Biapigenin Modulates the Activity of the Adenine Nucleotide Translocator in Isolated Rat Brain Mitochondria

ARTICLE *in* NEUROTOXICITY RESEARCH · AUGUST 2009

Impact Factor: 3.54 · DOI: 10.1007/s12640-009-9082-5 · Source: PubMed

5 AUTHORS, INCLUDING:

Paulo J Oliveira
University of Coimbra
229 PUBLICATIONS **3,137** CITATIONS
SEE PROFILE

Alberto C P Dias
University of Minho
72 PUBLICATIONS **1,210** CITATIONS
SEE PROFILE

João O Malva
University of Coimbra
112 PUBLICATIONS **2,833** CITATIONS
SEE PROFILE

Neurotox Res
DOI 10.1007/s12640-009-9082-5

Biapigenin Modulates the Activity of the Adenine Nucleotide Translocator in Isolated Rat Brain Mitochondria

Bruno A. Silva · Paulo J. Oliveira · Armando Cristóvão · Alberto C. P. Dias · João O. Malva

Received: 20 February 2009 / Revised: 1 May 2009 / Accepted: 30 June 2009

Abstract In this study, we investigated the effects of biapigenin, a biflavone present in the extracts of *Hypericum perforatum*, in rat brain mitochondrial bioenergetics and calcium homeostasis. We found that biapigenin significantly decreased adenosine diphosphate (ADP)-induced membrane depolarization and increased repolarization (by 68 and 37%, respectively). These effects were blocked by atractyloside and bongkrekic acid, but not oligomycin. In the presence of biapigenin, an ADP-stimulated state 3 respiration was still noticeable, which did not happen in the presence of adenine nucleotide translocator (ANT) inhibitors. Taking in consideration the relevance of the ANT in the modulation of the mitochondrial permeability transition pore (mPTP), mitochondrial calcium homeostasis was evaluated alone or in the presence of biapigenin. We found that biapigenin reduces mitochondrial calcium retention by increasing calcium efflux, an effect that was blocked by ADP plus oligomycin, an efficient blocker of the mPTP in brain mitochondria. Taken together, the results in this article suggest that biapigenin modulates mPTP opening, possibly by modulating ANT function, contributing for enhanced mitochondrial calcium efflux, thereby reducing calcium burden and contributing for neuroprotection against excitotoxicity.

Keywords *Hypericum perforatum* · Biapigenin · Mitochondria · Calcium homeostasis · Mitochondrial permeability transition pore

Abbreviations

$\Delta\Psi_m$	Mitochondrial transmembrane electric potential
ANT	Adenosine nucleotide translocator
CsA	Cyclosporin A
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
mPTP	Mitochondrial permeability transition pore
TPP	Tetraphenylphosphonium-chloride

Introduction

Calcium-mediated mitochondrial dysfunction results in sustained mitochondrial depolarization, affecting ATP synthesis, and increasing the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively). All these events contribute to the opening of the mitochondrial permeability transition pore (mPTP), and are interconnected in such a way that the onset of one of these events aggravates and facilitates the occurrence of the others (Brookes et al. 2004). The mPTP is a non-specific pore

B. A. Silva · P. J. Oliveira · A. Cristóvão · J. O. Malva
Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

J. O. Malva (✉)
Faculty of Medicine, Institute of Biochemistry, University of Coimbra, 3004-504 Coimbra, Portugal
e-mail: jomalva@fmed.uc.pt

A. Cristóvão
Department of Zoology, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

B. A. Silva · A. C. P. Dias
Department of Biology, Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Published online: 14 July 2009

resulting from the assembly of several mitochondrial proteins, and it is thought to be involved in both mitochondrial physiology and pathologic events (Kroemer et al. 2007). Under certain circumstances, the mPTP has been proposed to be a major player in the events associated with calcium-induced mitochondrial dysfunction (Ichas and Mazat 1998). Taking in consideration the functional relevance of the mPTP in the regulation of cellular energy status and in the decision of cell fate, potential therapeutic targets for the treatment of several neurodegenerative diseases have been considered in the pore complex (Parihar and Brewer 2007; Rasola and Bernardi 2007). In particular, compounds targeting the mPTP, either directly or indirectly, by reducing oxidative stress or mitochondrial calcium overload, are seen as potential source of future therapeutic tools.

Nowadays, *Hypericum perforatum* extracts are widely used as antidepressant agents being considered the Prozac from plants (Di Carlo et al. 2001). Moreover, some compounds present in these extracts were shown to possess neuroprotective properties against in vitro models of excitotoxicity (Kumar et al. 2006) and to afford neuroprotection against hypoxia-ischemic brain damage (Shin et al., 2006a). *H. perforatum* extracts are now recognized as potential beneficial products toward ischemia-reperfusion-related neurodegeneration (De Paola et al. 2005; Kumar et al. 2006).

Several flavonoids are present in significant amounts in *H. perforatum* extracts. Traditionally, flavonoids have been viewed as antioxidants (Rice-Evans 2001), but were also shown to be neuroprotective against glutamate-induced toxicity, by increasing intracellular glutathione (GSH) levels, lowering ROS levels, decreasing calcium influx, and inhibiting caspase activation (Ishige et al. 2001; Cho and Lee 2004; Lee et al. 2004; Yazawa et al. 2006; Shin et al. 2006b). Some studies have also reported the ability of flavonoids to inhibit mPTP opening (Santos et al. 1998; Park et al. 2003) and some studies report kaempferol and quercetin to directly activate the mitochondrial calcium uniporter in HeLa-transfected cells, suggesting a possible direct interaction with the uniporter or uniporter-associated proteins (Montero et al. 2004). Recently, we observed that biapigenin, a singular biflavone presents in *H. perforatum* extracts, affords neuroprotection to cultured hippocampal neurons in an in vitro model of excitotoxicity, and this effect was, in part, associated with a better control of calcium homeostasis (Silva et al. 2008). These results support the idea that mitochondria are a possible target for the neuroprotection exerted by biapigenin, and based on our previous results, we hypothesize that biapigenin modulates one component of the phosphorylative system, namely the adenine nucleotide translocator (ANT). In this study, we investigated putative mitochondrial targets of biapigenin with a critical role in the regulation of mitochondrial bioenergetics, calcium homeostasis, and cell survival.

