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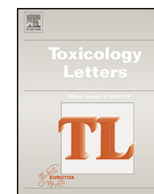

Cannabinoid-induced changes in respiration of brain mitochondria

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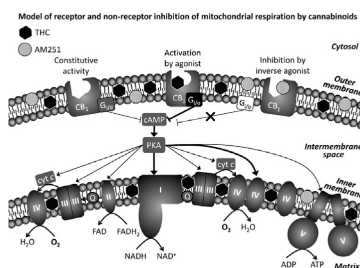
HIGHLIGHTS

- Cannabinoids are full or partial inhibitors of mitochondrial respiration.
- There is protective effect of antagonist/inverse agonist of cannabinoid receptor 1.
- Both receptor- and non-receptor action of cannabinoids influences respiration.

GRAPHICAL ABSTRACT



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ABSTRACT

Cannabinoids exert various biological effects that are either receptor-mediated or independent of receptor signaling. Mitochondrial effects of cannabinoids were interpreted either as non-receptor-mediated alteration of mitochondrial membranes, or as indirect consequences of activation of plasma membrane type 1 cannabinoid receptors (CB_1). Recently, CB_1 receptors were confirmed to be localized to the membranes of neuronal mitochondria, where their activation directly regulates respiration and energy production. Here, we performed in-depth analysis of cannabinoid-induced changes of mitochondrial respiration using both an antagonist/inverse agonist of CB_1 receptors, AM251 and the cannabinoid receptor agonists, Δ^9-tetrahydrocannabinol (THC), cannabidiol, anandamide, and WIN 55,212-2. Relationships were determined between cannabinoid concentration and respiratory rate driven by substrates of complex I, II or IV in pig brain mitochondria. Either full or partial inhibition of respiratory rate was found for the tested drugs, with an IC_{50} in the micromolar range, which verified the significant role of non-receptor-mediated mechanism in inhibiting mitochondrial respiration. Effect of stepwise application of THC and AM251 evidenced protective role of AM251 and corroborated the participation of CB_1 receptor activation in the inhibition of mitochondrial respiration. We proposed a model, which includes both receptor- and non-receptor-mediated mechanisms of cannabinoid action on mitochondrial respiration. This model explains both the inhibitory effect of cannabinoids and the protective effect of the CB_1 receptor inverse agonist.

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Abbreviations: ADP, adenosine diphosphate; AM251, 1-(2,4-dichlorophenyl)-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide; cAMP, cyclic adenosine monophosphate; CB, cannabinoid receptor; DMSO, dimethyl sulfoxide; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; IC_{50}, drug concentration that is required for 50% inhibition; K_i, affinity for receptor; MiR05, mitochondrial respiration medium; OXPHOS, oxidative phosphorylation; PKA, protein kinase A; THC, Δ^9-tetrahydrocannabinol; TMPD, tetramethyl-p-phenylenediamine; WIN 55,212-2, R-(+)-WIN 55,212, R-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone.

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1. Introduction

Besides their crucial role in the generation of adenosine-5′-triphosphate (ATP), mitochondria are involved in other important processes, such as regulation of free radicals, neurotransmitters, calcium, and apoptosis (Mattson et al., 2008). Thus, mitochondria represents a possible drug target with unexplored therapeutic and toxicological potential (Kanabus et al., 2014; Szeto, 2014). Cannabinoids, a class of chemical compounds that are specifically recognized by the cannabinoid receptors, are known to inhibit mitochondrial respiration (Bénard et al., 2012; Whyte et al., 2010). The physiological functions of the endocannabinoid system are complex and involve, among other functions, motor coordination, memory, appetite, pain modulation, neuroprotection, cognitive functions, affective states, and maintenance of energy homeostasis (Fišar, 2012; Marsicano and Lutz, 2006; Morena and Campolongo, 2014; Piscitelli and Di Marzo, 2012; Silvestri and Di Marzo, 2013; Trezza and Campolongo, 2013). Hence, research on the therapeutic potential of drugs modulating the endocannabinoid system is extensive (Aso and Ferrer, 2014; Maione et al., 2013; Pacher et al., 2006; Pacher and Kunos, 2013; Pertwee, 2009).

1.1. Cannabinoids

Cannabinoids can be categorized into (i) phytocannabinoids, e.g., Δ^9-tetrahydrocannabinol (THC) and cannabidiol; (ii) endocannabinoids, e.g., anandamide and 2-arachidonoylglycerol; and (iii) synthetic cannabinoids, e.g., WIN 55,212-2 and HU-210. Synthetic cannabinoids include potent cannabinoid receptor agonists and antagonists/inverse agonists. Antagonists were suggested to have the ability for minimization or prevention of side effects of phytocannabinoids. Rimonabant (SR141716A) and its analogue AM251 (Gatley et al., 1997) are examples of antagonist/inverse agonists of the type 1 cannabinoid receptor (CB_1).

The discovery of cannabinoid receptors (Devane et al., 1988) and the endocannabinoid system were the key events in recognizing cannabinoid receptors as the principal molecular target of cannabinoids (Pertwee et al., 2010). There are two types of G protein-coupled cannabinoid receptors, CB_1 and CB_2. CB_1 receptors are abundant in neurons, where they modulate neurotransmission. CB_2 receptors are expressed in cells of the immune system and mediate immunosuppressive effects; however, the functional presence of CB_2 receptors in central nervous system was demonstrated both in glia and at synapses (Morgan et al., 2009; Onaivi et al., 2006). Both receptor types are coupled to $G_{i/o}$ proteins, negatively to adenylyl cyclase and positively to mitogen-activated protein kinase. Furthermore, CB_1 receptors are connected *via* $G_{i/o}$ proteins to ion channels, negatively to N-type and P/Q-type calcium channels and positively to various types of potassium channels (Turu and Hunyady, 2010). The biochemical targets of cannabinoids include both CB_1 and CB_2 receptors along with downstream targets, which are regulated by chronic drug administration and receptor-independent effects.

