J Bioenerg Biomembr (2012) 44:273–280
DOI 10.1007/s10863-012-9431-6

The endocannabinoid 2-arachidonoylglicerol decreases calcium induced cytochrome c release from liver mitochondria

Patrizia Zaccagnino · Susanna D'Oria · Luigi Luciano Romano · Almerinda Di Venere · Anna Maria Sardanelli · Michele Lorusso

Received: 6 February 2012 / Accepted: 7 March 2012 / Published online: 22 March 2012

Abstract 2-Arachidonoylglicerol (2-AG) is an endocannabinoid that mimics the pharmacological effects of Δ^9 tetrahydrocannabinol, the psychoactive component of the plant *Cannabis sativa*. It is present in many mammalian tissues, such as brain, liver, spleen, heart and kidney, where it exerts different biological effects either receptor mediated or independently of receptor activation. This work analyzes the effects of 2-AG on liver mitochondrial functions. It is shown that 2-AG causes a relevant decrease of calcium induced cyclosporine A sensitive cytochrome c release from mitochondria, a process representing an early event of the apoptotic program. Cyclosporin sensitive matrix swelling and ROS production measured under the same conditions are, on the contrary, almost unaffected or even enhanced, respectively, by 2-AG. Furthemore, 2-AG is found to stimulate resting state succinate oxidase activity and to inhibit oligomycin sensitive F_oF_1 ATP synthase activity. All these effects are apparently associated with 2-AG dependent alteration in the fluidity of the mitochondrial membranes, which was measured as generalized polarization of laurdan fluorescence.

P. Zaccagnino · S. D'Oria · L. L. Romano · A. M. Sardanelli · M. Lorusso (✉)
Department of Basic Medical Sciences, University of Bari, Policlinico, Piazza G. Cesare,
70124 Bari, Italy
e-mail: m.lorusso@biochem.uniba.it

A. Di Venere
Department of Experimental Medicine and Biochemical Sciences, University of Tor Vergata,
00133 Rome, Italy

A. Di Venere
IRCCS Neuromed,
Pozzilli, Italy

Keywords Endocannabinoids · 2-Arachidonoylglicerol · Mitochondrial calcium overload · Mitochondrial membrane fluidity · Cytochrome c release · F_oF_1 ATP synthase

Introduction

The endocannabinoid system is an ubiquitous signalling network composed of specific receptors and their endogenous agonistic ligands, the so-called endocannabinoids. Endocannabinoids are bioactive lipid molecules, that comprise amides, esters and ethers of long chain polyunsaturated fatty acids and mimic the pharmacological actions of the exogenous cannabinoid Δ^9-tetrahydrocannabinol, the primary psychoactive ingredient of hashish and marijuana (Gaoni and Mechoulam 1971).

Endocannabinoids are involved in a different set of physiological and pathological processes such as immunomodulation, analgesia, cancer, appetite, epilepsy (Di Marzo and Petrocellis 2006; Zias et al. 1993; Di Marzo 1998; Piomelli 2005; Pacher et al. 2006) and produce their effects by binding to distinct G protein-coupled receptors identified as the cannabinoid CB1 and CB2 receptors (Devane et al. 1988; Matsuda et al. 1990; Munro et al. 1993). It has been recently reported that the actions of endocannabinoids may also be mediated by two orphan G protein-coupled non-CB1/CB2 receptors, GPR55 and GPR119 (Ryberg et al. 2007; Brown 2007). Endocannabinoids are produced on demand through cleavage of membrane phospholipids precursors. They are active near the site of their synthesis and, after exerting their action, are hydrolyzed by specific enzymes.

The two most studied members of the endocannabinoid family are N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG).

2-AG is a monoacylglycerol with an esterified arachidonic acid at the sn-2 position of glycerol backbone. The enzymatic cleavage of phosphatidylinositol by phospholipase C appears the major source of the 2-AG precursor diacylglycerol, that subsequently is hydrolyzed by a diacylglycerol lipase to produce 2-AG (Wang and Ueda 2009; Basavarajappa 2007; Bisogno et al. 2003; Di Marzo et al. 1999). 2-AG production is strongly stimulated by calcium (Kondo et al. 1998) or after membrane depolarization causing in turn an increase of intracellular Ca^{2+} levels (Kano et al. 2009).

2-AG first found in the central and peripheral nervous system (Sugiura et al. 1995; Stella et al. 1997), was subsequently demonstrated to be present in many other mammalian tissues such as liver, spleen, lung, heart, small intestine, kidney and colon (Kondo et al. 1998; Schmid et al. 2000; Pinto et al. 2002; Izzo et al. 2001). This broad distribution suggests it may play multiple roles with a number of target sites. Among the various receptor mediated biological responses produced, inhibition of adenylyl cyclase activity (Stella et al. 1997; Franklin et al. 2003; Mukherjee et al. 2004), modulation of lymphocyte proliferation (Sipe et al. 2005), inhibition of the hepatic fibroblast (Julien et al. 2005) and cancer growth (Melck et al. 2000; Ligresti et al. 2003) have been reported.

Recent reports have, however, shown that endocannabinoids exert definite biological effects within the cell, that are independent of cannabinoid receptor activation. In particular, mitochondria appear to be involved in the intracellular anandamide and 2-AG action (Zaccagnino et al. 2011; Catanzaro et al. 2009; Siegmund et al. 2007).