Experimental Procedures

Materials

Calcium Green-5N was supplied by Invitrogen (USA). Adenosine diphosphate monopotassium salt dihydrate, triphosphate magnesium salt, atractyloside, bongkrekic acid, bovine serum albumin fatty acid free, cyclosporin A, oligomycin, protease (Subtilisin, Carlsberg) type VIII, and tetraphenylphosphonium-chloride (TPP) were obtained from Sigma (Spain). Digitonin was obtained from Calbiochem (USA). Biapigenin was isolated by preparative HPLC from an *H. perforatum* extract, as described elsewhere (Dias et al. 1998). Purity was 98–99%. All the other chemicals used were of the highest grade of purity commercially available.

Animal Care

Male and female Wistar rats were purchased from Charles River (Barcelona, Spain) and maintained in local animal house facilities (CNC—Faculty of Medicine, University of Coimbra, Coimbra, Portugal). Animals were housed in groups of five animals per cage (max.), and had access to food (Ø15 mm pellets, 15–25 g/day, Charles River, Spain) and water (pH 3.5). Room temperature was kept at 21–23°C and the moisture at 50–60%. Animals were subjected to a 12-h day/night cycle. The research procedure was carried out in accordance with the European Directive 86/609/EEC. Animals were killed by cervical dislocation and then decapitated.

Mitochondrial Respiratory Chain and Mitochondrial Transmembrane Electric Potential Assays

Brain mitochondria were isolated from male Wistar rats (7–9 weeks old), using a method previously described and used to evaluate mitochondrial transmembrane electric potential ($\Delta\Psi_m$) and respiration (Silva et al. 2008). The $\Delta\Psi_m$ was monitored by evaluating transmembrane distribution of the lipophilic cation TPP ion (TPP^+) using a TPP-selective electrode with a calomel electrode as reference (Kamo et al. 1979). The difference in potential between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Kipp and Zonen recorder. Reactions were carried out at 30°C in a chamber with magnetic stirring in a 1 ml medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4,

10 μM EGTA, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, supplemented with 2 μM rotenone) and containing 3 μM TPP-Cl (Oliveira et al. 2004; Silva et al. 2008).

In a first approach, mitochondria (0.8 mg/ml) were incubated for 3 min with different concentrations of biapigenin. Reaction was started by adding 8 mM succinate to mitochondria in suspension. After reaching a steady-state distribution of TPP (plateau), 125 μM adenosine diphosphate (ADP) was added and alterations in $\Delta\Psi_m$ recorded. Adequate controls were performed without addition of biapigenin, but respecting the same 3 min lag phase used in the test conditions. Atractyloside (40 μM), bongkrekic acid (15 μM), oligomycin (5 μM), and ADP (125 μM) plus oligomycin were also tested, alone or in the presence of biapigenin (10 μM).

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in a 1 ml thermostatic, water-jacketed closed chamber with magnetic stirring (Estabrook 1967). State 3 respiration is defined as the consumption of oxygen in the presence of substrate and ADP, whereas state 4 respiration is defined as the consumption of oxygen after ADP consumption. Reactions were carried out at 30°C. Uncoupled respiration was assessed after the addition of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5 μM) to energized mitochondria. Mitochondrial respiration was not altered by the presence of TPP (data not shown). Respiratory control rate (RCR) values obtained were in accordance with the expected values for brain mitochondria and previously reported (Moreira et al. 2005).

In a second approach, mitochondria were energized with ATP (3 mM) in the presence of biapigenin (10 μM, 3 min pre-incubation). The effect of atractyloside (40 μM) and bongkrekic acid (15 μM) in ATP-induced energization was also evaluated, alone or in the presence of biapigenin.

Mitochondrial Calcium Loading Capacity and Calcium Efflux

Calcium retention was assessed by following Calcium Green-5N fluorescence. Appropriate calibration was performed with the pulses of 7 nmol Ca^{2+}/mg protein, added to mitochondria (0.2 mg/ml) in suspension. Reactions occurred at 30°C in a quartz cuvette with 2 ml reaction medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 10 μM EGTA, 5 mM HEPES, pH 7.4, supplemented with 2 μM rotenone) under magnetic stirring. Changes in fluorescence intensity were monitored using a fluorimeter Perkin-Elmer LS 50B (excitation at 506 nm, emission at 531 nm, 5 nm emission and excitation slits), and calcium uptake was assessed after energization with 4 mM succinate (Oliveira et al. 2003). When steady-state fluorescence was achieved, FCCP was added to evaluate total calcium release due to mitochondrial depolarization and, therefore, the amount of calcium that was accumulated due to mitochondrial transmembrane electric potential. Calcium uptake into mitochondria assessed with this method was 90–95% of the total calcium uptake (data not shown). Calcium efflux was evaluated in energized mitochondria upon the addition of a single calcium pulse (42 nmol Ca^{2+}/mg protein) added 3 min after the addition of mitochondria to reaction medium. The effect of biapigenin (10 μM) or drugs tested in calcium homeostasis was evaluated after 3 min incubation. Adequate controls were performed in order to assess possible interferences of biapigenin with the probe fluorescence, under either low or high calcium concentrations. No interference was observed for the experimental conditions used. When described, biapigenin or drugs were added after the calcium pulse to evaluate possible effects in calcium efflux. EGTA (40 μM) was added after achieving final steady-state fluorescence.