Cannabinoids are lipophilic molecules that are incorporated and accumulated into the cell membrane lipid bilayer. The physico-chemical properties of cannabinoids initially led to the assumption that they exert their effect *via* disturbance of the ordering of the lipid region of cell membranes (Leuschner et al., 1984; Mavromoustakos et al., 2001). Recently, it was confirmed that cannabinoids may influence lipid membrane domain formation (Tian et al., 2011) and activity of many membrane proteins in a receptor-independent mechanism (Maccarrone et al., 2011), including mitochondrial functions (Lenaz, 1987; Waczulikova et al., 2007 Zhang et al., 2013). The exact mechanisms of the action of cannabinoids have not been explained and the potential for modulators of the endocannabinoid system to induce both beneficial and harmful effects should be studied (Fowler et al., 2010; Sarne et al., 2011). In the brain, CB_1 receptor mediates the effects of endocannabinoids on neuronal transmission, plasticity and functions (Kano et al., 2009; Katona et al., 1999; Piomelli, 2003; Szabo et al., 1998). Functionally relevant levels of CB_1 receptors are also present in astrocytes (Stella, 2010) and participate in the release of inflammatory mediators, control of cellular metabolism, modulation of neuron–astrocytes communication and glutamate signaling.

1.2. Mitochondrial respiratory chain

Process of oxidative phosphorylation (OXPHOS) is performed by means of electron flow between complex I, III and IV or II, III and IV. Complex I, III and IV pump protons through inner mitochondrial membrane and proton gradient is utilized for ATP synthesis by ATP synthase (complex V). Respiratory complexes are multisubunit enzymes. There is electron transfer between the complexes mediated by lipid-soluble ubiquinone (between complex I or II and complex III) and water soluble cytochrome c (between complex III and complex IV). Complex III is known to exist in the membrane as a dimer. The OXPHOS protein complexes I, II, III and IV associate into stable respiratory supercomplexes (Chaban et al., 2014). In mammalian mitochondria, almost all the complex I are assembled into supercomplexes comprising complexes I and III_2 and up to four copies of complex IV (Schägger and Pfeiffer, 2000), these are called respirasomes. Recent studies have reported that dynamic supercomplex assembly determines optimal electron flux from different substrates through the respiratory chain (Acin-Perez and Enriquez, 2014; Chaban et al., 2014; Lapuente-Brun et al., 2013). It is proposed that the lipids (cardiolipin mainly) are required for the stability of the supercomplexes and also serve as a diffusion microdomain for the ubiquinone (Paradies et al., 2014; Pfeiffer et al., 2003).

Both, individual complexes and respirasome activities seem to be modulated by mitochondrial membrane potential and phosphorylation state of protein subunits (Genova and Lenaz, 2014). Cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA), present in subcellular compartments, including mitochondria (Sardanelli et al., 2006), phosphorylates mitochondrial proteins and plays a central regulatory role in energy-supplying and energy-requiring processes (Rosca and Hoppel, 2010). Complex I (Papa et al., 2012; Technikova-Dobrova et al., 2001; Yadava et al., 2008) and complex IV (Bender and Kadenbach, 2000) are the main targets for the regulation of oxidative phosphorylation by PKA.

1.3. Cannabinoids and mitochondria

Early studies suggest that cannabinoids affect mitochondrial function *via* lipophilic action (Bartova and Birmingham, 1976; Martin, 1986). Recently, endocannabinoid receptor signaling has been demonstrated to modulate numerous concomitant pathological processes, including neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress (Aso and Ferrer, 2014). Endocannabinoids, such as anandamide and 2-arachidonoylglycerol, were found to alter mitochondria-dependent signal transduction and thus participate in the regulation of energy homeostasis and apoptosis (Lipina et al., 2014). The upregulation of endocannabinoids may precede mitochondrial dysfunction in neurodegenerative disorders (Alger and Tang, 2012; Fagan and Campbell, 2014). Some effects of endocannabinoids are independent of their target cannabinoid receptors and are associated with a drug-induced increase in mitochondrial membrane fluidity (Catanzaro et al., 2009; Zaccagnino et al., 2012),

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dissipation of membrane potential, release of accumulated calcium, and opening of mitochondrial permeability transition pores (Wasilewski et al., 2004).

Cannabinoids are able to cause changes in integrated mitochondrial function, such as mitochondrial respiration and activities of individual respiratory complexes (Athanasiou et al., 2007). Recently, it was shown that CB_1 receptors are present in outer mitochondrial membranes, where their activation leads to decreased cAMP concentration, PKA activity, complex I activity, and mitochondrial respiration; i.e., activation of mitochondrial CB_1 receptors regulates energy metabolism (Bénard et al., 2012; Hebert-Chatelain et al., 2014). In this manner, exogenous and endogenous cannabinoids can activate mitochondrial CB_1 receptors and down-regulate mitochondrial respiration. It is hypothesized that mitochondrial CB_1 receptors depress mitochondrial respiration and enhance physiological responses mediated by endocannabinoids (Alger and Tang, 2012).

We hypothesize that the inhibitory effect of cannabinoids on mitochondrial respiratory rate is caused by the changes in dynamic properties of the lipid part of inner mitochondrial membrane, activation of mitochondrial CB_1 receptors, and assembly of respirasomes. The aim of our study was to confirm the roles of both receptor- and non-receptor-mediated mechanisms of action of cannabinoids with respect to changes in the energy states of cells characterized by the mitochondrial respiratory rate. We measured and analyzed the precise dependencies of cannabinoids concentration on mitochondrial respiratory rate using both agonists (THC, anandamide, WIN 55,212-2, and cannabidiol) and an inverse agonist (AM251) of CB_1 receptors (Fig. 1). Respiratory rate was driven by substrates of complex I, complex II, and complex IV, respectively. To discover the role of CB_1 receptors in the inhibition of mitochondrial respiration, titration was performed using cannabinoid receptor agonist THC in samples pretreated with antagonist/inverse agonist AM251 and *vice versa*. Data confirmed a model for effect of cannabinoids on mitochondrial respiration, which includes both receptor- and non-receptor-mediated mechanisms.

2. Materials and methods

2.1. Chemicals and solutions

The mitochondrial respiration medium (MiR05) consisted of sucrose 110 mM, K^+-lactobionate 60 mM, taurine 20 mM, $MgCl_2 \cdot 6H_2O$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA 1 g L^{-1} and HEPES 20 mM, adjusted to pH 7.1 with KOH (Pesta and Gnaiger, 2012). Substrates, inhibitors or cannabinoids were added to mitochondrial samples as described in the protocols below. The chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), except for anandamide and Tocrisolve from Tocris Bioscience (Tocris House, Bristol, UK).