These observations prompted us to carry out the present study aimed to better understand the effect of 2-AG on liver mitochondrial functions. We report here that 2-AG causes a substantial reduction of Ca^{2+} induced cytochrome c release from mitochondria, thus apparently conferring resistance of mitochondria to proapoptotic signals. This effect, together with those exerted on oxidative phosphorylation enzymes, is apparently associated with 2-AG induced alterations of lipid membrane ordering.

Materials and methods

Chemicals

Alamethicin, safranin-O, 6-Dodecanoyl-2-dimethylaminonaphthalene (Laurdan), p1,p2-Di (adenosine-5′) pentaphosphate pentalithium (Ap5) salt were purchased from Sigma (St. Louis, MO, USA). Cyclosporin A (CsA) was purchased from Calbiochem (San Diego, CA, USA). Hexokinase, phosphoenolpyruvate (PEP), L-lactate deydrogenase (LDH), pyruvate kinase (PK), glucose-6-phosphate deydrogenase (G6P-DH) and horseradish peroxidase (HRP) were from Roche Diagnostics Corporation (Indianapolis, IN, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) and calcium green-5N were purchased from Invitrogen–Molecular Probes (Eugene, OR, USA). 2-arachidonoylglycerol (2-AG) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Mouse anti-cytochrome c antibody was purchased from Zymed Laboratories Invitrogen Immunodetection (Carlsbad, CA, USA).

Isolation of rat liver mitochondria

Animal maintenance, handling and sacrifice were conducted as recommended by the institutional laboratory animal committee of the University of Bari.

Rat liver mitochondria were isolated by differential centrifugation using an isolation buffer containing 0.22 M mannitol, 75 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA and 0.25 mM PMSF as reported by Ito et al. (Ito et al. 1985). The final mitochondrial pellet was resuspended in the same isolation buffer at a protein concentration of 50–60 mg/ml as determined by the Biuret method, using bovine serum albumin as standard.

Detection of cytochrome c release

Cytochrome c release from freshly isolated mitochondria (0.25 mg/ml) was measured as previously described (Zaccagnino et al. 2009). Mitochondria were suspended in a medium containing 75 mM sucrose, 50 mM KCl, 30 mM TRIS-Cl, (pH 7.4), 2 mM KH_2PO_4, 5 mM $MgCl_2$ and 20 μM EGTA (Buffer A), supplemented with 10 mM succinate (plus 2 μg/ml rotenone) and incubated for 2 min at 25 °C. Where indicated 20 μM 2-AG, 180 μM Ca^{2+} and 2 μM CsA were added. Densitometric analysis of the bands were carried out by using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories).

Mitochondrial swelling

Mitochondria were suspended at 0.25 mg/ml in a medium containing 75 mM sucrose, 50 mM KCl, 30 mM TRIS-Cl, (pH 7.4), 2 mM KH_2PO_4, 10 mM succinate and 2 μg/ml rotenone. Swelling was triggered by the addition of 180 μM Ca^{2+} and changes in absorbance of the mitochondrial suspension were monitored at 540 nm in an Agilent 8453 diode-array spectrophotometer.

Detection of mitochondrial H_2O_2 production

The rate of mitochondrial H_2O_2 production was estimated by measuring the fluorescence increase (excitation at 475 nm, emission at 525 nm) induced by H_2O_2 oxidation

of dichlorofluorescin to the fluorescent compound dichlorofluorescein in the presence of horseradish peroxidase. 0.25 mg mitochondrial protein were suspended in Buffer A also containing 1.36 μM DCFH, 2 μg/ml rotenone and 0.4 μM HRP. The reaction was started by the addition of 10 mM succinate and the fluorescence signal was measured with a Jasco FP 6200 spectrofluorimeter. Conversion of fluorescence units to nmoles of H_2O_2 produced was performed by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

Measurement of extramitochondrial Ca^{2+} concentration

Mitochondria (0.25 mg protein/ml) were suspended in Buffer A supplemented with 10 mM succinate (plus rotenone) and 1 μM calcium green 5 N, at 25 °C. Ca^{2+} fluxes were measured spectrofluorimetrically as reported by Di Paola et al. (Di Paola et al. 2006) following the fluorescence changes at 505 nm (excitation), 535 nm (emission).

Mitochondrial respiration activity

The respiratory activity of mitochondria was measured polarographically with a Clark-type electrode, in an all-glass reaction chamber magnetically stirred, at 25 °C. Mitochondria were suspended at a final concentration of 0.25 mg/ml in a respiration medium containing 75 mM sucrose, 50 mM KCl, 30 mM TRIS-Cl, (pH 7.4), 2 mM KH_2PO_4, 2 mM $MgCl_2$ and 10 μM EGTA. Respiration was started by the addition of 10 mM succinate (plus rotenone). State 3 respiration was determined by adding 0.5 mM ADP.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured spectrofluorimetrically following the safranin-O fluorescence quenching at 525 nm (excitation), 575 nm (emission). Mitochondria (0.25 mg protein/ml) were suspended in Buffer A, supplemented with 2 μg/ml rotenone and 5 μM safranin-O at 25 °C (Di Paola et al. 2000). The transmembrane potential was generated by the addition of 10 mM succinate.

