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Anandamide increases swelling and reduces calcium sensitivity of mitochondria

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ABSTRACT

The endocannabinoid anandamide alters mitochondria-dependent signal transduction, thus controlling key cellular events like energy homeostasis and induction of apoptosis. Here, the ability of anandamide to directly affect the integrity of mitochondria was investigated on isolated organelles. We found that anandamide dose-dependently increases mitochondrial swelling, and reduces cytochrome *c* release induced by calcium ions. The effects of anandamide were independent of its target receptors (*e.g.*, cannabinoid or vanilloid receptors), and were paralleled by decreased membrane potential and increased membrane fluidity. Overall, our data suggest that anandamide can impact mitochondrial physiology, by reducing calcium sensitivity and perturbing membrane properties of these organelles.

Introduction

N-Arachidonoylethanolamine (anandamide, AEA) is a prototype member of fatty acid amides, a group of endocannabinoids (eCBs). These compounds represent an ever growing family of lipid signals with central and peripheral activity [1,2]. AEA binds to and activates type-1 (CB1R) and type-2 (CB2R) cannabinoid receptors, that are G protein-coupled receptors expressed in neurons and peripheral cells [3]. In addition, AEA is a low affinity ligand of the transient receptor potential vanilloid 1 (TRPV1), and thus it can be considered also a true "endovanilloid" [4].

AEA and other eCBs are involved in manifold pathophysiological processes, spanning from neurotransmission in the central nervous system [5], to immune regulation [6] and fertility control [7] in the periphery. Interestingly, many of the activities of eCBs relate to stress-recovery systems and to the maintenance of energy balance [8,9]. Additionally, AEA has emerged as a key-regulator of the cell choice between life and death, and indeed it is able to promote apoptosis of central and peripheral cells [10,11]. Both energy homeostasis and apoptosis largely depend on mitochondrial activity, and consistently AEA, eCBs, and plant-derived cannabinoids have been shown to affect the functionality of these organelles in sperm [12], and neurons [13,14], and to promote mitochondria-dependent apoptosis of various cell types [10,15,16].

A number of reports have described the ability of AEA to induce physiological and morphological changes of mitochondria [17,18], yet only recently clear evidence has been collected for an impact of AEA on mitochondrial membrane potential, oxygen consumption, and ATP production [19], possibly through inhibition of the mitochondrial respiratory chain [20]. On this basis, we sought to ascertain whether AEA could act directly on mitochondria, and treated isolated organelles to look for their swelling, calcium sensitivity, and cytochrome *c* release. All these are early effects of the apoptotic programme [10,14,21,22].

Materials and methods

Materials. Anandamide was purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide (SR141716) and *N*-[(1S)-endo-1,3,3-trimethyl-1-bicyclo [2.2.1]-heptan-2-yl]5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide (SR144528) were kind gifts of Sanofi-Aventis Recherche (Montpellier, France). Capsazepine (CPZ) was purchased from Tocris (Bristol, United Kingdom). All other chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Animals. Male Wistar rats (250–350 g), housed at 22 ± 2 °C under artificial light for 12-h light/dark cycle and with access to water and food *ad libitum*, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

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Isolation of mice liver mitochondria. Mice liver mitochondria were isolated as previously described [22], with slight modifications. Briefly, after cervical dislocation livers were rapidly removed and rinsed with ice-cold H buffer (300 mM sucrose, 5 mM TES, 0.2 mM EGTA, pH 7.2). Then minced, homogenized and centrifuged at 760 *g* for 10 min at room temperature (RT). The supernatant was recovered and further centrifuged at 8740 *g* for 10 min at RT. The pellet, suspended in H buffer, was added carefully onto the top of the 60/30/18% Percoll gradient. Following centrifugation at 8740 *g* for 10 min at RT, the mitochondrial fraction, located at the interface of the lower two layers, was collected, diluted with H buffer and centrifuged at 6800 *g* for 10 min at RT. The mitochondrial pellet resuspended in H buffer. Mitochondrial protein was determined by Bradford method using BSA as a standard.

Mitochondrial swelling. Changes in absorbance of mitochondria were monitored at 540 nm in an ELISA plate reader (Multiskan RC, ThermoLabsystem, Milan, Italy). Mitochondria were resuspended at 0.3 μg/200 μL in respiration buffer (100 mM KCl, 20 mM Tris–HCl, 20 mM Hepes, 10 mM NaCl, 1 mM KH_2PO_4, 20 μM EGTA, pH 7.2, supplemented with 2 μM rotenone and 5 mM succinate). Swelling was triggered by the addition of 50 μM AEA for 5 min alone, in the presence of 100 μM Ca^{2+} or after a pre-treatment with cannabinoid or vanilloid receptors antagonists (2 μM SR1, 2 μM SR2, 10 μM CPZ), or by 100 μM Ca^{2+} alone.

Cytosolic cytochrome c assay. The concentration of cytosolic cytochrome *c* in the essentially mitochondrial-free cytosolic protein fraction was measured with an ELISA assay, as described [23].