Stock solutions used include 10 mg mL^{-1} digitonin (in dimethyl sulfoxide, DMSO), 0.8 M malate (in water), 2 M pyruvate (in water), 0.5 M adenosine diphosphate (ADP, in 0.3 M $MgCl_2$), 1 M succinate (in water), 1 M ascorbate (in water), 200 mM tetramethyl-*p*-phenylenediamine (TMPD, in ethanol), 4 mg mL^{-1} oligomycin (in ethanol), 1 mM rotenone (in ethanol), 0.5 mg mL^{-1} antimycin A (antimycin; in ethanol), 1 mM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, in ethanol), 5 mM THC (in ethanol), 3.18 mM cannabidiol (in methanol), 5 mM anandamide (in Tocrisolve), 5 mM WIN 55,212-2 (in methanol) and 10 mM AM251 (in DMSO).

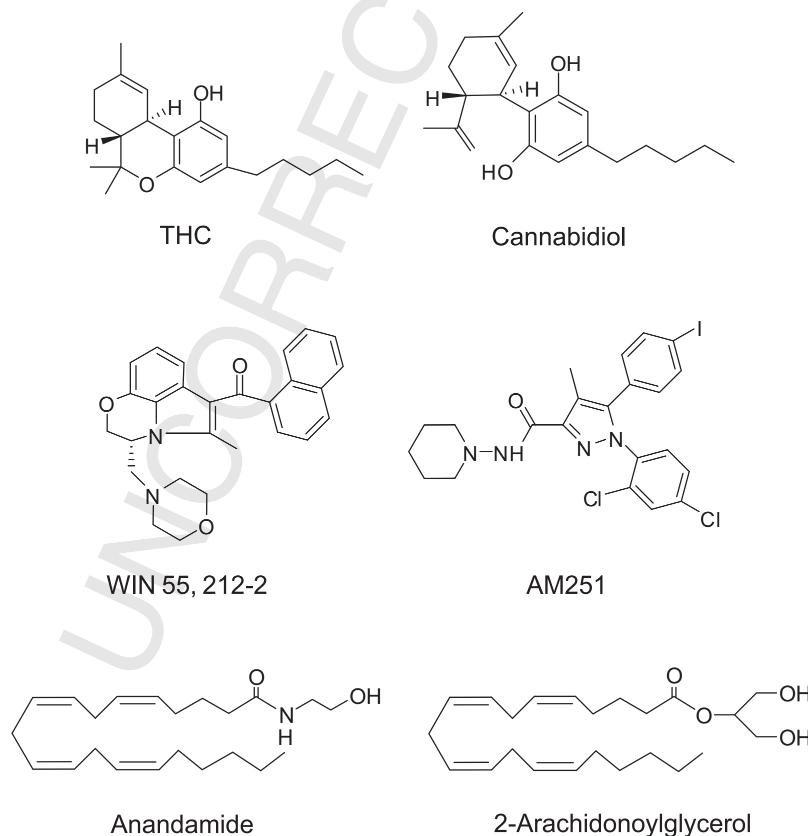

Fig. 1. Chemical structures of used cannabinoids. Phytocannabinoids, psychotropic Δ^9-tetrahydrocannabinol (THC) and non-psychoactive cannabidiol; endocannabinoids, anandamide and 2-arachidonoylglycerol; synthetic cannabinoid, WIN 55,212-2; and an antagonist/inverse agonist of cannabinoid receptors, AM251.

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2.2. Brain mitochondria

A high-quality preparation of crude mitochondrial fraction (mitochondria-enriched fraction) from tissue homogenate may represent an optimum compromise for a variety of respirometric, spectrophotometric and fluorometric studies (Brand and Nicholls, 2011). Crude mitochondrial fraction was isolated from pig brain cortex using homogenization and differential centrifugation, as previously described (Fišar, 2010). Brain cortex was separated without cerebellum, brain stem, and most of the midbrain in our experiments. Briefly, the brain cortex was gently homogenized in ten volumes (w/v) of ice-cold 0.32 M buffered sucrose supplemented with aprotinin, by means of a homogenizer with Teflon piston. The homogenate was centrifuged at 1000 g for 10 min to remove unbroken cells, nuclei and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions. Supernatants were collected and recentrifuged at 10,000 g for 15 min. The final pellet containing mitochondria was washed twice with buffered sucrose (10,000 g, 15 min), resuspended to a protein concentration of 20–40 mg mL^{-1}, and stored at 0–4 °C until the assay. The differential centrifugation guaranteed to remove whole cells or nuclei and to minimize possible contamination of crude mitochondria by microsomes, plasma membranes, lysosomes and cytosol (Whittaker, 1969; Brunner and Bygrave, 1969; Miller and Dawson, 1972; Wieckowski et al., 2009). To estimate effect of purity of mitochondria, we compared effect of THC on respiration in crude mitochondrial fraction and in mitochondria purified on sucrose (Pinna et al., 2003; Whittaker, 1969) or Ficoll density gradient (Clark and Nicklas, 1970). Enrichment of the sample by mitochondria was tested by monoamine oxidase activity (Fišar, 2010), citrate synthase activity (Srere, 1969), and respiratory rate. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

2.3. High-resolution respirometry

Mitochondrial respiratory rate was measured at 37 °C using a titration-injection high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) equipped with two closeable glass chambers with Clark-type electrodes. Experimental protocols were adapted from previous studies (Hroudová and Fišar, 2012; Pesta and Gnaiger, 2012).

The respiration medium MiR05 was added into chambers to a final volume of 2 mL. Following saturation of the medium with atmospheric oxygen, the chamber was closed using a stopper. Then, the crude mitochondrial fraction was added using a Hamilton syringe (final protein concentration was between 0.04 and 0.2 mg mL^{-1}) and both the oxygen concentration in μM and oxygen flux per mass (i.e., rate of oxygen consumption or respiratory rate) in pmol sec^{-1} mg^{-1} were monitored in the sample over time. Two parallel samples were titrated and measured simultaneously. The sample in the first chamber was titrated with the cannabinoid and the sample in the second chamber was titrated with the medium used as drug solvent.

The respiratory rate of mitochondria in several respiratory states related to electron entry through complex I, complex II or complex IV was assessed using specific mitochondrial substrates: malate + pyruvate for entry through complex I, succinate + rotenone for entry through complex II, and antimycin + ascorbate + TMPD for entry through complex IV. Oligomycin, rotenone, and antimycin were used as inhibitors of ATP synthase, complex I and complex III, respectively.

Several protocols using substrate control and the stepwise increase of cannabinoid concentrations were performed on digitonin-permeabilized (50 μg mL^{-1}) samples. A detailed description of experimental protocols and illustrative Oroboros Oxygraph-2k runs (Fig. S1) is provided in the Appendix A Supplemental methods, which is available online.