Measurement of F_oF_1 ATP synthase activity

F_oF_1 ATP synthase activity was measured in intact mitochondria as previously reported (Zaccagnino et al. 2011). 0.25 mg/ml of mitochondrial proteins were suspended in 200 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 10 mM KH_2PO_4, pH 7.4, 20 mM glucose, 5 units hexokinase, 300 μM Ap5, 2 μg/ml rotenone, 10 mM succinate and 0.3 mM ADP, at 25 °C. The concentration of glucose-6-phosphate was determined spectrophotometrically following NADP reduction at 340 nm.

Assessment of mitochondria membrane fluidity

Mitochondrial membrane fluidity was determined by measuring the fluorescence intensity of laurdan. Freshly prepared mitochondria were suspended (0.25 mg/ml) in Buffer A supplemented with 2 μg/ml rotenone, 10 mM succinate and incubated with the indicated concentrations of 2-AG for 8 minutes at 25 °C. Then, 0.25 μM laurdan was added and the samples were incubated for 30 min at 37 °C. Laurdan fluorescence emission generalized polarization readings (GP) were carried out in a thermostated computer-driven spectrofluorimeter ISS PC1 (ISS Inc, Urbana-Champaign, IL USA).

Results

Cytochrome c release and matrix swelling

In Fig. 1a the results of an experiment are reported aimed at analyzing the effects of 2-AG on calcium induced cytochrome c release from rat liver mitochondria.

The addition of 180 μM Ca^{2+} to mitochondria respiring with succinate (plus rotenone) caused a CsA sensitive cytochrome c release amounting to around 70% of the maximal release produced by alamethicin, a membrane channel-forming peptide that causes almost complete release of intermembrane proteins (Andreyev and Fiskum 1999). Preincubation of mitochondria with 20 μM 2-AG decreased by around 60% the amount of cytochrome c release caused by Ca^{2+} overload. 2-AG added to mitochondrial suspension in the absence of Ca^{2+} did not have any significant effect.

Figure 1b shows that mitochondria underwent CsA sensitive matrix swelling when pulsed with Ca^{2+}. 2-AG added alone was without effect, neither did it have appreciable effect on the rate and extent of Ca^{2+} induced swelling. Alamethicin induced swelling was used as internal positive control.

Release of accumulated Ca^{2+}

Figure 2 shows fluorescence traces monitoring extramitochondrial Ca^{2+} concentration changes. Four subsequent additions of 5 μM Ca^{2+} were required to determine a release of accumulated intra-mitochondrial Ca^{2+} (Fig. 2a). When mitochondria were preincubated with 2-AG their Ca^{2+} retention capacity was found to be diminished. In fact, no more than three additions of 5 μM Ca^{2+} were needed to cause Ca^{2+} release (Fig. 2b). CsA prevented this spontaneous Ca^{2+} release, which was, however, produced by the addition of the protonophore CCCP (Fig. 2c).

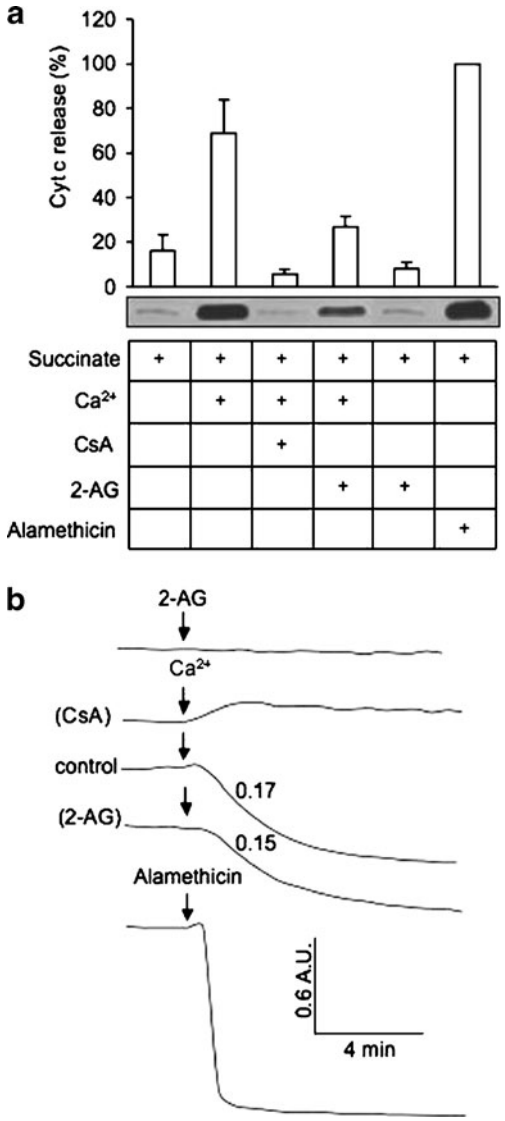

Fig. 1 Cytochrome c release and matrix swelling in rat liver mitochondria. (**a**) Cytochrome c release analysis was carried out as described under Materials and Methods. Values from densitometric measurements of the bands are expressed as the percentage of the maximal release induced by Alamethicin. Ca^{2+} and 2-AG were used respectively at 180 and 20 μM. Values reported are means ± SEM from five separate experiments. Where indicated 2 μM CsA was present. (**b**) Mitochondrial swelling was followed by absorbance changes at 540 nm in succinate-energized mitochondria. Mitochondrial suspension was pulsed with 180 μM Ca^{2+} in the absence (control) and in the presence of 2 μM CsA or 20 μM 2-AG. Values on the traces represent rate of absorbance change min^{-1}. Where indicated mitochondria suspension was pulsed with 20 μM 2-AG or 75 μg/mg protein Alamethicin

