAH.

Discussion

These results demonstrate that SKM 4-45-1 is a substrate for the AEA transmembrane carrier and its presence in cells can be detected by fluorescence. We have demonstrated that SKM 4-45-1 is nonfluorescent extracellularly; its fluorescence is dependent on cellular uptake and nonspecific esterase cleavage of the diacetates. SKM 4-45-1 inhibits the cellular accumulation of [3H]AEA and conversely, the uptake of SKM 4-45-1 into C6 glioma cells is inhibited by AEA. In addition, SKM 4-45-1 uptake into C6 glioma is inhibited by analogs of AEA with IC50 values similar to the IC50 values for their inhibition of [3H]AEA uptake. Similar to [3H]AEA accumulation, SKM 4-45-1 uptake into CGCs is inhibited by
AEA but not by PEA. Moreover, SKM 4-45-1 does not bind the cannabinoid CB1 receptor, and it is not a substrate or competitor for FAAH. Therefore, our data demonstrate that SKM 4-45-1 is a substrate for the AEA carrier and should be a useful molecule for the investigations of the AEA uptake mechanism.

Our previous study indicated that AEA transport in CGCs is via a protein-mediated facilitated diffusion that is bidirectional (Hillard et al., 1997). The bidirectionality of AEA transport raises the interesting possibility of the presence of endogenous intracellular AEA that could affect SKM 4-45-1 transport into cells. However, we have been unable to measure endogenous AEA in cells due to its low amounts; therefore, we do not expect endogenous AEA to influence the transport of SKM 4-45-1 into cells under our experimental conditions. Furthermore, we have previously determined that AEA is concentrated inside cells beyond its concentration gradient (unpublished data), therefore, any undetectable presence of endogenous AEA may be a negligible factor with regard to SKM 4-45-1 transport into cells. The mechanism for this intracellular accumulation of AEA is yet unknown.

In the last several years, many laboratories have provided evidence for AEA as an endogenous neurotransmitter or neu...
romodulator. AEA is a natural constituent of brain that is biologically active (Devane et al., 1992) and is synthesized and released from neurons in a calcium-dependent manner (DiMarzo et al., 1994). Administration of AEA to animals produces physiological effects that are characteristic of the cannabinoids (Fride and Mechoulam, 1993). The biochemical effects of AEA administration include inhibition of adenyl cyclase activity (Felder et al., 1993; Vogel et al., 1993) and the inhibition of the opening of voltage-operated calcium channels (Mackie et al., 1993). However, an important criterion that must be met by any neurotransmitter/neurotransmitter is that there must exist a mechanism to terminate its activity. There are two documented mechanisms of AEA inactivation act in series, such that AEA from the extracellular milieu is taken up into the cells to be catabolized by intracellular FAAH.

Transport of polar molecules across cell membranes have been well studied and characterized. Lipid molecule transport however, has been more difficult to study. It has been hypothesized that lipid transport into cells occurs by simple diffusion: adsorption to cell membrane, transmembrane movement, and desorption into the cytosol (Hamilton, 1998). Recent studies however, have identified and cloned protein carriers for lipids, including carriers for the prostaglandins (Kanai et al., 1995) and long-chain fatty acids (LCFAs) (Schaffer and Lodish, 1994). Therefore, protein carriers exist and function to transport lipid molecules across cell membranes. The availability of a fluorescent substrate for the AEA carrier may aid in its molecular characterization and identification.

The purpose of our study was to design a tool with which to study the AEA uptake mechanism. Similar tools have been used by others to study various transport processes, including the characterization of the kinetics of nucleotide transport into synaptic vesicles with a fluorescent analog of ATP (Gualix et al., 1999) and excretion transport with a fluorescently labeled version of the anthelmintic drug ivermectin (Fricker et al., 1999). In another study, Schaffer and Lodish (1994) used a fluorescent analog of LCFAs to measure uptake and ultimately clone the LCFA transporter by an elegant functional assay. Therefore, there are several potential uses for a fluorescent substrate in the study of transport mechanisms.

In this report, we describe the synthesis and characterization of an analog of AEA, SKM 4-45-1, that is transported into the cell by the same mechanism that transports AEA. Furthermore, SKM 4-45-1 is specific for the AEA transporter and does not interact with either the cannabinoid CB1 receptor or FAAH at micromolar concentrations. Therefore, SKM 4-45-1 may be used to study AEA transport in ways similar to the examples described above. For example, SKM 4-45-1 could be used to identify cells that transport AEA and potentially identify intracellular sites of AEA sequestration, if they exist. Furthermore, similar to the study of Schaffer and Lodish (1994), SKM 4-45-1 also may prove advantageous in the identification and cloning of the AEA transporter. In addition, because SKM 4-45-1 is only fluorescent after activation by intracellular esterases, it also could potentially be used to study the bidirectionality of the AEA transporter based on the accumulation of extracellular fluorescence. One limitation of SKM 4-45-1, however, is that the manifestation of the accumulation of extracellular fluorescence occurs as a result of two kinetic processes, uptake and intracellular de-esterification. Therefore, the utility of SKM 4-45-1 in kinetic studies of AEA transport is limited. In conclusion, SKM 4-45-1 is a fluorescent analog of AEA that interacts selectively with the AEA transporter and hence, will be a useful tool for the study of AEA movement across cell membranes.

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References


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