2.4. Data analysis

Data were collected and analyzed using DatLab 4.3 software (Oroboros Instruments, Innsbruck, Austria). Oxygen solubility factor relative to distilled water was set at 0.92. Respiratory rates (oxygen flux per mg protein) were expressed as pmol O_2 consumed per second relative to 1 mg of protein in the sample. Following subtraction of residual oxygen consumption from the other total respiratory rate values, the relative respiratory rates were determined after normalization to the rate in the absence of drug. All data presented are expressed as means ± standard error of the mean. Inhibition of respiratory rate with drugs was analyzed using a four-parameter logistic regression (SigmaPlot, Systat Software Inc., Richmond, CA, USA) to quantify the IC_{50} (the concentration of a drug that is required for 50% inhibition), the Hill slope (quantifies the steepness of a dose-response curve at its midpoint, which was used to determine the degree of cooperativity of the ligand binding to the enzyme) and the residual (residual activity at high drug concentration).

3. Results

The effects of several cannabinoids, both agonists of cannabinoid receptors (THC, cannabidiol, anandamide, and WIN 55,212-2) and an antagonist/inverse agonist of CB_1 receptor (AM251), on the respiration rate of pig brain mitochondria were assessed. Electron entry into OXPHOS was provided through complex I, complex II or complex IV. The combined effect of the CB_1 receptor agonist THC and the antagonist/inverse agonist AM251 was also determined.

3.1. Inhibition of respiration by cannabinoids

Data illustrating the inhibition of the respiratory rate by cannabinoids are shown in Fig. 2, and the parameters characterizing the strength of the tested cannabinoids in inhibiting the mitochondrial respiratory rate are summarized in Tables 1–3.

The phytocannabinoids THC and cannabidiol as well as endocannabinoid anandamide were confirmed to be full inhibitors of mitochondrial respiration supported by substrates of either complex I or complex II. In contrast, both WIN 55,212-2 and AM251 were revealed as partial inhibitors of the respiratory rate linked to complex I or complex II (Tables 1 and 2). Taking into account both IC_{50} and residual respirations at high cannabinoid concentrations, cannabidiol and THC exhibited the highest efficacy of inhibition amongst all the tested cannabinoids. There was strong correlation between inhibitory effects of cannabinoids on respiration supported through complex I and complex II: correlation coefficients were found 0.980 ($p = 0.003$) for IC_{50} and 0.973 ($p = 0.005$) for residual activity at high drug concentration.

Inhibitory effects of THC, cannabidiol, anandamide, and AM251 on mitochondrial respiration supported through complex IV (Table 3) were smaller compared to inhibition of respiration supported through complex I or complex II. WIN 55,212-2 induced small increase of respiratory rate linked with electron input through complex IV (Fig 2C).

Based on the IC_{50}, the strength of cannabinoids to inhibit mitochondrial respiration decreased in the following order: cannabidiol > WIN 55,212-2 > THC ≥ AM251 > anandamide for respiration supported by complex I substrates, WIN 55,212-2 = cannabidiol > AM251 = THC > anandamide for respiration supported by complex II substrates, and THC > cannabidiol > anandamide > AM251 for electron input

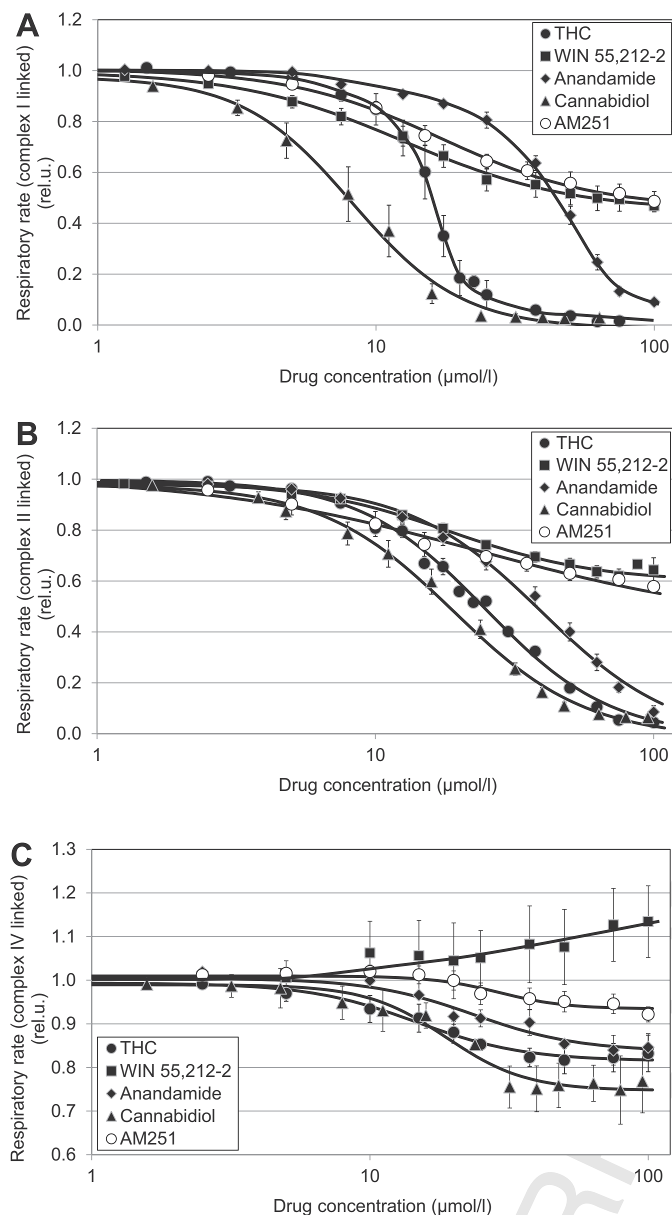

Fig. 2. Cannabinoid-induced inhibition of the respiratory rate in pig brain mitochondria incubated in substrates of electron supply through (**A**) complex I, (**B**) complex II, and (**C**) complex IV. Dose-response curves are displayed as plots of the respiratory rate against the drug concentration, presuming that the relative respiratory rate equals to 1 at zero drug concentration. Lines represent the best fitted curves using the four-parameter logistic function. Values are means from *n* independent experiments ± standard error of the mean (*n* values are shown in Tables 1–3). AM251, antagonist/inverse agonist of cannabinoid receptor type 1; THC, Δ^9-tetrahydrocannabinol.

