Binding, degradation and apoptotic activity of stearoylethanolamide in rat C6 glioma cells

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Stearoylethanolamide (SEA) is present in human, rat and mouse brain in amounts comparable with those of the endocannabinoid anandamide (arachidonoylethanolamide; AEA). Yet, the biological activity of SEA has never been investigated. We synthesized unlabelled and radiolabelled SEA to investigate its binding, degradation and biological activity in rat C6 glioma cells. We report that SEA binds to a specific site distinct from known cannabinoid or vanilloid receptors, and that AEA and capsazepine partly (approx. 50 %) antagonized this binding. Treatment of C6 cells with SEA inhibits cellular nitric oxide synthase and does not affect adenylate cyclase, whereas treatment with cannabinoid type 1 agonist 2-arachidonoylglycerol activates the former enzyme and inhibits the latter. C6 cells also have a specific SEA membrane transporter, which is inhibited by NO, and a fatty acid amide hydrolase capable of cleaving SEA. In these cells, SEA shows pro-apoptotic activity, due to elevation of intracellular calcium, activation of the arachidonate cascade and mitochondrial uncoupling. NO further enhances SEA-induced apoptosis. Moreover, the cannabinoid type 1 receptor-mediated decrease in cAMP induced by AEA in C6 cells is potentiated by SEA, suggesting that this compound also has an 'entourage' effect. Taken together, this study shows that SEA is an endocannabinoid-like compound which binds to and is transported by new components of the endocannabinoid system. It seems noteworthy that degradation and pro-apoptotic activity of SEA are regulated by NO in a way opposite to that reported for AEA.

Key words: arachidonate cascade, calcium, endocannabinoids, mitochondria, nitric oxide.

INTRODUCTION

Endocannabinoids include amides, esters and ethers of long chain polyunsaturated fatty acids, with anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) as the prototypal endogenous agonists of cannabinoid (CB) receptors [1,2]. They bind both the brain (CB1) and the peripheral (CB2) receptors, mimicking several actions of the marijuana constituent Δ^9-tetrahydrocannabinol [3,4]. Endocannabinoids play a number of roles in the nervous system, including regulation of dopaminergic neurotransmission, inhibition of glutamate-induced excitotoxicity and pain modulation [3], whereas peripherally they exert cardiovascular actions [5]. To date, other endocannabinoid-like compounds have been described in the brain of mammals, namely palmitoylethanolamide (PEA), oleoylethanolamide and stearoylethanolamide (SEA). PEA was shown to protect cerebellar granule neurons from excitotoxic death, to prevent antigen-induced mast-cell activation and to exert peripheral analgesic effects [6]. The activity of oleoylethanolamide as an anorexic lipid mediator has recently been characterized [7], whereas no pathophysiological roles have been reported for SEA till now. This is somewhat surprising, since SEA in rat [8], mouse and human [9] brain amounts to 11–14 % of the total *N*-acylethanolamines, whereas AEA represents 7–8 % only. These data have also been extended to mouse epidermal cells in culture, where SEA and AEA represent 38 and 4.5 % of the total *N*-acylethanolamines respectively [10]. Previously, we have found that SEA has cannabimimetic properties, as assessed by the tetrad of behavioural tests highly predictive of cannabimimetic compounds [11]. In the present study, we sought to investigate the ability of rat C6 glioma cells to bind, take up and hydrolyse SEA. We also characterized the pro-apoptotic potential of SEA, by analogy with recent data on the ability of AEA to induce this type of cell death [12–14]. Overall, the results suggest that SEA is an endocannabinoid-like compound with pro-apoptotic activity, which is regulated by NO in a way opposite to that reported for AEA.

MATERIALS AND METHODS

Materials

Chemicals were of the purest analytical grade. Stearic acid, oxalyl chloride, dry dichloromethane, ethanolamine, AEA, eicosa-5,8,11,14-tetraynoic acid (ETYA), indomethacin, PMSF, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), pertussis toxin (PTX), resinferatoxin, wortmannin, sodium nitroprusside (SNP) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma. 2-AG, arachidonoyl-trifluoromethyl-ketone (ATFMK), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *N*-(4-hydroxyphenyl) arachidonoylamide (AM404) were from Research Biochemicals International (Natick, MA, U.S.A.). Capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide, Caps) and

Abbreviations used: AC, adenylate cyclase; AEA, anandamide (arachidonoylethanolamide); 2-AG, 2-arachidonoylglycerol; AMT, AEA membrane transporter; Caps, capsazepine; CB, cannabinoid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ETYA, eicosa-5,8,11,14-tetraynoic acid; FAAH, fatty acid amide hydrolase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAP, mitogen-activated protein; NOS, nitric oxide synthase; PEA, palmitoylethanolamide; PTX, pertussis toxin; SEA, stearoylethanolamide; SBS, SEA-binding site; SIN-1, 3-morpholinosydnonimine; SMT, SEA membrane transporter; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside.

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PEA were purchased from Calbiochem (San Diego, CA, U.S.A.). VDM11 and HU-210 were from Tocris-Cookson (Bristol, U.K.), 3-morpholinosydnonimine (SIN-1) and 2′-amino-3′-methoxyflavone (PD98059) were from Alexis Corporation (Läufelfingen, Switzerland). [^{3}H]AEA (223 Ci/mmol) and [^{3}H]CP55,940 (126 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA, U.S.A.) and L-[2,3,4,5-^{3}H]arginine (64 Ci/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide (SR141716) and *N*-[1(*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528) were a gift from Sanofi-Synthelabo (Montpellier, France). [^{3}H]Resinferatoxin (48 Ci/mmol) was a gift from Dr V. Di Marzo (Consiglio Nazionale delle Ricerche, Pozzuoli, Italy), and cannabidiol was a gift from Dr M. Van der Stelt (Utrecht University, The Netherlands). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxy]-2-[2-amino-5-methyl-phenoxy]ethane-*N*,*N*,*N*′,*N*′-tetra-acetoxymethyl ester (Fluo-3 AM) were from Molecular Probes (Eugene, OR, U.S.A.).