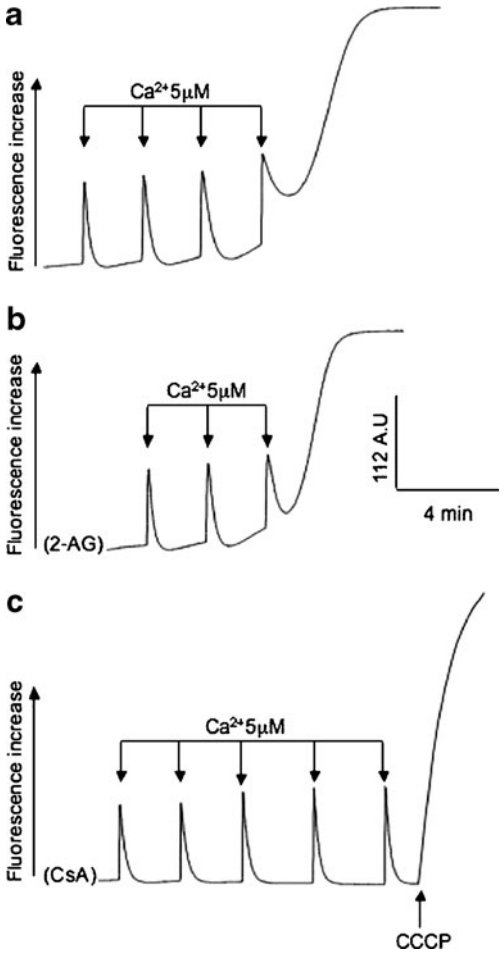

Fig. 2 Ca^{2+} retention capacity measurements in rat liver mitochondria. Ca^{2+} fluxes were followed as described under Materials and Methods. Were indicated (**b** and **c**) the mitochondrial suspension was preincubated with 20 μM 2-AG and 2 μM CsA, respectively. CCCP was used at concentration of 0.25 μM

Reactive oxygen species production

Ca^{2+} overload of mitochondria oxidizing succinate (plus rotenone) caused a large CsA sensitive ROS production whose rate and extent were much higher than those observed in the presence of antimycin. Ca^{2+} induced ROS production was significantly enhanced, by around 35%, when mitochondria were preincubated with 2-AG (Fig. 3).

Mitochondrial respiration and F_oF_1 ATP synthase activities

The effects of 2-AG on oxygen consumption and transmembrane potential in mitochondria oxidizing succinate are reported in Fig. 4.

A micromolar concentration of 2-AG caused a significant stimulation of succinate (plus rotenone) supported respiration (resting state) and a considerable inhibition of ADP stimulated respiration (State III) (Fig. 4a). As a consequence, 2-AG determined a marked drop of the respiratory control ratio (RCR) calculated as ADP-stimulated vs succinate supported respiration (Fig. 4b). These effects appeared to be concentration dependent. BSA did and atractyloside did not reverse the 2-AG stimulation of mitochondrial respiration under resting conditions (not shown). The increase

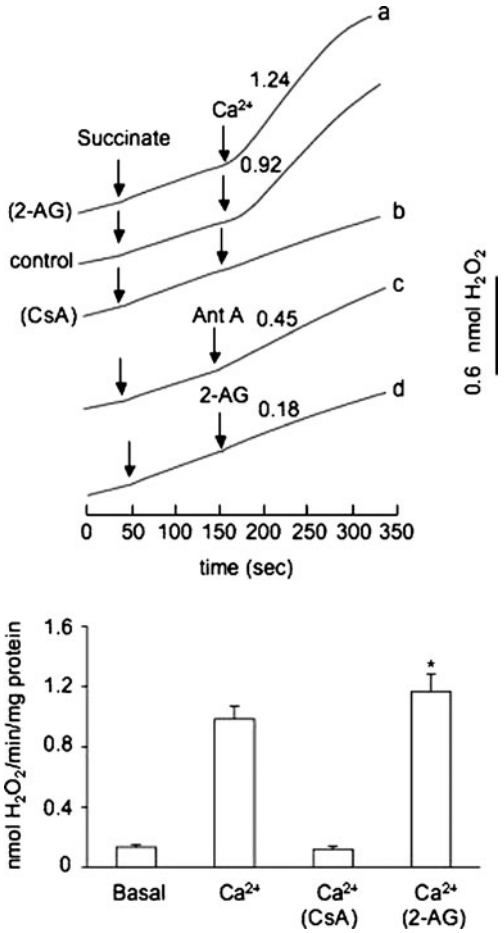

Fig. 3 H_2O_2 production in respiring liver mitochondria, effect of calcium overload. Mitochondrial suspension (0.25 mg/ml) was preincubated with 20 μM 2-AG (trace a), 2 μM CsA (trace b) or vehicle in the control. The reaction was started by the addition of succinate in the presence of rotenone. Where indicated 180 μM Ca^{2+} (traces a, b and control), 2 μg/ml Antimycin A (Ant A, trace c) and 20 μM 2-AG (trace d) were subsequently added. Numbers on the traces refer to the rate of H_2O_2 production as nmol min mg $protein^{-1}$. In the bottom of the Figure a statistical analysis of the effect of 2-AG on calcium induced ROS production is reported. Where indicated 180 μM Ca^{2+}, 2 μM CsA and 20 μM 2-AG were used. Values reported are means ± SEM from three separate experiments (*p<0.05)