Measurement of membrane potential. The mitochondrial membrane potential ($\Delta\Psi_m$) was measured as previously described [32], by using the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine (JC-1), which exhibits a potential-dependent accumulation in mitochondria [24]. The fluorescence of JC-1 is influenced by its concentration with a green light (530 nm) being emitted in dilute solutions, and a red light (595 nm) when molecules aggregate at larger concentrations. This method has been validated for a reliable analysis of $\Delta\Psi_m$ changes in mitochondria [25]. For our experiments, mitochondrial suspension (0.3 μg/μL) was incubated with 1 μM JC-1 in M buffer (220 mM sucrose, 68 mM mannitol, 10 mM KCl, 5 mM KH_2PO_4, 5 mM succinate, 10 mM Hepes, pH 7.2, and 2 μM rotenone just before use) for 15 min at RT in the dark. After washing in PBS, the mitochondria were suspended in the M buffer and left untreated or treated with 50 μM AEA for 5 min and examined by using a spectrofluorimeter LS50 at 590 nm (PerkinElmer Life and Analytical Science, Boston, MA). Carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP), a protonophore and uncoupler of oxidative phosphorylation in mitochondria [26] was used as positive control at 5 μM [31].

Assessment of mitochondrial membrane fluidity. Mitochondrial membrane fluidity was determined by means of the fluorescent probe 6-dodecanoyl-2-dimethylamino-naphtalene (laurdan), as already described [21]. Briefly, the freshly isolated mitochondria (125 μg) suspended in 250 μL of respiration buffer were left untreated or incubated with 50 μM AEA for 5 min at RT. Then, 0.5 μM laurdan was added and the suspension was kept for 30 min at 37 °C. Fluorescence readings were carried out in a thermostated (27 ± 0.1 °C) computer-driven spectrofluorimeter LS50 B model (PerkinElmer Life and Analytical Science, Boston, MA). Blank spectra were measured on unlabeled cells and were subtracted from the spectra of labeled cells [27]. Membrane fluidity is inversely proportional to the ratio of fluorescence at 440 nm *versus* that at 490 nm (F_{440}/F_{490}): the higher the ratio, the lower the fluidity [35]. From the spectroscopy data, laurdan emission generalized polarization (GP) was calculated as follows:

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}).$$

Statistical analysis. Data reported in this paper are the means ± SD of at least three independent experiments, each performed in triplicate. Unless stated otherwise, statistical analysis was performed through unpaired *t* test (GraphPAD Software for Science, San Diego, CA).

Results and discussion

In order to evaluate the effect of AEA on mitochondrial swelling, in the first series of experiments we isolated mitochondria from rat livers [22], and measured their absorbance value at 540 nm (A_{540}): the smaller the A_{540} value, the larger the extent of swelling [28]. We used AEA in a range (0–100 μM) that has been recently used to investigate the effects of (endo)cannabinoids on mitochondria [19]. Moreover, low micromolar concentrations of AEA can be detected in neuronal cells and in brain slices under normal conditions [29], and they markedly increase *in vivo* in cells or tissues subjected to various insults, as well as in primary central and peripheral cells of patients suffering from neurodegenerative or neuroinflammatory diseases [6]. Treatment with AEA increased mitochondrial swelling in a time- (0–15 min range) and dose- (0–100 μM range) dependent manner, reaching a maximum after 10 min of treatment with 50 μM AEA (Fig. 1A, and data not shown). To further corroborate our data, the effect of 50 μM AEA was compared with that of 100 μM Ca^{2+}, a well-known inducer of swelling [30]. Interestingly, the time-course of Ca^{2+}-induced swelling was characterized by a relatively short lag phase, followed by a fast decrease in A_{540} down to a ~60% of the initial value. Instead, AEA

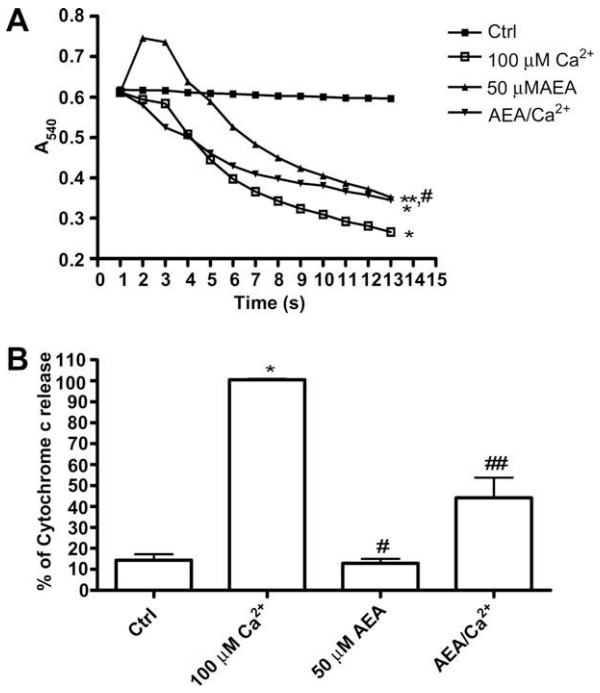

Fig. 1. Effect of AEA on rat liver mitochondria. (A) Effect of AEA on mitochondrial swelling. Intact mitochondria were resuspended and their swelling was monitored fluorimetrically by measuring the decrease of the absorbance at 540 nm (A_{540}), in response to 100 μM Ca^{2+} or 50 μM AEA, alone or in combination. *Denotes $p < 0.001$ and **$p < 0.05$ *versus* controls (Ctrls), and #denotes $p < 0.05$ *versus* Ca^{2+}-treated samples. (B) Effect of AEA on cytochrome *c* release. Cytochrome *c* release from mitochondria treated as reported in panel A was determined by means of ELISA. *Denotes $p < 0.01$ *versus* Ctrl, #$p < 0.001$, and ##$p < 0.05$ *versus* Ca^{2+}-treated samples. Data are representative of at least three independent experiments, each performed in triplicate. Statistical analysis was performed through unpaired *t* test (GraphPAD Software for Science, San Diego, CA).