Synthesis of SEA

Since SEA was not commercially available when this work started, we synthetized it from stearoyl chloride, prepared by adding oxalyl chloride (4 eq.) to stearic acid (1 eq.) in anhydrous dichloromethane under nitrogen atmosphere in dry glassware [15]. The end of the reaction was checked by TLC on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), using ethyl acetate as eluent and spraying the layer with ammonium molybdate–cerium (IV) sulphate; a single spot was observed with an $R_f = 0.12$ different from that of stearic acid ($R_f = 0.50$). The radiochemical synthesis of *N*-[9,10-^{3}H]SEA ([^{3}H]SEA) was performed, following the same procedure, with [9,10-^{3}H] stearic acid (60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO, U.S.A.) with an initial radioactivity of 0.5 mCi/mmol. The specific radioactivity of [^{3}H]SEA was 0.3 mCi/mmol, corresponding to a radiochemical yield of 60 %. The identity and chemical purity of synthesized SEA were determined by ^{1}H and ^{13}C NMR spectroscopy, GC–MS and MS, as reported [9]. The synthesis of SEA yielded a compound > 98 % pure (both for unlabelled and radiolabelled forms), with a product yield of 60 %.

Cell culture and determination of apoptosis

Rat C6 glioma cells were cultured in Ham's F-12 medium supplemented with 10 % fetal calf serum as described previously [12]. Cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and were fed every 3 days. After incubation for 48 h with the indicated compounds, floating and enzymically detached cells were collected together by centrifugation at 200 ***g*** for 5 min. Viability was estimated by the Trypan Blue dye-exclusion method in a Neubauer hemocytometer. Apoptosis was estimated by the cell-death detection–ELISA kit (Boehringer Mannheim, Mannheim, Germany), based on the evaluation of DNA fragmentation by an immunoassay for histone-associated DNA fragments in the cell cytoplasm. This method has recently been validated for C6 cells in comparison with cytofluorimetric analysis [12], performed in a FACS calibur Flow Cytometer (Becton Dickinson, Lincoln Park, NJ, U.S.A.). This latter technique quantifies apoptotic body formation in dead cells by staining with propidium iodide (50 μg/ml). Control cells contained less than 4.0 ± 1.0 apoptotic bodies every 100 cells analysed [12].

Binding assays

Binding of [^{3}H]SEA to C6 cells (200×10^6/test) was performed by rapid filtration assays as described previously [12]. Binding data were elaborated by non-linear regression analysis, using the Prism 3 program (GraphPAD Software for Science, San Diego, CA, U.S.A.), to calculate the kinetic constants of [^{3}H]SEA binding, i.e. maximum binding B_{max} and dissociation constant K_d. Binding of 100 pM [^{3}H]resinferatoxin was evaluated by rapid filtration assays, performed as described previously [16]. In all binding experiments, non-specific binding was determined in the presence of 1 μM 'unlabelled' agonist [12,16].

Enzymic assays

C6 cells (5×10^6/test) were incubated for 15 min at 37 °C with SEA or 2-AG, then they were washed, homogenized and subjected to enzymic assays. Nitric oxide synthase (NOS; EC 1.14.13.39) was assayed by incubating cell extracts with the radiolabelled substrate [^{3}H]arginine, and then measuring the reaction product [^{3}H]citrulline as described previously [17]. NOS activity was expressed as pmol of citrulline released per min per mg of protein. Forskolin (1 μM)-stimulated adenylate cyclase (AC; EC 4.6.1.1) activity was determined according to the amount of cAMP [18] detected in cell extracts with the cAMP Enzyme Immunoassay kit, as described below. AC activity was expressed as pmol cAMP per min per mg of protein. The effect of PTX on enzymic activities was determined by preincubating C6 cells for 3 h at 37 °C with 5 μg/ml of PTX before addition of SEA or 2-AG, or vehicle in control experiments [19].

Determination of SEA uptake and hydrolysis

The activity of the SEA membrane transporter (SMT) in C6 cells was measured as described previously [17]. Cells (2×10^6/test) were incubated for different time intervals, at 37 or 4 °C, with 300 nM [^{3}H]SEA. Then they were washed three times in 2 ml of PBS containing 1 % BSA and were finally resuspended in 200 μl of PBS. Membrane lipids were then extracted [17], resuspended in 0.5 ml methanol, mixed with 3.5 ml of Sigma–Fluor liquid-scintillation cocktail for non-aqueous samples (Sigma), and radioactivity was measured in an LKB1214 Rackbeta scintillation counter (Amersham Pharmacia Biotech). To discriminate non-carrier-mediated from carrier-mediated transport of SEA through cell membranes, [^{3}H]SEA uptake at 4 °C was subtracted from that at 37 °C [20]. Incubations (15 min) were also performed with different concentrations of [^{3}H]SEA, in the range 0–1000 nM, to determine the apparent Michaelis–Menten constant K_m and the maximum velocity V_{max} of the uptake by non-linear regression analysis, performed by the Prism 3 program (also in this case, the uptake at 4 °C was subtracted from that at 37 °C). SMT activity was expressed as pmol of SEA taken up per min per mg of protein. Q_{10} value was calculated as the ratio of SEA uptake at 30 and 20 °C [20]. The effect of various compounds on SMT activity was determined by adding directly each substance to the assay buffer, at the indicated concentrations, and incubating for 15 min at 37 °C. In the case of CCCP, cells were preincubated with 50 μM CCCP for 15 min at 37 °C before the addition of [^{3}H]SEA, to abolish the mitochondrial transmembrane potential [21]. Uptake of [^{3}H]AEA by C6 cells was measured as described previously [17].

Fatty acid amide hydrolase (FAAH; EC 3.5.1.4) activity was assayed in C6 cell extracts by measuring the release of [^{3}H]stearic acid from [^{3}H]SEA at pH 9.0, using reversed-phase HPLC [12]. FAAH activity was expressed as pmol of stearate released per min per mg of protein. The apparent K_m and V_{max} values of the FAAH-catalysed hydrolysis of [^{3}H]SEA were calculated by non-linear regression analysis, performed by the Prism 3 program. The effect of various compounds on FAAH activity was determined by adding each substance directly to the assay buffer, at the indicated concentrations, and incubating for 15 min at 37 °C. The FAAH-catalysed hydrolysis of [^{3}H]AEA in C6 cells was studied with the same methodology described for the hydrolysis of [^{3}H]SEA.