Table 1
Inhibition of the respiratory rate in the pig brain crude mitochondrial fraction incubated in substrates of electron supply through complex I.

Drug	IC_{50}(μM)	Hill slope	Residual(rel.u.)	*n*
THC	15.1 ± 0.2	4.74 ± 0.30	0.028 ± 0.013	14
Anandamide	43.1 ± 1.4	2.65 ± 0.18	−0.015 ± 0.026	8
Cannabidiol	8.2 ± 0.6	2.20 ± 0.35	−0.014 ± 0.034	5
WIN 55,212-2	12.6 ± 1.8	1.45 ± 0.33	0.448 ± 0.037	8
AM251	17.2 ± 2.2	1.58 ± 0.31	0.459 ± 0.025	4
THC at 50 μM AM251	55.6 ± 3.1	2.31 ± 0.24	−0.029 ± 0.037	6
THC at 25 μM AM251	36.9 ± 2.6	2.40 ± 0.29	−0.054 ± 0.038	3
THC at 1 μM AM251	12.8 ± 1.2	2.58 ± 0.62	−0.004 ± 0.045	3

Values are means ± standard error calculated using SigmaPlot software. IC_{50}, half maximal inhibitory concentration; Hill slope, characterizes the slope of the inhibitory curve at its midpoint; *n*, number of independent experiments; residual, residual activity at high drug concentration; AM251, antagonist/inverse agonist of cannabinoid receptor type 1; THC, Δ^9-tetrahydrocannabinol.

through complex IV. Generally, anandamide was a less potent inhibitor of respiratory rate compared to THC and cannabidiol and inhibition by WIN 55,212-2 and AM251 was very low even at high drug concentrations. The Hill slopes of the most of inhibitory curves were significantly higher than 1.0, indicating a positively cooperative reaction.

We did not observe significant competition between cannabinoid THC and uncoupler FCCP (using protocol D in the Supplemental methods). Respiratory control ratio, calculated as ratio of respiratory rate after oligomycin addition to capacity of electron transport system (determined by titration with FCCP), was decreased from 7.9 to 4.3 in presence of 12.5 μM THC, which matches to the THC-induced inhibition of mitochondrial respiration supported through complex I. In other word, both respiratory rate and capacity of electron transport system were decreased by THC.

3.2. Combined effect of THC and AM251 on respiration

The role of the activation of CB_1 receptors in inhibition of the mitochondrial respiratory rate was examined in two ways: (1) the effect of THC was measured in a sample preincubated in the antagonist/inverse agonist AM251; (2) the effect of AM251 was measured in a sample preincubated in THC.

(1) The sample was preincubated in a fixed concentration (1 μM, 25 μM or 50 μM) of the antagonist/inverse agonist AM251 for 20 min followed by measurement of dose-response curves to determine the effects of AM251 on THC-induced inhibition of respiration driven by complex I or complex II substrates (Fig. 3). The inhibitory curves at 25 or 50 μM AM251 displayed a biphasic decrease in respiratory rate linked to complex I and a shift in the IC_{50} values, as well as the realization of full inhibition at a significantly higher THC concentration (Fig. 3, Tables 1 and 2).

(2) The sample was preincubated in THC (50 μM, 100 μM or 200 μM) for 20 min followed by measurement of dose-response curves to determine the effects of AM251 on respiration of the sample inhibited by THC (Fig. 4). The effect of AM251 was strongly dependent on the degree of THC-induced inhibition of respiratory rate. We detected an AM251-induced increase in respiratory rate in the case of preincubation in a high THC concentration (when respiration was below 15% of the rate before addition of THC).

Table 2
Inhibition of the respiratory rate in the pig brain crude mitochondrial fraction incubated in substrates of electron supply through complex II

Drug	IC_{50}(μM)	Hill slope	Residual(rel.u.)	*n*
THC	24.8 ± 0.4	1.91 ± 0.06	−0.013 ± 0.011	8
Anandamide	39.6 ± 1.4	1.82 ± 0.09	−0.023 ± 0.023	6
Cannabidiol	19.1 ± 1.1	1.83 ± 0.18	−0.017 ± 0.034	5
WIN 55,212-2	18.5 ± 2.0	1.64 ± 0.28	0.597 ± 0.023	8
AM251	23.6 ± 7.3	0.86 ± 0.19	0.424 ± 0.062	4
THC at 50 μM AM251	65.1 ± 65.6	0.79 ± 0.22	0.045 ± 0.039	4

Values are means ± standard error calculated using SigmaPlot software. IC_{50}, half maximal inhibitory concentration; Hill slope, characterizes the slope of the inhibitory curve at its midpoint; *n*, number of independent experiments; residual, residual activity at high drug concentration; AM251, antagonist/inverse agonist of cannabinoid receptor type 1; THC, Δ^9-tetrahydrocannabinol.

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Table 3
Inhibition of the respiratory rate in the pig brain crude mitochondrial fraction with electron entry through complex IV

Drug	IC_{50}(μmol/L)	Hill slope	Residual(rel.u.)	n
THC	14.7 ± 2.8	2.23 ± 1.00	0.815 ± 0.016	6
Anandamide	23.7 ± 3.9	2.30 ± 0.85	0.837 ± 0.018	3
Cannabidiol	18.8 ± 3.4	3.02 ± 1.36	0.747 ± 0.025	3
WIN 55,212-2	n.d.	n.d.	n.d.	
AM251	29.1 ± 6.8	3.59 ± 2.48	0.934 ± 0.012	3

Values are means ± standard error calculated using SigmaPlot software. IC_{50}, half maximal inhibitory concentration; Hill slope, characterizes the slope of the inhibitory curve at its midpoint; *n*, number of independent experiments; residual, residual activity at high drug concentration; AM251, antagonist/inverse agonist of cannabinoid receptor type 1; THC, Δ^9-tetrahydrocannabinol.