ATPase Activity

The ATPase activity was determined by following the production of protons resulting from ATP hydrolysis, in accordance with the potentiometric method described by Madeira et al. (1974). Reactions were carried out at 30°C in an open chamber with magnetic stirring in 2 ml of reaction medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 0.5 mM HEPES-K, 10 μM EGTA, pH 7.3). Mitochondria (0.8 mg/ml) were incubated for 3 min with 10 μM biapigenin. Reactions were started by adding 2 mM ATP-Mg to mitochondria in suspension, and changes in pH were recorded for 5 min. The addition of oligomycin at the end of the experiment completely abolished proton release. Adequate controls were performed without addition of compounds. The pH changes were continuously monitored with a pH meter, with the electrode inserted in the reaction medium under magnetic stirring. pH variations were registered in a Perkin-Elmer recorder (Model 56) connected to the pH set through a circuit of compensation of basal-voltage. Internal calibration was performed at the end of each experiment, with the addition of adequate (600 nmol) amounts of NaOH, and counter-titrated by adding the same amount of HCl (due to instability of NaOH in solution).

ATP-Synthase Activity

The ATP-synthase activity was measured by following the pH variations associated to ATP-synthesis, through the use of the potentiometric method described previously (Madeira et al. 1974). Reactions were carried out at 30°C in an open chamber with magnetic stirring in 2 ml of reaction

medium without HEPES (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 10 μM EGTA, pH 7.3). Mitochondria (0.8 mg/ml) were incubated for 3 min with 10 μM biapigenin. Reactions were started by adding 8 mM succinate to mitochondria in suspension. One minute after energization, an aliquot of 125 μM ADP was added and changes in pH were recorded. Adequate controls were performed without the addition of compounds. Internal calibration was performed at the end of each experiment, with the addition of adequate amounts of HCl (5 nmol).

Adenylate Nucleotide Quantification

Adenylate nucleotides were recovered by using an acidic extraction procedure and separated by reverse-phase liquid chromatography, as described previously with some minor modifications (Stocchi et al. 1985). All the extraction procedures were carried out at 0–4°C to minimize nucleotides degradation. Adenylate nucleotides were extracted from succinate-energized brain mitochondria incubated with either biapigenin alone or in combination with other drugs, after a complete phosphorylation cycle (1 min after total ADP phosphorylation). From the reaction medium, 300 μl was removed from an eppendorf tube containing oligomycin plus ice-cold 0.5 M perchloric acid ($HClO_4$). The mixture was then centrifuged (14,000 rpm at 4°C, for 5 min). The pellets were stored at −80°C for protein quantification (using BioRad protein assay). The supernatant was recovered and the pH set to 6.5 with ice-cold 2.5 M KOH in 1.5 M KH_2PO_4 and centrifuged (14,000 rpm at 4°C, for 2 min). The new obtained supernatant was recovered with extreme caution, in order to avoid the soluble permanganate salts produced, and stored at −80°C for further chromatographic analysis.

Samples were centrifuged at 14,000 rpm for 5 min before injecting into the HPLC system. Adenine nucleotides (ATP, ADP, and AMP) concentration in each sample were determined by HPLC according to a method previously described (Ferreira et al. 1997) in a Gilson-Asted system, consisting of a Pump model 305 coupled to a computer, by an interface system model 506C and Gilson Software 715 HPLC Controller. In brief, adenine nucleotides were separated on a Lichrospher 100 RP-18 (5 μm, 125 mm) from Merck (Darmstadt, Germany), protected by a guard column Lichrospher 100 RP-18 (5 μm, 4 mm). During each run, an isocratic elution with 100 mM potassium phosphate buffer (pH 6.0) and 1% methanol was performed for 8 min, at a flowrate of 1.1 ml/min. Detection was performed using a Gilson UV detector Model 116, at 254 nm. The concentration of the nucleotides was determined after running standard nucleotide solutions in the same conditions, and chromatograms were later analyzed with Gilson software. The detection limit for each analyte was 1–2 pmol per injection.

Data Analysis and Statistics

Results are presented as means ± SE of the indicated number of experiments, usually run in triplicate or quadruplicate unless otherwise stated. Statistical significance was determined using the Student's *t*-test or the one-way ANOVA test for multiple comparisons, with Tukey's post-hoc test.

Results

Biapigenin Decreases the ADP-Induced Dissipation of the Mitochondrial Transmembrane Electric Potential, But Potentiates ADP-Induced Hyperpolarization

The effect of biapigenin in mitochondrial bioenergetics was evaluated by following TPP^+ uptake in isolated rat brain mitochondria, which is an indirect estimation of the mitochondrial transmembrane electric potential ($\Delta\Psi_m$), and by following mitochondrial respiration assessed by monitoring oxygen consumption. Isolated rat brain mitochondria (0.8 mg/ml) were incubated with several concentrations of biapigenin. Resting mitochondria exhibited a $\Delta\Psi_m$ of −180.7 ± 2.3 mV. At a concentration of 10 μM, biapigenin significantly reduced ADP (125 μM)-induced depolarization (by 67.8%; $P < 0.05$, when compared to the control) and significantly increased mitochondrial repolarization (by 371.7%; $P < 0.01$, when compared to the control). Results are shown in Fig. 1 and summarized in Table 1.