Determination of cAMP, mitochondrial uncoupling and intracellular calcium

C6 cells (5×10^6 cells/test) were treated with 1 μM forskolin in the presence of different compounds (or vehicle alone in the controls) for 15 min; then the medium was discarded and trypsinized cells were homogenized as described previously [12]. The cAMP levels in acetylated cell extracts were determined with the Cayman Chemical cAMP enzyme immunoassay kit (Alexis Corporation, Läufelfingen, Switzerland), as described previously [12]. cAMP in the extracts was within the linearity range of the method, calibrated with acetylated cAMP in accordance with the manufacturer's instructions. Mitochondrial uncoupling and intracellular calcium concentration were evaluated by flow cytometric analysis in a FACS calibur Flow Cytometer (Becton Dickinson). Mitochondrial uncoupling was measured using the fluorescent probe JC-1, as described previously [22]. JC-1 (dissolved in DMSO) was used at a final concentration of 20 μM. Control cells were treated with vehicle alone (1 % of the final volume). After the treatment, C6 cells were washed in PBS and incubated for 20 min at 37 °C. Cells were then analysed in an FL1/FL2 dot plot (530 nm/570 nm), gating on morphologically normal cells. Cytoplasmic-free calcium was measured using the fluorescent Ca^{2+} indicator Fluo-3 AM, as described previously [23]. C6 cells were collected by centrifugation and washed twice in Ca^{2+}- and Mg^{2+}-free PBS. Then, Fluo-3 AM (10 μM dissolved in DMSO) was added and cells were incubated for 40 min at 37 °C in the dark and frequently shaken manually. Control cells were treated with vehicle alone (1 % of the final volume). Cells were then collected by centrifugation and resuspended in culture medium without FBS. Fluo-3 AM fluorescence was recorded on a linear scale at 530 nm (bandwidth 30 nm) at a flow rate of approx. 1000 cells/s. Mean fluorescence values for 3000 events were registered every 10 s.

Statistical analysis

Data reported in this paper are the means ± S.D. for at least three independent experiments, each performed in duplicate. Statistical analysis was performed by the non-parametric Mann–Whitney test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science).

RESULTS

Properties of SEA binding

C6 cell membranes were able to bind [^{3}H]SEA according to a saturable process, and 1 μM 'unlabelled' SEA fully prevented this binding (Figure 1A). Analysis of the binding data yielded a K_d of 264 ± 27 pM and a B_{max} of 343 ± 11 fmol · mg protein^{-1}. AEA, but not 2-AG, PEA, SR141716 or SR144528 (each used at 1 μM), partly (approx. 50 %) displaced 500 pM [^{3}H]SEA

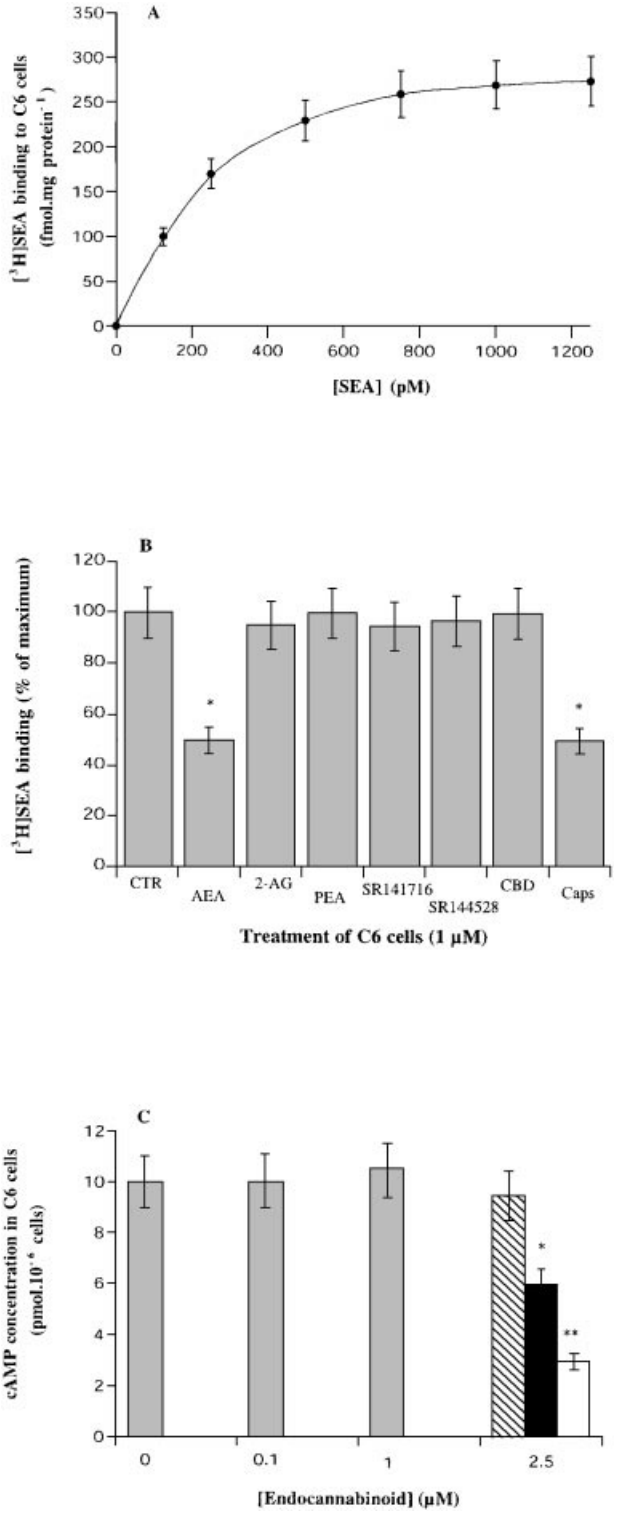

Figure 1 SEA binding to C6 cells

(**A**) Dose dependence of the binding of [^{3}H]SEA to C6 cell membranes. * Indicates the binding in the presence of 1 μM of 'unlabelled' SEA. (**B**) Effect of various compounds (each used at 1 μM) on the binding of 500 pM of [^{3}H]SEA to C6 cell membranes. Values were expressed as percentage of the untreated cells (100 % = 230 ± 23 fmol · mg protein^{-1}). CTR, control. *$P < 0.01$ compared with CTR. (**C**) Effect of SEA (hatched bar), AEA (black bar) or their combination (white bar) on cAMP concentration in C6 cells. *$P < 0.05$ and **$P < 0.01$ compared with control; $^{\#}P < 0.01$ compared with AEA-treated cells.