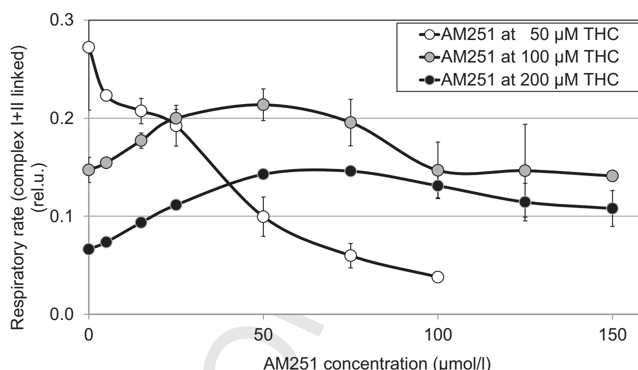

Fig. 4. AM251-induced changes in the respiratory rate in pig brain mitochondria incubated in substrates of electron supply through both complex I and complex II. The samples were preincubated in 50 μM (*n* = 3 independent experiments), 100 μM (*n* = 3) or 200 μM (*n* = 4) THC for 20 min and titration of AM251 followed. Dose-response curves are displayed as plots of the respiratory rate against the AM251 concentrations, presuming that the relative respiratory rate equals to 1 before addition of THC. Values are means ± standard error of the mean. AM251, antagonist/inverse agonist of cannabinoid receptor type 1; THC, Δ^9-tetrahydrocannabinol.

4. Discussion

This study presents for the first time data concurrently pointing to receptor and non-receptor mode of action of cannabinoids on mitochondria. The results suggested that both non-receptor- and receptor-mediated mechanisms are involved in the regulation of mitochondrial respiration by cannabinoids (Fig. 5). Non-receptor-mediated mechanisms are evidenced by the different potencies of several cannabinoids to inhibit the mitochondrial respiratory rate (Fig. 2, Tables 1–3). CB_1 receptor-controlled mechanisms are evidenced by the changes in respiratory rate induced by the interaction of an agonist and an inverse agonist of the CB_1 receptor (Figs. 3A and 4).

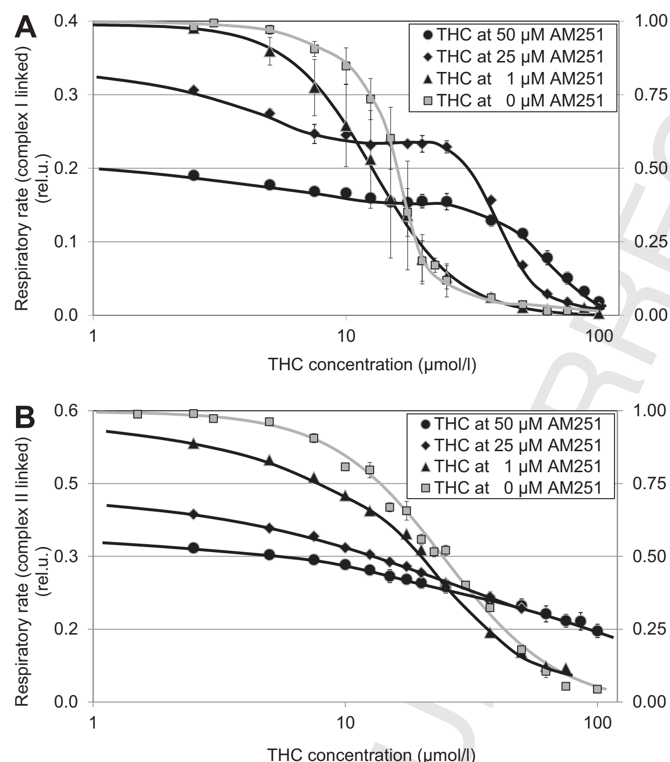

Fig. 3. THC-induced inhibition of the respiratory rate in pig brain mitochondria incubated in substrates of electron supply through (**A**) complex I or (**B**) complex II. The samples were preincubated in 1 μM, 25 μM or 50 μM antagonist/inverse agonist AM251 for 20 min and titration of THC followed. Dose-response curves are displayed as plots of the respiratory rate against the THC concentrations, presuming that the relative respiratory rate equals to 1 before addition of AM251. Values are means from *n* independent experiments ± standard error of the mean (*n* values are shown in Tables 1 and 2). Inhibitory curves at 1 μM AM251 or without preincubation in AM251 are displayed using the secondary axis on the right. THC, Δ^9-tetrahydrocannabinol.

Cannabinoid-induced changes in mitochondrial membrane dynamic properties have been suggested to explain the inhibition of mitochondrial functions by cannabinoids; however, the recent discovery that CB_1 receptors are localized to neuronal mitochondria (Bénard et al., 2012) supported the participation of receptor-mediated mechanisms. Discussion is in progress about possible technical issues that can occur when existence of CB_1 receptor on mitochondria is evidenced (Hebert-Chatelain et al., 2014b; Morozov et al., 2013, 2014). Recent publications (Hebert-Chatelain et al., 2014a; Vallée et al., 2014) evidenced the existence of mitochondrial CB_1 receptors and our results supported their role in regulation of mitochondrial respiration.

4.1. Non-receptor mechanisms

All the tested cannabinoids were found to inhibit the mitochondrial respiratory rate. Relatively small cannabinoid-induced inhibition of respiratory rate supported through complex IV as compared to complex I or II supported respiration (Fig. 2, Tables 1–3) indicates minor role of complex IV in mechanisms of action of THC.

The effective doses of the cannabinoids reaching half the maximal respiratory rate were in the range of 8 to 43 μM, which is similar to the data from previous study (Athanasiou et al., 2007). However, higher doses of cannabinoids were necessary to see the respiration effects compared to recent studies (Bénard et al., 2012; Hebert-Chatelain et al., 2014a) that can be related to the procedure of sample preparation. Purity of mitochondrial fraction and concentration of endocannabinoids may modify effect of exogenously added cannabinoids. Endocannabinoids are known to increase in the brain after death (Schmid et al., 1995). However, we do not suppose that high levels of endocannabinoids were responsible for lower inhibitory potency of *in vitro* added cannabinoids because pig brains were stored in ice-cold medium and processed during 2 h after death. Purity of mitochondrial sample might be more important (Hebert-Chatelain et al., 2014a; Morozov et al., 2014).