ANT Blockers Inhibit the Hyperpolarizing Effect of Biapigenin in the Presence of ADP

The results reported above suggest that biapigenin can exert a modulatory effect in the phosphorylative system. To identify a possible target for biapigenin, we compared the effects of biapigenin with known modulators of the mitochondrial phosphorylative system, the ATP synthase and the adenine nucleotide translocator (ANT). Typical behavior of isolated mitochondria is represented in Fig. 2a. The effect of added biapigenin to isolated mitochondria is represented in Fig. 2b, whereas oligomycin, atractyloside, or bongkekic acid were also added individually to isolated mitochondria in order to test their modulatory effect (Fig. 2c, e, and g, respectively). We observed that biapigenin-induced ADP-dependent hyperpolarization was not affected by oligomycin (1 μg/ml, Fig. 2d), a drug that

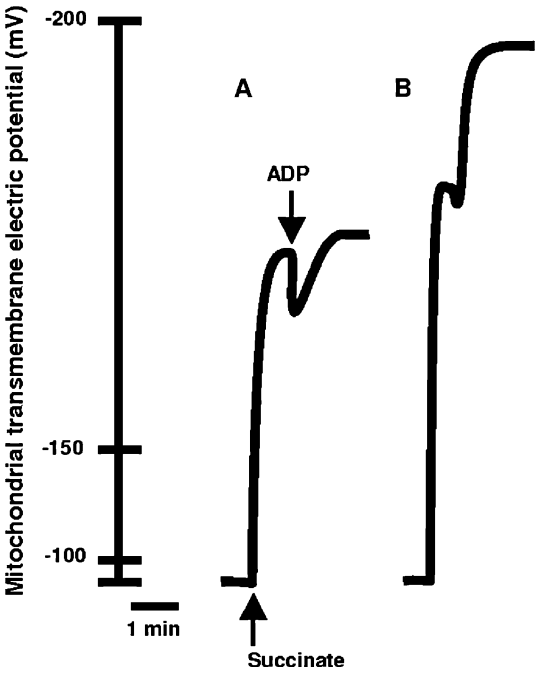

Fig. 1 Biapigenin significantly reduces ADP (125 μM)-induced depolarization and causes ADP-induced hyperpolarization in isolated rat brain mitochondria. Representative traces of mitochondrial TPP^+ uptake by isolated mitochondria energized with succinate (8 mM) in **a** control and **b** following incubation with biapigenin (10 μM) (added 3 min before energization with succinate). Values shown in Table 1 are shown as mean ± SEM of five to six independent experiments. * $P < 0.05$, ** $P < 0.01$ (when compared with control)

Table 1 Effect of different concentrations of biapigenin in mitochondrial transmembrane electric potential ($\Delta\Psi_m$)—assessed by energization with succinate, after ADP addition (depolarization) and in the repolarization phase

Biapigenin (μM)	$\Delta\Psi_m$ (mV)		
	Energization	Depolarization	Repolarization
0	−180.7 ± 2.3	20.2 ± 3.7	−2.0 ± 0.6
0.3	−181.3 ± 1.6	12.9 ± 0.3	−2.1 ± 0.6
1	−184.1 ± 0.1	14.0 ± 0.7	−2.3 ± 1.1
3	−186.8 ± 2.7	10.6 ± 0.7	−5.3 ± 1.2
10	−188.1 ± 3.1	6.5 ± 1.4*	−9.5 ± 1.0**
30	−186.6 ± 3.4	4.0 ± 2.1*	−8.3 ± 1.3**

inhibits proton flow through the F_O subunit of the ATP synthase, although inhibitors of the ANT, atractyloside (40 μM), and bongkrekic acid (16 μM) were able to prevent the observed effect (Fig. 2f and h, respectively).

Biapigenin Does Not Significantly Affect ADP-Induced Increase in Respiration

Mitochondrial respiration was assessed by monitoring oxygen consumption in isolated rat brain mitochondria. Mitochondria (0.8 mg/ml) were energized with succinate (8 mM). No significant differences were observed in state 2 respiration (substrate-driven basal respiration) after 3 min pre-incubation with biapigenin (10 μM), atractyloside (40 μM), bongkrekic acid (16 μM), or oligomycin (1 μg/ml). Despite the notorious effect of biapigenin on ADP-induced depolarization, stimulation of mitochondrial respiration with ADP (state 3 respiration) was not blocked by biapigenin. As expected, atractyloside and bongkrekic acid blocked state 3 respiration. Representative recordings for control and for biapigenin-treated mitochondria are shown in Fig. 3a. Atractyloside-mediated inhibition of ADP-stimulated oxygen consumption was decreased in the presence of biapigenin (Fig. 3b, c), an effect which was not observed with bongkrekic acid (Fig. 3c). In the presence of biapigenin, the inhibition of state 3 respiration mediated by atractyloside was decreased (Fig. 3b, c). However, the inhibitory effect of bongkrekic acid was not altered in the presence of biapigenin (Fig. 3c).

Biapigenin Inhibits FCCP-Uncoupled Respiration in Energized Mitochondria

Maximal uncoupled respiration was monitored after FCCP (1 μM) addition to the reaction medium (representative traces are shown in Fig. 4a). Biapigenin (10 μM) significantly decreased FCCP-induced stimulation of mitochondrial respiration (32% reduction, $P < 0.05$ when compared to control; Fig. 4a, b). Interestingly, the effect of biapigenin on uncoupled respiration was comparable to that of atractyloside (40 μM, Fig. 4b). In contrast, although bongkrekic acid (16 μM) per se did not affect FCCP-induced uncoupled respiration, co-incubation with biapigenin (10 μM) significantly decreased maximal respiration induced by FCCP (59%, $P < 0.001$ when compared to control; Fig. 4b).