Table 1 Effect of SEA and 2-AG on NOS and AC activity in C6 cells

Treatment of cells with every compound listed, in the absence of SEA or of 2-AG, did not significantly affect NOS or AC activity under the same experimental conditions (100% = 120 ± 10 pmol of citrulline · min^{-1} · mg $protein^{-1}$ for NOS, and 60 ± 5 pmol cAMP · min^{-1} · mg $protein^{-1}$ for AC).

Compound	NOS activity (% of control)	AC activity (% of control)
Vehicle	100	100
SEA (0.1 μM)	70 ± 7*	95 ± 10
SEA (0.5 μM)	55 ± 5*	105 ± 10
SEA (1 μM)	35 ± 4†	95 ± 10
SEA (1 μM) + SR141716 (10 μM)	35 ± 4†	95 ± 10
SEA (1 μM) + Caps (10 μM)	70 ± 7†‡	95 ± 10
SEA (1 μM) + PTX (5 μg/ml)	40 ± 4†	105 ± 10
2-AG (1 μM)	250 ± 25†	30 ± 3†
2-AG (1 μM) + SR141716 (0.1 μM)	120 ± 12§	90 ± 10§
2-AG (1 μM) + Caps (10 μM)	260 ± 25†	34 ± 3†
2-AG (1 μM) + PTX (5 μg/ml)	240 ± 24†	87 ± 9§

* $P < 0.05$.
† $P < 0.01$ compared with vehicle-treated controls.
‡ $P < 0.01$ compared with SEA (1 μM).
§ $P < 0.01$ compared with 2-AG (1 μM).

from the binding site (Figure 1B). Also, 1 μM cannabidiol, a selective antagonist of a newly discovered 'endothelial' type of cannabinoid receptor [5], did not affect the binding of 500 pM [^{3}H]SEA to C6 cells, whereas 1 μM Caps, an antagonist of vanilloid receptors [24], partly (approx. 50%) displaced it (Figure 1B). Moreover, 1 μM SEA was unable to displace 100 pM [^{3}H]CP55.940, a high-affinity ligand for both CB1 and CB2 receptors [25], or 100 pM [^{3}H]resinferatoxin, a selective agonist of vanilloid receptors [24], from C6 cells (results not shown). Consistently, up to 2.5 μM, SEA failed to induce the CB1 receptor-mediated decrease in forskolin-induced cAMP concentration in C6 cells, which express CB1 receptors [12], at variance with 2.5 μM AEA (Figure 1C). Interestingly, although 2.5 μM AEA alone reduced cAMP concentration from 10 ± 1 to 6.0 ± 0.5 pmol/10^6 cells, the addition of 2.5 μM SEA together with AEA further reduced cAMP to 3.0 ± 0.3 pmol/10^6 cells (Figure 1C).

Signalling pathways regulated by the activation of CB1 receptors include stimulation of NOS and inhibition of AC, the latter effect being mediated by G_i/G_o proteins (see for a review [3]). In the present study, we sought to investigate the effect of SEA on NOS and AC activity in C6 cells, as compared with the effect of the CB1 agonist 2-AG [1,26]. The latter compound was used instead of AEA, because it does not interfere with the binding of SEA (Figure 1B). SEA was found to inhibit NOS activity in a dose-dependent manner, whereas it did not affect AC (Table 1). The inhibition of NOS was not prevented by 10 μM SR141716 or by pretreatment with 5 μg/ml PTX, an inhibitor of G_i/G_o proteins [19], whereas 10 μM of Caps partly (approx. 50%) prevented it at the same concentration (Table 1). In contrast to SEA, 2-AG enhanced NOS activity and 0.1 μM of SR141716, but not 10 μM of Caps or 5 μg/ml PTX, counteracted this effect (Table 1). Remarkably, 2-AG also reduced AC, and this effect was prevented by 0.1 μM SR141716 or 5 μg/ml PTX, but not by Caps. These findings suggest that activation of SEA-binding site (SBS) inhibits NOS-mediated signalling pathways, but is ineffective on signal transduction mediated by AC, whereas activation of CB1 receptors by 2-AG exhibits the typical features of CB1-mediated signalling [3].