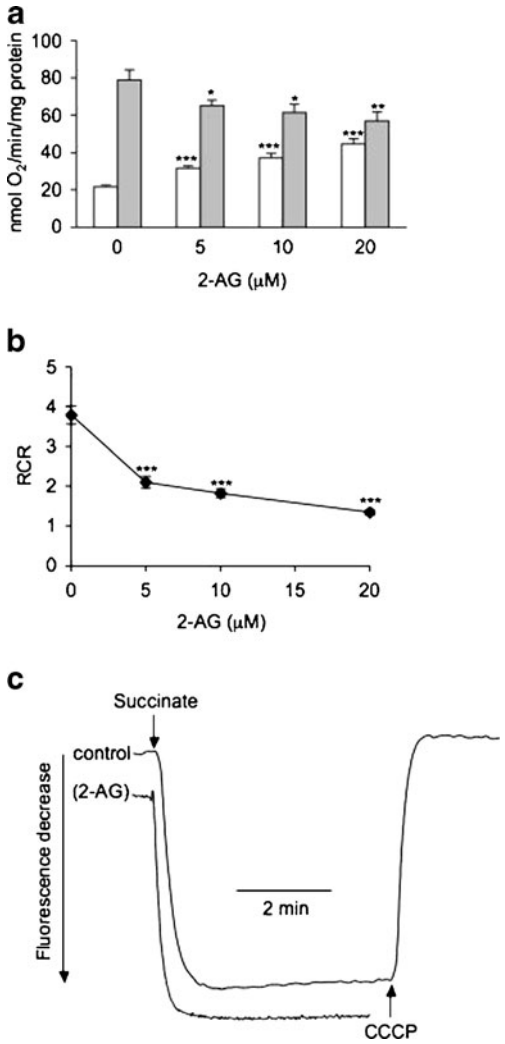

Fig. 4 Oxygen consumption and membrane potential measurements in rat liver mitochondria. (**a**) Resting state (white bars) and ADP stimulated respiration (gray bars) were measured in mitochondria preincubated with the indicated concentration of 2-AG. (**b**) Respiratory control ratio (RCR) values are reported as ADP-stimulated vs succinate supported respiration. Values reported are means ± SEM from five to ten separate experiments (*p<0.05; **p<0.01; ***p<0.001). (**c**) Membrane potential was generated by the addition of succinate to mitochondria preincubated with or without 20 μM 2-AG. Vehicle in the control. CCCP was used at concentration of 0.25 μM

of the resting state respiration was not a consequence of a 2-AG dependent destabilizing effect of the inner membrane, that might have caused uncoupling. In fact, 2-AG did not affect the succinate generated CCCP sensitive transmembrane potential measured as safranin fluorescence quenching (Fig. 4c).

The inhibition by 2-AG of ADP stimulated succinate respiration, that was even observed in spite of the 2-AG dependent stimulation of the resting state oxygen consumption, prompted us to analyze the effect of 2-AG on the F_oF_1 ATP synthase activity. It was found that the oligomycin sensitive ATP synthesis was progressively inhibited by 2-AG in the concentration range of 5–20 μM, with IC_{50} value of 10 μM (Fig. 5).

Membrane fluidity

Figure 6 shows the effect of 2-AG on the fluidity of mitochondrial membranes, measured as Generalized Polarization (GP) of laurdan fluorescence. Membrane fluidity is inversely proportional to the ratio of laurdan fluorescence at 440 nm vs that at 490 nm (F_{440}/F_{490}): the higher the ratio, the lower the membrane fluidity (Palleschi and Silvestroni

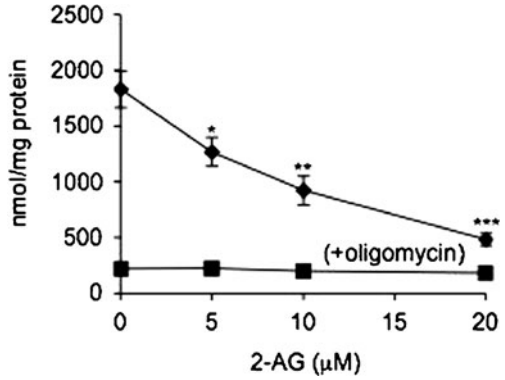

Fig. 5 F_oF_1 ATP synthase activity measurement in liver mitochondria. ATP synthesis was measured as nmol of glucose 6-phosphate produced during 5 min incubation of mitochondria respiring with succinate (plus rotenone) in the presence of 2-AG (5–20 μM). Where indicated 2 μg/ml oligomycin was present. Values reported are means ± SEM from three separate experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$). For other details see under Materials and Methods

1996). From the emission fluorescence spectra (excitation wavelength=360 nm), laurdan GP was calculated as follows:

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

where I_{440} and I_{490} are the laurdan emission intensities at 440 nm and 490 nm respectively.

It was found that 2-AG caused a concentration dependent significant decrease in GP values, i.e. an increase in mitochondrial membrane fluidity (Fig. 6).