Biapigenin Potentiates ATP-Induced Hyperpolarization

ATPase activity was evaluated by monitoring TPP^+ uptake upon addition of ATP (3 mM) in the presence of 2 μM rotenone, reflecting membrane potential induced by ATP hydrolysis. Representative recordings of TPP^+ uptake are shown in Fig. 5a. Biapigenin (10 μM) significantly increased $\Delta\Psi_m$ generated by adding ATP (3 mM) to the reaction medium (30%, $P < 0.01$ when compared to control; Fig. 5a, b). Atractyloside (40 μM) and bongkrekic acid (16 μM) significantly reduced ATP-mediated energization (12 and 30%, respectively). Interestingly, atractyloside was unable to block the effect of biapigenin, whereas bongkrekic acid significantly inhibited the hyperpolarizing effect of biapigenin in the presence of ATP

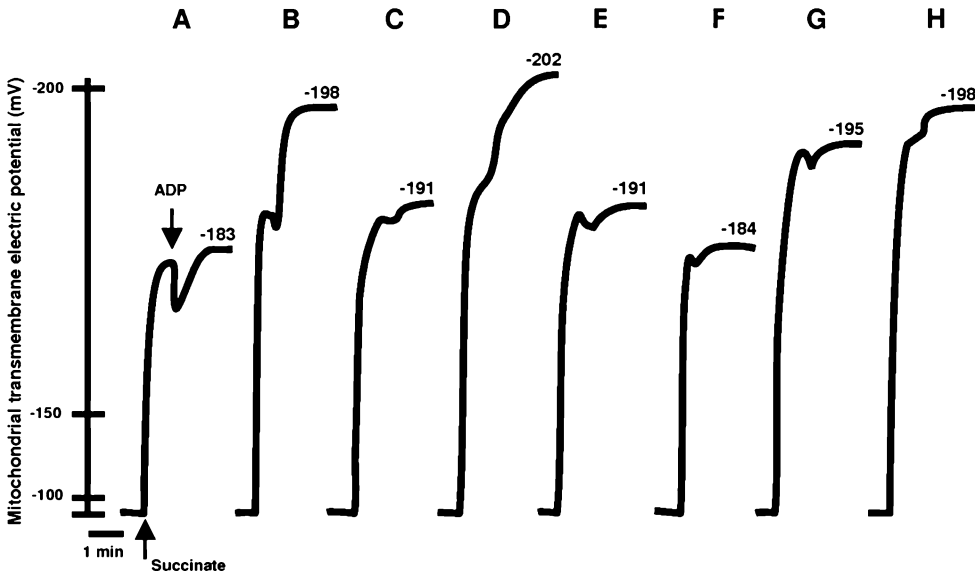

Fig. 2 Inhibitors of the ANT block the biapigenin-mediated ADP-induced hyperpolarization. Mitochondrial transmembrane potential ($\Delta\Psi_m$) (indirectly evaluated by TPP^+ uptake) was monitored in isolated rat brain mitochondria energized with succinate. Representative traces are shown; values of $\Delta\Psi_m$ after repolarization (post-ADP) are indicated. **a** control, **b** biapigenin (10 μM), **c** oligomycin (1 μg/ml), **d** oligomycin plus biapigenin, **e** atractyloside (40 μM), **f** atractyloside plus biapigenin, **g** bongkrekic acid (16 μM), **h** bongkrekic acid plus biapigenin

(Fig. 5b). Oligomycin (1 μg/ml) also inhibited the effect of biapigenin on ATP-induced $\Delta\Psi_m$ generation (Fig. 5b).

The ATP-hydrolyzing (ATPase) activity was also assessed indirectly by monitoring pH changes in freeze-thaw mitochondria. The release of protons resulting from ATP hydrolysis due to the ATPase activity was followed after the addition of 3 mM ATP to mitochondria. In clear opposition with the previous results, ATPase activity was significantly reduced in the presence of biapigenin (16%, $P < 0.05$ when compared to control; Fig. 5c).

Biapigenin Reduces ATP-Synthase Activity in Intact Mitochondria

The ATP-synthase activity was assessed in energized mitochondria in the presence of biapigenin, atractyloside, bongkrekic acid, and oligomycin. Biapigenin (10 μM) significantly reduced ATP synthase activity (40% reduction; $P < 0.01$ when compared to the control; Fig. 6b). Atractyloside (40 μM), bongkrekic acid (16 μM), and oligomycin (1 μg/ml) were more powerful inhibitors of ATP-synthase activity than biapigenin (91, 96, and 99% reduction, respectively; $P < 0.001$ when compared to control; Fig. 6a). Accordingly, biapigenin (10 μM) reduced the ADP/O ratio and the lag phase (measurement of time required for recovery from ADP-induced depolarization) by 26 and 79%, respectively.

Biapigenin and ANT Inhibitors Decrease ATP Synthesis

To confirm a possible inhibitory effect of biapigenin on ATP synthesis, we measured the levels of adenine nucleotides after a complete phosphorylative cycle; which roughly translated into 1 min after ADP addition to energized mitochondria (the average time required by control mitochondria to phosphorylate added ADP). Biapigenin (10 μM) significantly inhibited ATP-synthesis (82% reduction; $P < 0.001$, when compared to the control). Atractyloside (40 μM), bongkrekic acid (16 μM), and oligomycin (1 μg/ml) significantly inhibited ATP synthesis (83, 95, and 94% reduction, respectively; $P < 0.001$, when compared to the control). Results are shown in Table 2.