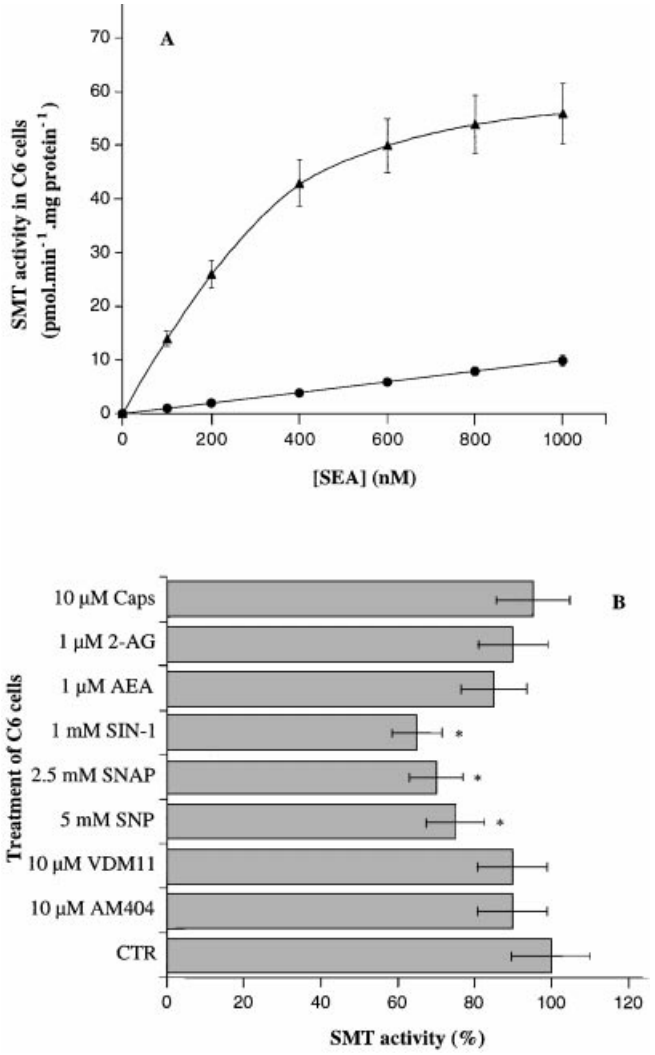

Figure 2 SEA uptake by C6 cells

(**A**) Dose dependence of the activity of the SMT in intact C6 cells at 37 °C (▲) or at 4 °C (●). (**B**) Effect of different compounds on the uptake of 200 nM of [^{3}H]SEA by SMT, expressed as percentage of the untreated cells (100% = 26 ± 3 pmol · min^{-1} · mg $protein^{-1}$). CTR, control. *$P < 0.05$ compared with CTR.

Degradation of SEA by uptake and hydrolysis

The uptake of [^{3}H]SEA by intact C6 cells was dependent on temperature (Q_{10} approx. 1.5), time ($t_{1/2}$ = approx. 5 min) and concentration (Figure 2A; results not shown), suggesting that an SMT was responsible for [^{3}H]SEA uptake [17,20]. The import of [^{3}H]SEA at 37 °C was a saturable process, showing apparent K_m and V_{max} values of 398 ± 58 nM and 82 ± 5 pmol · min^{-1} · mg $protein^{-1}$ respectively. The uptake of 200 nM [^{3}H]SEA by SMT was not affected by *N*-(4-hydroxyphenyl) arachidonoylamide [27] or VDM11 [28], specific inhibitors of AEA transporter, or by excess AEA or 2-AG (up to 1 μM), or by 10 μM Caps (Figure 2B). The lack of effect of AEA and Caps on SMT rules out that the transporter might contribute to the binding of [^{3}H]SEA to SBS (Figure 1B). However, SMT was significantly ($P < 0.05$) inhibited by NO donors SNP and SNAP, and by peroxynitrite (ONOO-)-donor SIN-1, which instead increased

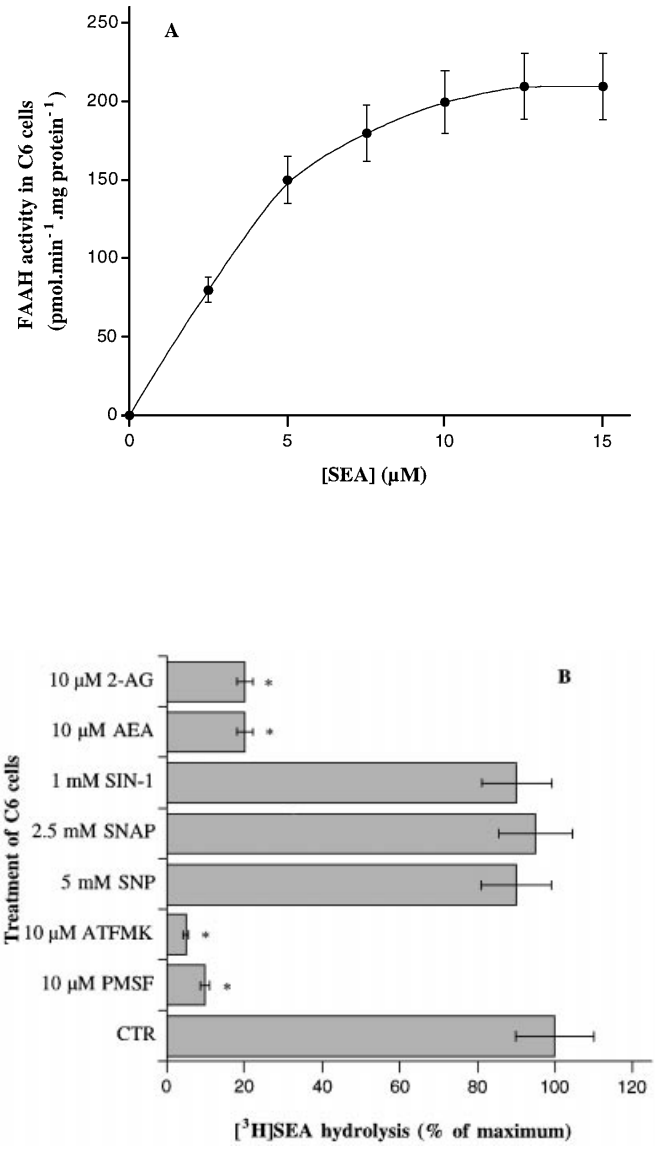

Figure 3 SEA hydrolysis by C6 cells

(**A**) Dose dependence of SEA hydrolysis by FAAH in C6 cells. (**B**) Effect of different compounds on the hydrolysis of 5 μM [^{3}H]SEA by FAAH, expressed as percentage of the untreated cells (100% = 150 $\pm$ 15 pmol · min^{-1} · mg protein^{-1}). CTR, control. *$P < 0.01$ compared with CTR.

AEA transport through the AEA membrane transporter (AMT) already described in these cells [12]. Moreover, up to 1 μM, SEA did not inhibit the uptake of [^{3}H]AEA (200 nM) by AMT. Altogether, these results demonstrate that SMT and AMT are different. Finally, pretreatment of C6 cells with the uncoupling agent CCCP [21] did not affect SMT activity, suggesting that it was not energy-dependent (results not shown).