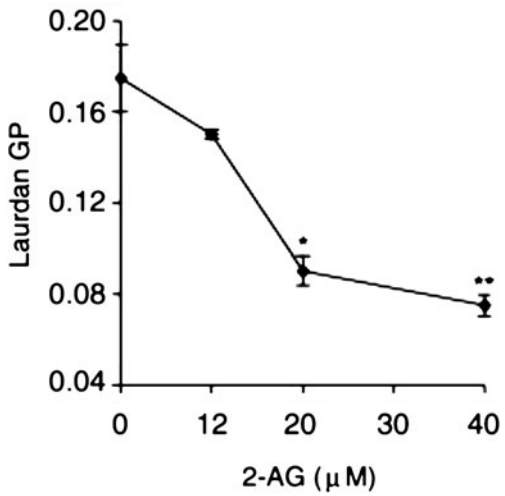

Fig. 6 Mitochondrial membrane fluidity measurements. The effect of 2-AG on the mitochondrial membrane fluidity was evaluated in rat liver mitochondria labeled with 0.25 μM laurdan. Generalized polarization (GP) was calculated as follows: $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$ where I_{440} and I_{490} are the laurdan emission intensities at 440 nm and 490 nm respectively. The intensities at 440 nm and 490 nm were corrected by subtracting the values obtained for unlabeled mitochondria. GP values are expressed as means ± SEM from three to five independent experiments (*$p<0.05$; **$p<0.01$)

Discussion

Cytosolic calcium increase and ROS production are essential factors for opening of MPTP (Mitochondrial Permeability Transition Pore), a non specific pore of the inner mitochondrial membrane, which is permeant to molecules up to 1.5 kDa of M.W. and opens under conditions of elevated matrix Ca^{2+} concentrations. Pore opening causes the release into the cytosol of intermembrane proteins, such as cytochrome c, a process which represents an early event of the apoptotic program execution. The current view is that oxidative stress plays an important role in promoting the membrane permeability transition (MPT). In particular oxidation of SH groups in the adenine nucleotide translocator (ANT) would stimulate pore opening and MPT (McStay et al. 2002). CsA sensitive mitochondrial ROS are in turn produced by Ca^{2+} overload (Fig. 3), this would indicate that MPT itself causes mitochondrial oxidative stress. Since this Ca^{2+} dependent ROS production is larger than that measured in the presence of antimycin, i.e. with the respiratory chain completely inhibited, it can be concluded that inhibition of electron transfer is not the only factor stimulating ROS production. Loss of the mitochondrial GSH and NADPH dependent antioxidant defenses upon MPTP opening was suggested to dysregulate ROS balance under these conditions (Maciel et al. 2001; Hansson et al. 2008).

The main finding of the present study is that 2-AG markedly inhibits CsA sensitive Ca^{2+} dependent cytochrome c release (Fig. 1). This aspect may be of pathophysiological relevance considering that Ca^{2+} overload is typical of several pathological states such as reperfusion injury (Crompton et al. 1988; Broekemeier et al. 1989; Halestrap and Davidson 1990) and both acute and chronic experimentally induced diabetes (Chan and Junger 1984; Studer and Ganas 1989). Sustained elevation of cytosolic Ca^{2+} is deleterious as it can cause cell death by promoting cytochrome c release from mitochondria. 2-AG generation which is dependent on/or stimulated by calcium (Kondo et al. 1998) may play an important role in reducing the susceptibility of mitochondria to release proapoptotic proteins, thus exerting a feed-back control and protecting the cell from death.

The inhibition exerted on the cytochrome c release process cannot be explained in terms of 2-AG dependent decreased Ca^{2+} sensitivity of mitochondria, for the following reasons: i) Ca^{2+} retention capacity was actually decreased by 2-AG, this indicating an increase of Ca^{2+} sensitivity (Fig. 2); ii) only a negligible decrease of Ca^{2+} dependent matrix swelling was observed in mitochondria supplemented with 2-AG (Fig. 1b); iii) CsA sensitive MPT dependent ROS production was even increased by 2-AG (Fig. 3). The conclusion is that 2-AG, although decreasing cytochrome c release, does not appear to inhibit MPT induced by Ca^{2+} overload.

Many membrane associated enzymes and processes are known to be affected by cannabinoids (Martin 1986), whose lipophilicity plays an important role (Thomas et al. 1990). As far as the cytochrome c release is concerned, it is conceivable that the process is associated with changes in the organization of membrane lipids (and proteins). It may thus be hypothesized that 2-AG distribution in the membrane causes lipid rearrangement leading to hindrance of the release process. Interestingly, the other endocannabinoid anandamide was found similarly to increase rat liver mitochondrial membrane fluidity and to decrease Ca^{2+} dependent cytochrome c release (Catanzaro et al. 2009). Thus endocannabinoids appear to function as antiapoptotic agents inducing resistance of mitochondria to proapoptotic signals.

While decreasing MPT dependent cytochrome c release, 2-AG caused a significant increase of CsA-sensitive ROS production subsequent to MPTP opening (Fig. 3). The increased mitochondrial membrane fluidity we measured upon treatment of mitochondria with 2-AG (Fig. 6) may explain this finding. The effect resembles that elicited by free fatty acids and by the endocannabinoid-like N-arachidonylglicine, which have been reported to cause ROS production in excess of that produced in mitochondria inhibited by antimycin. This was attributed to their properties to increase membrane fluidity and explained that under these conditions the accessibility of oxygen to electron donating sites of the respiratory chain and, consequently, the superoxide anion release are facilitated (Zaccagnino et al. 2009; Schonfeld and Wojtczak 2007).