It is difficult to isolate very pure mitochondrial fractions from the brain using current methods, i.e., mitochondrial fraction can be contaminated with synaptosomes and fragments of plasma membranes. It was previously reported that WIN 55,212-2, at concentration 100 nM, reduced mitochondrial respiration in brain purified mitochondria but not in mitochondria-enriched fraction

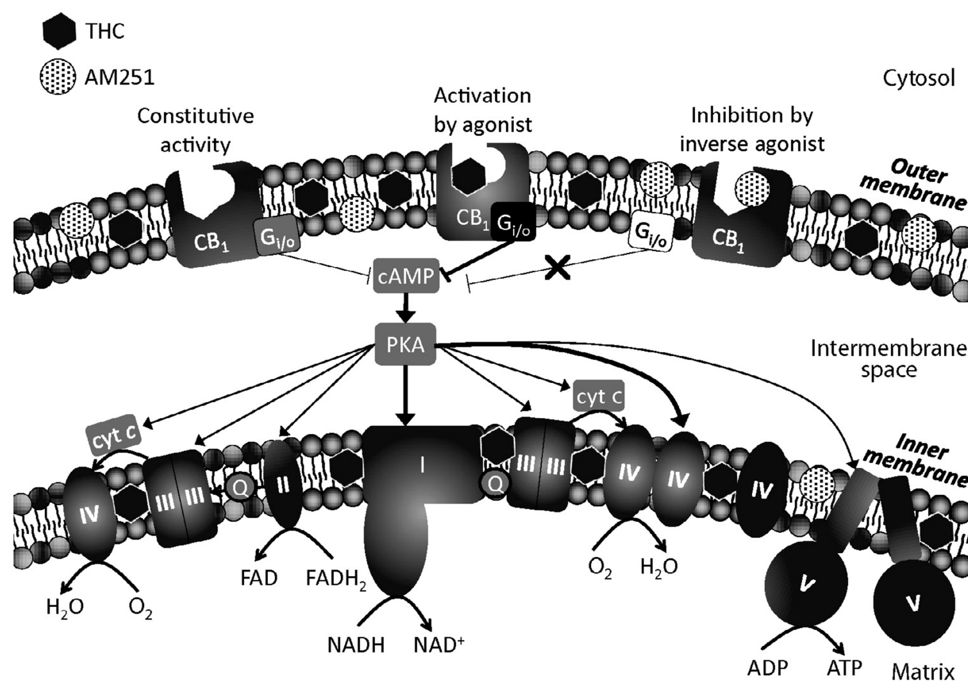

Fig. 5. Model representing receptor and non-receptor inhibition of mitochondrial respiration by cannabinoids. Receptor mechanism is based on the finding that cannabinoid receptors type 1 (CB_1) are localized in outer mitochondrial membrane and activation of these $G_{i/o}$ protein coupled receptors induce decreased phosphorylation and altered activity of components of respiratory chain, complex I especially (Alger and Tang, 2012; Bénard et al., 2012). Constitutive activity of CB_1 receptors causes low $G_{i/o}$ activation also in the absence of agonist. Antagonist/inverse agonist of CB_1 receptor (AM251) eliminates the intrinsic receptor activity. Δ^9-tetrahydrocannabinol (THC)-induced activation of CB_1 leads to decrease in cyclic adenosine monophosphate (cAMP) production, protein kinase A (PKA) activity, and phosphorylation of all respiratory complexes, which affect their assembly and the activity. Non-receptor mechanism of inhibition of mitochondrial respiration by cannabinoids is based on accumulation of cannabinoids in hydrophobic parts of inner mitochondrial membrane, which can impair molecular interactions and assembly of respiratory chain. Model of supramolecular organization of respiratory complexes was under consideration, in our model. ATP synthase is marked as complex V.

(Hebert-Chatelain et al., 2014a). We verified that effective doses of the cannabinoids reaching half the maximal respiratory rate decreased in purified mitochondria, while IC_{50} remained in micromolar region (Supplemental methods, Fig. S2, Table S1). Thus, we suppose that apparently low inhibitory potencies of cannabinoids are associated with their high lipophilicity and accumulation in cell membranes. It results in much lower free cannabinoid concentration compared to bulk cannabinoid concentration. This approach is advisable in such type of experiments when effects on mitochondrial respiration are measured without artefacts due to sample purification and when relative inhibitory potency of cannabinoids is the determining factor for data interpretation.

According to PubChem Compound database, atomic based prediction (Xlog *P*), a conventional method for predicting log *P*, was determined 7.0 for THC, 6.5 for cannabidiol, 5.4 for anandamide, 4.4 for WIN 55,212-2, and 6.5 for AM251. We did not find any significant correlation between respiratory parameters (Tables 1–3) and Xlog *P* values. It indicates that differences in potency of various cannabinoids to inhibit mitochondrial respiration are not related to their lipophilicity.

It is evident that, considering the affinity and mode of action of tested cannabinoids (Mechoulam et al., 2007; Pertwee et al., 2010), the receptor-mediated mechanism cannot explain (i) similar inhibitory effects of THC and cannabidiol, (ii) different effects of WIN 55,212-2 compared to THC, cannabidiol or anandamide, and (iii) similar effects of WIN 55,212-2 and AM251 (Fig. 2, Tables 1 and 2). Consequently, the present investigation confirmed a significant role of non-receptor-mediated mechanisms in the inhibition of mitochondrial respiration by cannabinoids.

Value of Hill slope higher than 1 (Tables 1–3) indicates that once the cannabinoid molecule is bound to the membrane, binding affinity for other cannabinoid molecules increases. The effect was the most marked for THC; we suppose that it reflects increased binding affinity of THC into THC-disturbed membrane. Low Hill slopes might be expected if two distinct sites (membrane and receptor) are involved. Thus, high Hill slopes indicate that an effect of binding to CB_1 receptor is too small and/or there is no overlap in binding affinities of cannabinoids to membrane and to CB_1 receptor. Decrease of Hill slopes of THC-induced inhibitory curves in AM251-princubated samples support the view that the inhibition of respiratory rate is caused by competitive binding of both the drugs to the inner mitochondrial membrane.

Drug metabolism by some microsomal and mitochondrial enzymes can impact the pharmacological response to cannabinoids. Brain mitochondria have substantial levels of endocannabinoid-degrading activity. While fatty-acid amide hydrolase, the primary degradative enzyme for AEA, was known to be densely present in the mitochondria, Bénard et al. (2012) found that purified mitochondria also have substantial 2-arachidonoylglycerol degrading activity attributable to monoacylglycerol lipase. THC, cannabidiol and synthetic cannabinoids are primarily metabolized by cytochrome P450 enzymes, which are abundant in the liver and are also expressed in the brain (Ferguson and Tyndale, 2011; Huestis, 2007). We did not observe any change in percent of inhibition, when crude mitochondrial preparation was incubated with cannabinoid for tens of minutes. It indicates that either metabolism of cannabinoids is very low or metabolites show the same inhibition as original cannabinoids. However, we cannot completely exclude that Hill slope is affected due to more effective metabolism of cannabinoids at lower than at higher drug concentrations.