C6 cells have an FAAH, whose activity was identified for the first time by Deutsch and Chin [29]. These cells were able to hydrolyse [^{3}H]SEA according to a Michaelis–Menten kinetics, with apparent K_m and V_{max} of 6 $\pm$ 1 μM and 300 $\pm$ 23 pmol · min^{-1} · mg protein^{-1} respectively (Figure 3A). Inhibitors of FAAH activity, PMSF and arachidonoyl trifluoromethyl ketone [30] fully inhibited the hydrolysis of 5 μM [^{3}H]SEA when used at 10 μM, as much as 10 μM of AEA or 2-AG (Figure 3B). NO donors SNP or SNAP, and $ONOO^-$ donor SIN-1, were

Table 2 SEA-induced programmed cell death in C6 glioma cells

Treatment of C6 cells for 48 h with every compound listed, in the absence of SEA, did not significantly affect cell death under the same experimental conditions. Values in parantheses represent the percentage with reference to samples treated with 0.1 μM of SEA.

Sample	Apoptotic bodies (fold over control)
Control	–
SEA (0.1 μM)	2.0 $\pm$ 0.2* (100)
SEA (0.5 μM)	3.2 $\pm$ 0.3* (160)
SEA (1 μM)	4.4 $\pm$ 0.5* (220)
SEA (0.1 μM) + HU-210 (1 μM)	3.8 $\pm$ 0.4*‡ (190)
SEA (0.1 μM) + HU-210 (1 μM) + L-NAME (500 μM)	2.2 $\pm$ 0.2*‖ (110)
SEA (0.1 μM) + SIN-1 (1 mM)	3.8 $\pm$ 0.4*‡ (190)
SEA (0.1 μM) + Caps (10 μM)	1.5 $\pm$ 0.1†§ (75)
SEA (0.1 μM) + PD98059 (10 μM)	1.9 $\pm$ 0.2* (95)
SEA (0.1 μM) + wortmannin (10 μM)	2.1 $\pm$ 0.2* (105)
SEA (0.1 μM) + ETYA (10 μM)	1.1 $\pm$ 0.1‡ (55)
SEA (0.1 μM) + indomethacin (10 μM)	1.2 $\pm$ 0.1‡ (60)

* $P < 0.01$ compared with control.
† $P < 0.05$ compared with control.
‡ $P < 0.01$ compared with SEA (0.1 μM)-treated samples.
§ $P < 0.05$ compared with SEA (0.1 μM)-treated samples.
‖ $P < 0.05$ compared with SEA + HU210-treated samples.

ineffective at the same concentrations that inhibited SMT activity (Figure 2B), as already reported for AEA hydrolysis [12]. At pH 5.0, any hydrolysis of [^{3}H]SEA by C6 cell homogenates was hardly detectable (results not shown), ruling out the involvement of the amidase described recently in lysosomes and mitochondria [30]. AEA competitively inhibited [^{3}H]SEA hydrolysis by C6 FAAH (inhibition constant $K_i = 7 \pm 1$ μM), and, conversely, SEA competitively inhibited [^{3}H]AEA hydrolysis by the same cells ($K_i = 5 \pm 1$ μM). This latter finding corroborates the hypothesis that SEA might potentiate the activity of AEA (Figure 1C) by inhibiting its degradation.

Apoptotic activity of SEA towards C6 glioma cells

In preliminary experiments, we found that 1 μM SEA induced apoptosis in C6 cells in a time-dependent manner, reaching a level of 2-fold over controls at 24 h and a maximum of 4-fold at 48 h. This period of time was used to characterize further SEA-induced apoptosis, and showed that apoptotic body formation induced by SEA was also dose-dependent (Table 2). Apoptosis was apparent already at 0.1 μM SEA (2-fold over the control) and reached a level of approx. 4-fold over the control at 1 μM SEA (Table 2). These concentrations of SEA are in the physiological range [9,10]. CB1 receptor agonist HU-210 [25], used at 1 μM, almost doubled apoptotic body formation induced by 0.1 μM of SEA, but co-incubation with the NOS inhibitor L-NAME at 500 μM prevented the additive effect of HU-210 (Table 2). Also co-incubation with 1 mM SIN-1 almost doubled the apoptotic body formation induced by 0.1 μM SEA, whereas 10 μM Caps reduced it by 50% (Table 2). Mitogen-activated protein (MAP) kinase inhibitor PD98059 [19] and the phosphoinositide 3′-kinase inhibitor wortmannin [31], both used up to 10 μM, did not affect apoptosis induced by 0.1 μM SEA, whereas the lipoxygenase inhibitor ETYA [32] or the cyclo-oxygenase inhibitor indomethacin [33] fully prevented it at the same concentration (Table 2). Overall, SEA-induced apoptosis does not involve MAP kinase or phosphoinositide 3′-kinase activation, unlike apoptosis triggered by

Table 3 Effect of SEA on mitochondrial uncoupling and intracellular calcium concentration in C6 glioma cells

Treatment of C6 cells with every compound listed, in the absence of SEA, did not significantly affect mitochondrial uncoupling or intracellular calcium under the same experimental conditions. Values refer to measurements performed 6 h (mitochondrial uncoupling) or 6 min (intracellular calcium) after the addition of each compound.

Compound	Mitochondrial uncoupling (fold over control)	Intracellular calcium (fold over control)
None	–	–
SEA (0.1 μM)	2.3 ± 0.3*	2.4 ± 0.3*
SEA (0.5 μM)	4.5 ± 0.5*	2.6 ± 0.3*
SEA (1 μM)	6.3 ± 0.6*	3.0 ± 0.3*
SEA (0.1 μM) + HU-210 (1 μM)	5.3 ± 0.5*‡	3.8 ± 0.4*§
SEA (0.1 μM) + HU-210 (1 μM) + L-NAME (500 μM)	2.7 ± 0.3*	2.2 ± 0.3*
SEA (0.1 μM) + SIN-1 (1 mM)	5.7 ± 0.6*‡	4.0 ± 0.4*§
SEA (0.1 μM) + Caps (10 μM)	1.7 ± 0.2†§	1.8 ± 0.2†§

* $P < 0.01$ compared with control.
† $P < 0.05$ compared with control.
‡ $P < 0.01$ compared with SEA (0.1 μM).
§ $P < 0.05$ compared with SEA (0.1 μM).