We have shown here that 2-AG also affects the activity of oxidative phosphorylation enzymes. The consequences of fluidity changes on the properties of membrane bound enzymes have been widely studied and reported to be not uniform, yielding either an increase or a decrease of their activity (Spector and Yorek 1985). In particular, it has been reported that the succinate oxidase and F_oF_1 ATP synthase activities of liver mitochondria are differently regulated by changes in mitochondrial membrane fluidity occurring in alloxan induced diabetes rats (Patel and Katyare 2006a, b). We have confirmed here these data and suggest that the observed increase of resting state succinate respiration and the inhibition of the oligomycin sensitive ATP synthase activity could be explained in terms of 2-AG dependent lipid rearrangement and consequent alteration of lipid-protein interaction.

In conclusion, the decrease by 2-AG of calcium induced cytochrome c release reveals a protective effect that would be exerted by the endocannabinoid towards apoptotic signals such as the cytosolic calcium concentration increase, which is associated with several pathological conditions. On the other hand, the F_oF_1 ATP synthase inhibition by 2-AG, which is also replicated by endocannabinoid anandamide (Zaccagnino et al. 2011), could cause a decline of ATP production and, possibly, mitochondrial dysfunctions. Thus, the balance of various and not uniform effects produced by 2-AG on the mitochondrial functions could determine the fate of the cell in response to the endocannabinoid signal.

Acknowledgements This work was financially supported by a grant from the National Research Project (PRIN) on "Mitochondrial Bioenergetics: Redox Mechanisms, ROS Production, Redox Control of Cell Differentiation" and FIRB Project on "Italian Human ProteomNet". We thank Prof. Sergio Papa for suggestions and critical reading of the manuscript and Dr Roberta Bernardini of the "Centro di Servizi Interdipartimentale, Stazione per la Tecnologia Animale" University of Tor Vergata, Rome, for technical assistance.

References

Andreyev A, Fiskum G (1999) Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver. Cell Death Differ 6(9):825–832. doi:10.1038/sj.cdd.4400565

Basavarajappa BS (2007) Critical enzymes involved in endocannabinoid metabolism. Protein Pept Lett 14(3):237–246

Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A et al (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163(3):463–468. doi:10.1083/jcb.200305129

Broekemeier KM, Dempsey ME, Pfeiffer DR (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 264(14):7826–7830

Brown AJ (2007) Novel cannabinoid receptors. Br J Pharmacol 152 (5):567–575. doi:10.1038/sj.bjp. 0707481

Catanzaro G, Rapino C, Oddi S, Maccarrone M (2009) Anandamide increases swelling and reduces calcium sensitivity of mitochondria. Biochem Biophys Res Commun 388(2):439–442. doi:10.1016/j.bbrc.2009.08.037

Chan KM, Junger KD (1984) The effect of streptozocin-induced diabetes on the plasma membrane calcium uptake activity of rat liver. Diabetes 33(11):1072–1077

Crompton M, Ellinger H, Costi A (1988) Inhibition by cyclosporin A of a Ca2+ −dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 255(1):357–360

Devane WA, Dysarz FA 3rd, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34(5):605–613

Di Marzo V (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. Biochim Biophys Acta 1392(2–3):153–175

Di Marzo V, Petrocellis LD (2006) Plant, synthetic, and endogenous cannabinoids in medicine. Annu Rev Med 57:553–574. doi:10.1146/annurev.med.57.011205.135648

Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA et al (1999) Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. Eur J Biochem 264(1):258–267

Di Paola M, Cocco T, Lorusso M (2000) Ceramide interaction with the respiratory chain of heart mitochondria. Biochemistry 39 (22):6660–6668

Di Paola M, Zaccagnino P, Oliveros-Celis C, Lorusso M (2006) Arachidonic acid induces specific membrane permeability increase in heart mitochondria. FEBS Lett 580(3):775–781. doi:10.1016/j.febslet.2005.12.090

Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. J Neurosci 23 (21):7767–7775

Gaoni Y, Mechoulam R (1971) The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish. J Am Chem Soc 93(1):217–224

Halestrap AP, Davidson AM (1990) Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 268(1):153–160

Hansson MJ, Mansson R, Morota S, Uchino H, Kallur T, Sumi T et al (2008) Calcium-induced generation of reactive oxygen species in brain mitochondria is mediated by permeability transition. Free Radic Biol Med 45(3):284–294. doi:S0891-5849(08)00208-6

Ito A, Ogishima T, Ou W, Omura T, Aoyagi H, Lee S et al (1985) Effects of synthetic model peptides resembling the extension peptides of mitochondrial enzyme precursors on import of the precursors into mitochondria. J Biochem 98(6):1571–1582

Izzo AA, Fezza F, Capasso R, Bisogno T, Pinto L, Iuvone T et al (2001) Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. Br J Pharmacol 134(3):563–570. doi:10.1038/sj.bjp. 0704293

Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M et al (2005) Antifibrogenic role of the cannabinoid receptor CB2 in the liver. Gastroenterology 128(3):742–755