Further argument for non-receptor mechanism of action of cannabinoids on mitochondrial respiration states that only approximately 30% of neuronal mitochondria contain CB_1

receptors (Bénard et al., 2012). It can be concluded that non-receptor changes in the properties of the lipid region of membranes play a crucial role in full inhibition of respiration at high concentrations of THC, cannabidiol and anandamide (Fig. 2).

The significantly higher IC_{50} of anandamide compared to THC (Fig. 2, Tables 1 and 2) can be accounted on the basis of different effects of THC and anandamide on the membrane microviscosity: higher anandamide concentrations caused increased fluidity, whereas a decrease in fluidity was induced by THC at concentrations above 10 μM (Velenovská and Fišar, 2007). The different effect of anandamide on membrane dynamics can be explained by different chemical structure of endocannabinoids compared to both phytocannabinoids and synthetic cannabinoids (Barnett-Norris et al., 1998). Different inhibitory effect of WIN 55,212-2 (compared to THC) can reflect major structural differences existing between aminoalkylindoles and other cannabinoid receptor agonists (Pertwee et al., 2010). The antagonist/inverse agonist of the CB_1 receptor, AM251, was found to be a weak partial inhibitor of mitochondrial respiration. Due to the mode of pharmacological action of AM251, its inhibitory effect can be fully attributed to the non-receptor-mediated mechanism.

4.2. Receptor mechanisms

Processes leading to decreased mitochondrial respiration due to activation of mitochondrial CB_1 receptors contribute to the "depolarization-induced suppression of inhibition", a process that is initiated by retrograde activation of presynaptic CB_1 receptors at GABAergic terminals (Kano et al., 2009; Katona et al., 1999; Piomelli, 2003; Szabo et al., 1998). Thus, inhibition of mitochondrial respiration supports the endocannabinoid-dependent form of synaptic plasticity (Bénard et al., 2012). Model was introduced, which describes the role of presynaptic plasma membrane CB_1 receptors as well as mitochondrial CB_1 receptors in cannabinoid-induced suppression of complex I and inhibition of respiration (Alger and Tang, 2012; Bénard et al., 2012). The model includes processes initiated by the stimulation of mitochondrial CB_1 receptors followed by inhibition of the cAMP/PKA pathway, complex I activity and mitochondrial respiration. In this manner, mitochondrial CB_1 receptors are involved in the regulation of energy metabolism and contribute to a cannabinoid-dependent form of short-term plasticity of inhibitory GABAergic neurotransmission. We implicated the effect of PKA mediated phosphorylation of several components of respiratory chain in our model (Fig. 5), although an effect of phosphorylation on function of these components is generally unknown (Rosca and Hoppel, 2010).

Cannabinoids in micromolar concentration were required to observe their receptor-mediated effect on mitochondrial respiration (Figs. 3 and 4). It might be due to low purity of mitochondria (Hebert-Chatelain et al., 2014a), when accumulation of cannabinoids occurs in plasma and mitochondrial membranes and CB_1 receptors are activated in both synaptosomal and mitochondrial membranes. We suppose that relatively high drug concentration, at which we observed receptor-mediated effects, is related to the very high nonspecific to specific binding ratios of cannabinoids. However, while brain mitochondrial purification is difficult, we maintain that when a drug produces a significant effect on mitochondrial respiratory rate, one can conclude that observed effect is related to processes on the inner mitochondrial membrane, independently of purity of mitochondrial fraction.

Nonexistent or opposing receptor-mediated effects of an inverse agonist might be expected as compared to agonists. It should be noted that the inverse agonist actions are based solely on its ability to reverse the constitutive activity (signal transduction in the absence of exogenously applied agonists) of the CB_1 receptor (Howlett et al., 2011). Involvement of the effects of both CB_1 receptor activity and non-receptor-mediated mechanism is necessary to interpret the inhibitory curves in Fig. 3A:

1. The initial decrease in biphasic course of respiratory rate can be ascribed to the reactivation of CB_1 receptors by THC, i.e., to the change in CB_1 receptor activity when the antagonist/inverse agonist AM251 is displaced by the agonist THC.
2. Full inhibition of the respiratory rate is reached at significantly higher THC concentration compared to the sample without preincubation in AM251, which reflects the protective role of AM251 in the non-receptor-mediated mechanism of action of THC. We propose that (i) the effect of AM251 on the structure and ordering of the lipid bilayer is rather different from the effects of THC (Fig. 5); (ii) a higher THC concentration is required for full inhibition of the respiratory rate because THC must displace AM251 from the CB_1 receptors to reach full inhibition.

In contrast, the inhibitory curve in Fig. 3B does not display a biphasic course, which may reflect an independence of the respiratory rate linked to complex II from the activation of the CB_1 receptor. The higher THC concentration required to inhibit respiration linked to complex II in the sample preincubated in AM251, reflects a protective action of AM251. This result confirmed that there is the same or a similar protective role of AM251 in the non-receptor-mediated mechanism of inhibition of respiration linked to both complex II and complex I.

We propose that the AM251-induced increase in the respiratory rate of mitochondria preincubated in a high concentration of THC (Fig. 4) may occur due to partial competitive displacement of THC from the mitochondrial CB_1 receptor binding sites by AM251; i.e., the increase in the respiratory rate *via* AM251 reflects the reversion of CB_1 receptor activation. The subsequent decrease in the respiratory rate at higher AM251 concentrations can be ascribed to the cumulative non-receptor-mediated mechanisms of inhibition *via* both AM251 and THC.

5. Conclusions

The effects of cannabinoids with different affinity for CB_1 receptors and different modes of action suggested a significant role of non-receptor-mediated mechanisms in inhibition of mitochondrial respiration. Concurrently, our data supported some role of receptor-mediated mechanisms in effects of cannabinoids on respiration, because participation of CB_1 receptor activation/inhibition can elucidate both (i) biphasic course of respiratory rate and the decrease in potency of THC to inhibit the respiratory rate in samples preincubated in the antagonist/inverse agonist AM251 and (ii) the AM251-induced increase in respiratory rate in samples preincubated in a high concentration of THC. Our work provides rationale for both receptor- and non-receptor-mediated mechanisms of cannabinoid inhibitory action on mitochondrial respiration.

Conflict of interest

The authors declare that they have no conflict of interest.

Uncited reference

Q4

Watanabe et al. (1988).

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2014.09.002.

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