CB1 activation [31], but rather it proceeds through activation of the arachidonate cascade. SEA led to a dose-dependent mitochondrial uncoupling, which was most evident 6 h after treatment of C6 cells (Table 3). At this time interval, the increase in mitochondrial uncoupling was already significant with 0.1 μM SEA, a concentration that also induced significant apoptotic body formation (Table 2). Treatment with SEA also caused a dose-dependent, rapid (within 6 min) increase in intracellular calcium concentration (Table 3). The potent CB1 agonist HU-210 (1 μM) [1,2] enhanced NOS activity in C6 cells approx. 3-fold over the control (results not shown), corroborating the data obtained with 2-AG (Table 1) and extending previous observations in endothelial cells [17]. HU-210 (1 μM) as well as SIN-1 (1 mM) further enhanced the effect of 0.1 μM SEA on both mitochondrial uncoupling and calcium level. L-NAME (500 μM) counteracted the effect of HU-210, and Caps (10 μM) inhibited the increase in intracellular calcium and mitochondrial uncoupling induced by 0.1 μM of SEA by approx. 50% (Table 3).

DISCUSSION

In the present paper, we report evidence that rat C6 glioma cells bind and degrade SEA, which has pro-apoptotic activity towards these cells. Recently, we have shown that in mice SEA behaves similarly to AEA in the tetrad of tests which, when performed together, are highly indicative of cannabimimetic activity [2,11]. Yet, SEA did not bind to CB1 or VR1 receptors within mouse central nervous system, suggesting that SEA, unlike AEA and 2-AG, is an 'endocannabinoid-like' compound. Here, we show that SEA also has a pro-apoptotic potential, which might be relevant *in vivo*, because SEA is present in rat [8], mouse and human [9] brain at even higher amounts than AEA. In an attempt to elucidate the mechanisms of SEA activity, we synthesized tritium-labelled SEA and used it to investigate binding, transport and hydrolysis in C6 cells. Taken together, biochemical assays and pharmacological experiments demonstrated that SEA: (i) binds to specific sites different from known cannabinoid or vanilloid receptors, (ii) is taken up by a specific SMT, and (iii) is hydrolysed by FAAH. Notably, SEA potentiates the activity of AEA and inhibits its degradation by FAAH, suggesting that the biological activity of SEA might also involve an indirect 'entourage' effect on AEA.

We show that SEA did not bind to CB1 receptors in C6 cells; indeed it was unable to displace [^{3}H]CP55.940, its binding was not prevented by SR141716 and its effect on NOS and AC was different from that elicited by 2-AG through CB1 receptors. These findings are consistent with the observation that SEA does not have the structural requirements necessary for *N*-acyl-ethanolamines to occupy CB1 receptors [34]. However, SEA did bind to specific sites, as suggested by the observation that the binding was saturable, was fully displaced by an excess of 'unlabelled' SEA (Figure 1A) and was partly (approx. 50%) antagonized by 1 μM AEA or 1 μM Caps (Figure 1B). In this context, it should be recalled that Caps inhibits VR1 receptors with IC_{50} of approx. 0.02 μM [28], and SEA failed to displace [^{3}H]resinferatoxin from C6 cell membranes. Taken together, these observations rule out that SEA might bind to VR1 receptors, as does AEA [12,28]. On the other hand, mounting evidence suggests that the number of cannabinoid and vanilloid receptors still has to increase [35,36], therefore it can be suggested that SBS might be a new member of this receptor family. It seems noteworthy that CB1 agonists have been reported to activate inducible NOS in endothelial cells [17], and to inhibit the increased expression of this enzyme triggered in C6 cells by lipopolysaccharide and interferon-γ [37]. Therefore, it is tempting to suggest that SEA binding to SBS also might inhibit the inducible type of NOS in C6 cells (Table 1). Finally, the 'entourage effect' of SEA, i.e. the ability to potentiate the effects of AEA via several mechanisms, including the inhibition of its degradation [6,38], might contribute to the biological activity of this endocannabinoid-like compound. The physiological relevance of entourage compounds has recently been underlined for PEA [39].

In order to be a good signalling molecule, a substance has to be rapidly cleared after activation of its target. Consistently, C6 cells have a selective SMT and an FAAH capable of degrading SEA (Figures 2 and 3). It can be proposed that SMT and subsequently FAAH regulate the activity of SEA at SBS by controlling its life span in the extracellular matrix. Remarkably, SBS and SMT activity have recently been found also in mouse brain (M. Maccarrone, A. Cartoni, D. Parolaro, A. Margonelli, P. Massi, M. Bari, N. Battista and A. Finazzi-Agrò, unpublished work). In C6 cells, the kinetic properties and the regulation of SMT were quite different from those of the AMT in these cells [12]. In particular, SMT was inhibited by NO and even more by $ONOO^-$ (Figure 2B), which instead are known to activate AMT under the same experimental conditions [17]. On the other hand, the hydrolysis of SEA was catalysed by an FAAH showing the same features already described for the hydrolysis of AEA [12] (Figure 3). However, the catalytic efficiency (i.e. the V_{max}/K_m ratio) of the SEA hydrolysis by FAAH was approx. 8-fold lower than that of AEA, suggesting that SEA, with a saturated ($C_{18:0}$) fatty acid, was a poorer FAAH substrate than AEA, an unsaturated ($C_{20:4}$) fatty acid derivative. This finding recalls previous observations on PEA [30,40].