Biapigenin Decreases Calcium Accumulation

The ability of mitochondria to accumulate calcium was evaluated in energized mitochondria by the use of a low affinity calcium-sensitive probe, Calcium Green-5N. Mitochondria (0.2 mg/ml) were energized with succinate in the presence of 15 μM $CaCl_2$ and 100 nM Calcium Green-5N in the assay medium. Mitochondrial calcium accumulation was recorded by following the decrease in fluorescence intensity (reflecting decrease calcium concentration in the medium and accumulation into mitochondria). The concentration of biapigenin, atractyloside, and bongkrekic acid was adjusted to the amount of protein used, so that data could be compared between different experimental protocols with different protein concentrations. Representative traces are shown in Fig. 7a–c. Biapigenin (10 μM) significantly reduced calcium accumulation. Upon incubation with cyclosporin A (0.6 μM), an increase in calcium-loading capacity was observed. Interestingly, biapigenin was able to prevent the effect of cyclosporin A on calcium accumulation (Fig. 7a, d).

To ascertain if the effect of biapigenin in maximal calcium accumulation was due to decreased calcium uptake or

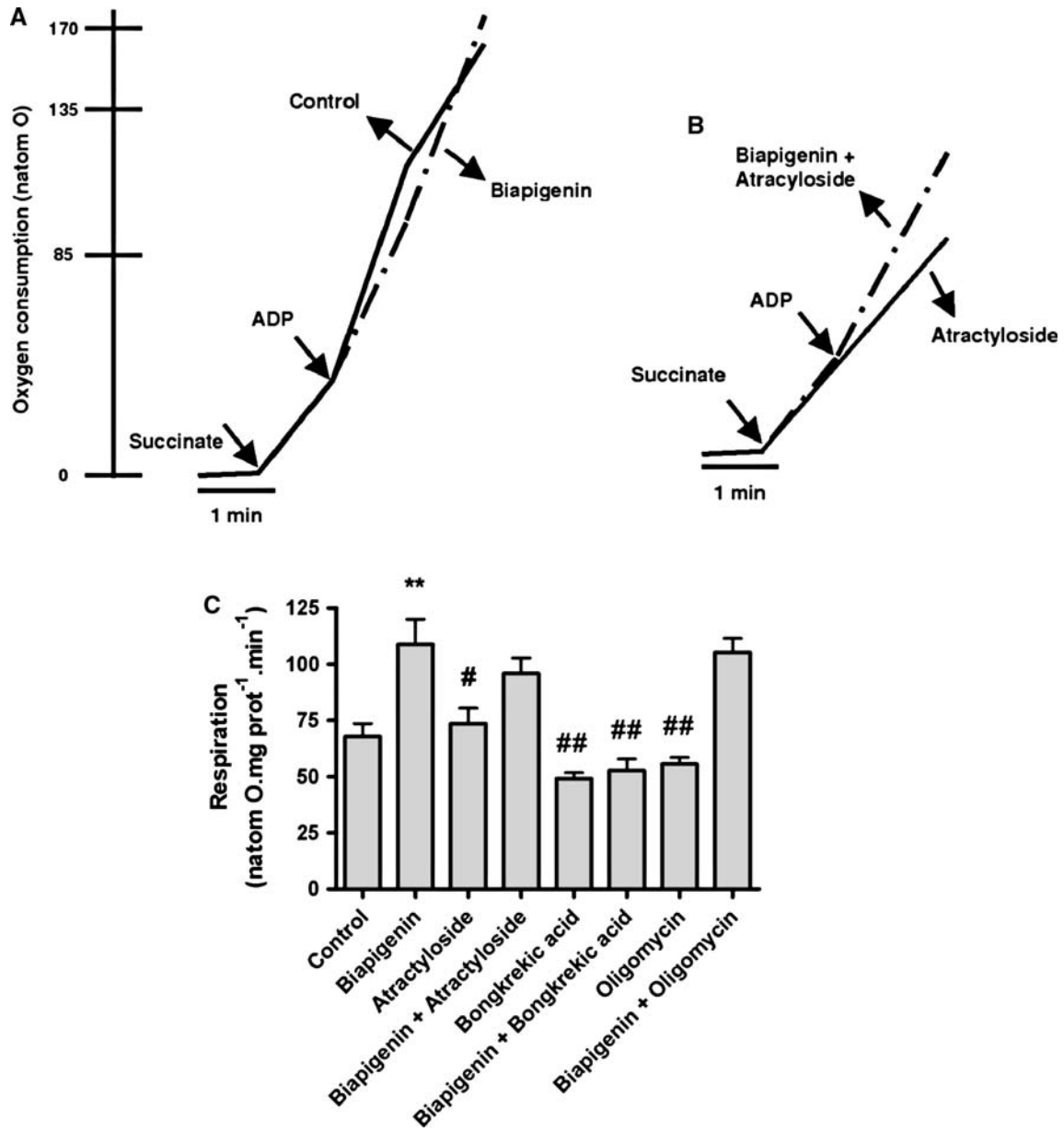

Fig. 3 State 3 respiration is not significantly blocked by biapigenin. Isolated rat brain mitochondria (0.8 mg/ml) were incubated for 3 min with biapigenin (10 μM), before energization with succinate (8 mM). Addition of 125 μM ADP was performed 1 min after energization. **a** Representative recordings of mitochondrial respiration for a control, and for mitochondria pre-incubated with biapigenin (10 μM). **b** Pre-incubation with atractyloside (40 μM) inhibited ADP-induced stimulation of respiration, which was preserved in the presence of biapigenin (10 μM). **c** Respiration was evaluated after the addition of 125 μM ADP to energized mitochondria. Values are presented as mean ± SEM from three to four independent experiments. ** $P < 0.01$ (comparatively to control); $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ (comparatively to biapigenin)