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89(1):309–380. doi:10.1152/physrev.00019.2008

Kondo S, Kondo H, Nakane S, Kodaka T, Tokumura A, Waku K et al (1998) 2-Arachidonoylglycerol, an endogenous cannabinoid receptor agonist: identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through CA2+ −dependent and -independent mechanisms. FEBS Lett 429(2):152–156

Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio MG, Cosenza V et al (2003) Possible endocannabinoid control of colorectal cancer growth. Gastroenterology 125(3):677–687

Maciel EN, Vercesi AE, Castilho RF (2001) Oxidative stress in Ca(2+)-induced membrane permeability transition in brain mitochondria. J Neurochem 79(6):1237–1245

Martin BR (1986) Cellular effects of cannabinoids. Pharmacol Rev 38 (1):45–74

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346(6284):561–564. doi:10.1038/346561a0

McStay GP, Clarke SJ, Halestrap AP (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem J 367(Pt 2):541–548. doi:10.1042/BJ20011672

Melck D, De Petrocellis L, Orlando P, Bisogno T, Laezza C, Bifulco M et al (2000) Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. Endocrinology 141(1):118–126

Mukherjee S, Adams M, Whiteaker K, Daza A, Kage K, Cassar S et al (2004) Species comparison and pharmacological characterization of rat and human CB2 cannabinoid receptors. Eur J Pharmacol 505(1–3):1–9. doi:10.1016/j.ejphar.2004.09.058

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365 (6441):61–65. doi:10.1038/365061a0

Pacher P, Batkai S, Kunos G (2006) The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 58(3):389–462. doi:10.1124/pr.58.3.2

Palleschi S, Silvestroni L (1996) Laurdan fluorescence spectroscopy reveals a single liquid-crystalline lipid phase and lack of thermotropic phase transitions in the plasma membrane of living human sperm. Biochim Biophys Acta 1279(2):197–202

Patel SP, Katyare SS (2006a) Effect of alloxan diabetes and subsequent insulin treatment on temperature kinetics properties of succinate oxidase activity in rat kidney mitochondria. J Membr Biol 213 (1):31–37. doi:10.1007/s00232-006-0041-2

Patel SP, Katyare SS (2006b) Insulin-status-dependent modulation of FoF1-ATPase activity in rat liver mitochondria. Lipids 41(7):695–703

Pinto L, Izzo AA, Cascio MG, Bisogno T, Hospodar-Scott K, Brown DR et al (2002) Endocannabinoids as physiological regulators of colonic propulsion in mice. Gastroenterology 123(1):227–234

Piomelli D (2005) The endocannabinoid system: a drug discovery perspective. Curr Opin Investig Drugs 6(7):672–679

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J et al (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 152(7):1092–1101. doi:10.1038/sj.bjp. 0707460

Schmid PC, Schwartz KD, Smith CN, Krebsbach RJ, Berdyshev EV, Schmid HH (2000) A sensitive endocannabinoid assay. The simultaneous analysis of N-acylethanolamines and 2-monoacylglycerols. Chem Phys Lipids 104(2):185–191

Schonfeld P, Wojtczak L (2007) Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport. Biochim Biophys Acta 1767(8):1032–1040. doi:10.1016/j.bbabio.2007.04.005

Siegmund SV, Qian T, de Minicis S, Harvey-White J, Kunos G, Vinod KY et al (2007) The endocannabinoid 2-arachidonoyl glycerol induces death of hepatic stellate cells via mitochondrial reactive oxygen species. FASEB J 21(11):2798–2806. doi:10.1096/fj.06-7717com

Sipe JC, Arbour N, Gerber A, Beutler E (2005) Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders. J Leukoc Biol 78(1):231–238. doi:10.1189/jlb.0205111

Spector AA, Yorek MA (1985) Membrane lipid composition and cellular function. J Lipid Res 26(9):1015–1035

Stella N, Schweitzer P, Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388 (6644):773–778. doi:10.1038/42015

Studer RK, Ganas L (1989) Effect of diabetes on hormone-stimulated and basal hepatocyte calcium metabolism. Endocrinology 125 (5):2421–2433

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K et al (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215(1):89–97

Thomas BF, Compton DR, Martin BR (1990) Characterization of the lipophilicity of natural and synthetic analogs of delta 9-tetrahydrocannabinol and its relationship to pharmacological potency. J Pharmacol Exp Ther 255(2):624–630

Wang J, Ueda N (2009) Biology of endocannabinoid synthesis system. Prostaglandins Other Lipid Mediat 89(3–4):112–119. doi:10.1016/j.prostaglandins.2008.12.002

Zaccagnino P, Saltarella M, D'Oria S, Corcelli A, Saponetti MS, Lorusso M (2009) N-arachidonylglycine causes ROS production and cytochrome c release in liver mitochondria. Free Radic Biol Med 47(5):585–592. doi:10.1016/j.freeradbiomed.2009.05.038

Zaccagnino P, Corcelli A, Baronio M, Lorusso M (2011) Anandamide inhibits oxidative phosphorylation in isolated liver mitochondria. FEBS Lett 585(2):429–434. doi:10.1016/j.febslet.2010.12.032

Zias J, Stark H, Sellgman J, Levy R, Werker E, Breuer A et al (1993) Early medical use of cannabis. Nature 363(6426):215. doi:10.1038/363215a0