Recently, attention has been focused on the regulation of cell growth and differentiation by endocannabinoids, which might account for some pathophysiological effects of these lipids. AEA, unlike 2-AG, PEA or oleoylethanolamide, has been shown to induce apoptosis both *in vitro* [12,14] and *in vivo* [13], extending to endocannabinoids previous observations made with Δ^9-tetrahydrocannabinol [41]. In the present study, even SEA is shown to induce programmed cell death in C6 glioma cells,

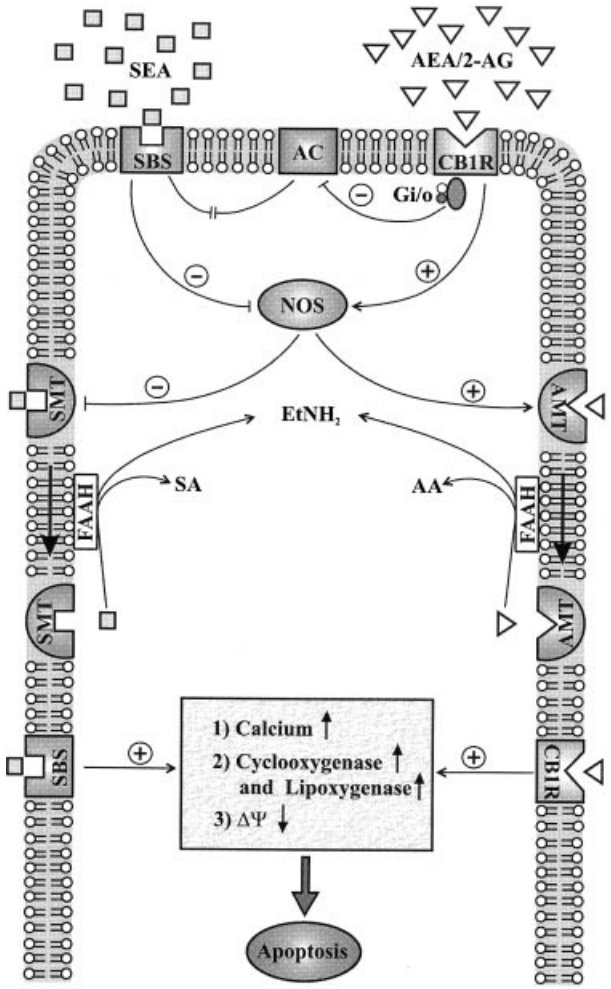

Figure 4 Binding, degradation and apoptotic activity of SEA in C6 cells

Binding of SEA to its site-specific binding site (SBS) inhibits NOS activity, whereas it does not affect AC. Conversely, binding of AEA or 2-AG to CB1 receptor activates NOS and, through G_i/G_0 proteins, inhibits AC. NO generated by NOS inhibits SMT but activate AMT. Once taken up, both SEA and AEA are hydrolysed by FAAH, which releases ethanolamine ($EtNH_2$), stearic acid (SA) and arachidonic acid (AA). Binding of SEA to SBS triggers a series of events starting with an increase in intracellular calcium followed by the activation of cyclo-oxygenase and lipoxygenase, and decrease in mitochondrial membrane potential ($\Delta\Psi$), ultimately leading to apoptosis. Activation of CB1 receptors further potentiates the pro-apoptotic activity of SEA, because it enhances the release of NO by NOS, thus inhibiting SMT and prolonging the half-life of SEA in the extracellular space.

paralleled by increased intracellular calcium (within 6 min) and mitochondrial uncoupling (within 6 h) (Tables 2 and 3). Caps partly (approx. 50 %) reverted these effects of SEA and partly (approx. 50 %) displaced it from its binding sites in C6 cells, suggesting that the pro-apoptotic activity of SEA was mediated by its binding to SBS. Also AEA-induced apoptosis in C6 and other cells is known to be paralleled by a rapid increase in calcium levels and disruption of the mitochondrial membrane potential, due to the activation of vanilloid receptors [12]. However, a major difference in the pro-apoptotic activities of SEA through SBS and of AEA through vanilloid receptors is that CB1 receptor activation increased the former (Tables 2 and 3) while reducing the latter [12]. Since both effects of CB1 receptors were abolished by L-NAME, and mimicked by SIN-1 (the present study and [12]), it can be suggested that the NO release associated with CB1 activation was responsible for the regulation of the apoptotic activity of both endocannabinoids. In fact, NO, and, even more, peroxynitrite, reduced SMT activity (Figure 2B), thus leading to (i) slow SEA degradation, (ii) high SEA extracellular concentration, and (iii) higher activity of SEA at its binding site. On the other hand, NO, and, even more, peroxynitrite, are known to increase AEA import, thus reducing the extracellular concentration of AEA and its activity at the receptor. The interplay between vanilloid and cannabinoid receptors in AEA-induced apoptosis has been depicted previously [12]. Remarkably, SEA-induced apoptosis occurred through activation of the arachidonate cascade; indeed it was fully prevented by lipoxygenase (ETYA) or cyclo-oxygenase (indomethacin) inhibitors (Table 2). On the other hand, the lack of effect of PD98059 and wortmannin suggests that the apoptotic pathway triggered by SEA did not involve MAP kinase or phosphoinositide 3′-kinase (Table 2), which instead are involved in CB1 receptor-mediated death programmes [31]. In this context, it should be recalled that lipoxygenase activity, which might be enhanced as a consequence of an increase in intracellular calcium [32], has been shown to trigger programmed organelle degradation [42] and apoptosis in different cell types, by directly uncoupling the mitochondria and facilitating the release of cytochrome *c* [32]. The binding and degradation of SEA, its effect on NOS and AC activity, and on apoptosis, are depicted in Figure 4. The scheme also shows how CB1 receptor signalling differs from that triggered by SEA, and the impact of activation of CB1 receptors on SEA degradation and activity.

In conclusion, the results reported here show that SEA is a biologically active endocannabinoid-like compound, and that C6 cells have the machinery to bind, take up and hydrolyse it. They also show that the pro-apoptotic activity of SEA is regulated by NO in a way opposite to that of AEA, suggesting that a different regulation of SEA and AEA might co-ordinate their activity in neuronal cells.

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