due to increased efflux, we evaluated calcium efflux in energized mitochondria. Mitochondria energized with succinate (8 mM) were exposed to a single pulse of 40 μM $CaCl_2$ (Fig. 8a–e, indicated by full arrow). Two approaches were used to test the effect of biapigenin. In the first one, 10 μM biapigenin was pre-incubated for 3 min before to the calcium pulse (bright green); in a second approach, a pulse of biapigenin was added 1 min after the calcium pulse (to assess a direct effect in calcium efflux; dark green—Fig. 8a).

Pre-incubation with 10 μM biapigenin reduced maximal calcium accumulation (Fig. 8a, bright green), whereas addition of biapigenin (10 μM) to calcium-loaded mitochondria induced calcium efflux as can be observed in Fig. 8a (dark green). Although bongkrekic acid (16 μM) increased maximal calcium accumulation in the presence

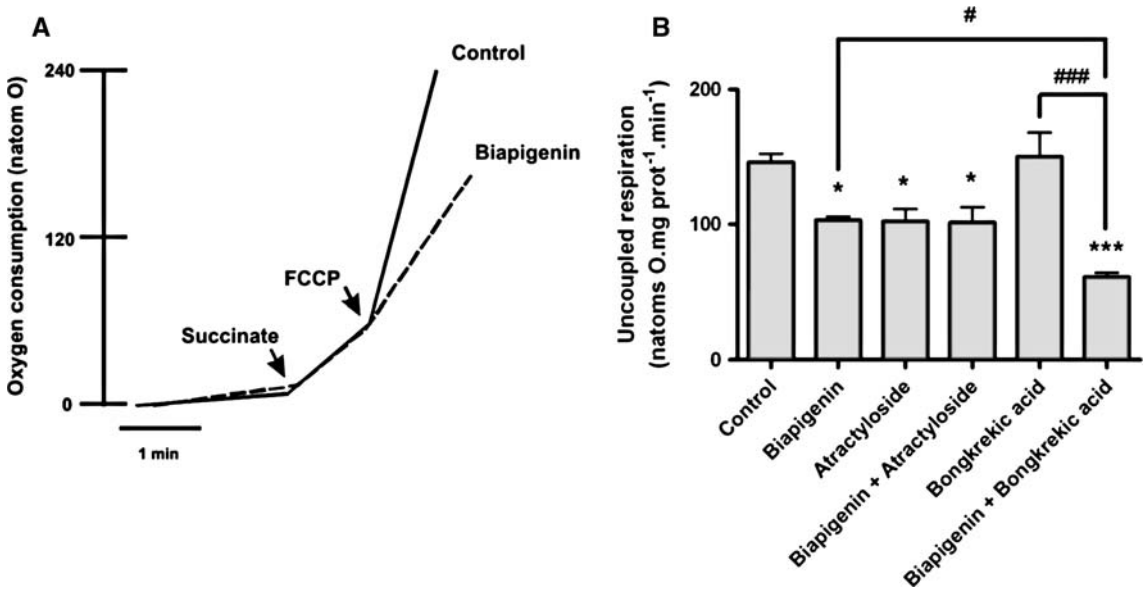

Fig. 4 Biapigenin reduces mitochondrial respiration stimulated by FCCP. Isolated rat brain mitochondria (0.8 mg/ml) were incubated for 3 min with biapigenin (10 μM) before energization. FCCP (1 μM) addition was performed 1 min after energization. **a** Representative traces of mitochondrial respiration in the control and in mitochondria pre-incubated with 10 μM biapigenin. **b** Mitochondrial uncoupled respiration was assessed by monitoring oxygen consumption after addition of 1 μM FCCP to energized mitochondria. Values are presented as mean ± SEM from three to four independent experiments. * $P < 0.05$, *** $P < 0.001$ (comparatively to control); # $P < 0.05$, ### $P < 0.001$

of biapigenin, it was insufficient to prevent biapigenin-induced calcium efflux (Fig. 8b). Atractyloside (40 μM) alone increased calcium efflux (Fig. 8c) and when co-incubated with biapigenin the effect was apparently additive (Fig. 8c, dark red). Moreover, cyclosporin A (0.6 μM) reduced biapigenin-induced calcium efflux (Fig. 8d), whereas ADP (125 μM) plus oligomycin (1 μg/ml) completely abolished the effects of biapigenin in calcium accumulation and efflux (Fig. 8e).

Discussion

Novel therapeutic strategies against several forms of neuronal or cardiac injury (Belisle and Kowaltowski 2002; Kristal et al. 2004; Wu et al. 2006; Halestrap et al. 2007) may involve direct inhibition of mPTP by agents such as cyclosporin A and, possibly, diazoxide, or indirect inhibition, by reducing oxidative stress and/or mitochondrial calcium overload. Cyclosporin A binds to cyclophilin D, which is known to interact with the ANT facilitating a calcium-induced rearrangement of the ANT into a pore-forming conformation (Halestrap et al. 2002). Therefore, cyclosporin A increases mitochondrial calcium-loading capacity by inhibiting mPTP opening. Diazoxide targets the phosphorylative system and it has been reported to be neuroprotective in in vitro models of neurotoxicity (Kowaltowski et al. 2006) and in in vivo models of ischemia-reperfusion (Murata et al. 2001; Teshima et al. 2003). Moreover, diazoxide also inhibits ATP degradation during the ischemic phase, while it has no deleterious effects in normal mitochondria (Comelli et al. 2007). It might seem somehow controversial the idea that inhibition of mitochondrial phosphorylation can result in neuroprotection, especially because under physiological conditions cells critically require ATP synthesis. However, under stressful conditions, with impairment of mitochondrial function, such as in excitotoxic or ischemia-reperfusion events, it has been described that inhibitors of the phosphorylative system can be neuroprotective.

Naturally occurring flavonoids are able to prevent mitochondrial lipid peroxidation and can inhibit mPTP opening (Santos et al. 1998). Moreover, flavonoids are endowed with free radical scavenging and antioxidant properties (Schroeter et al. 2000; Rice-Evans 2001), which can also contribute to the inhibitory effect of flavonoids toward mPTP opening. These studies suggest that some flavonoids are able to interact with mitochondrial physiology, exerting neuroprotective actions, especially when able to target the mPTP complex.

Biapigenin Inhibits Proton Flow Through the ANT

Biapigenin (10 μM) significantly inhibited ADP-induced depolarization, and significantly increased repolarization following ADP addition. Inhibitors of the ATP synthase

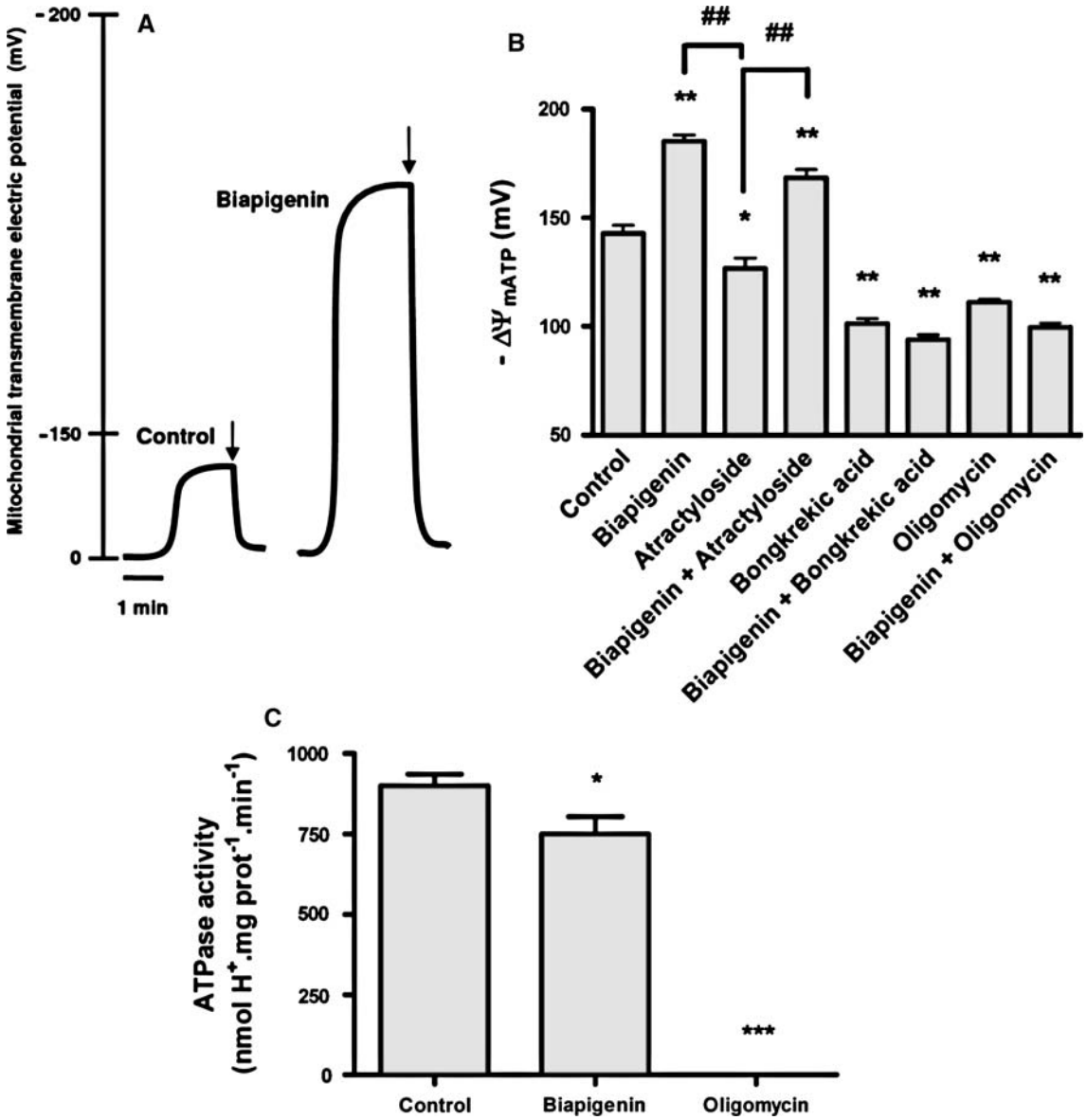

Fig. 5 Effect of biapigenin in ATP-induced mitochondrial energization. $\Delta\Psi_m$ was measured by monitoring TPP^+ uptake by mitochondria upon addition of 3 mM ATP to the reaction medium. Biapigenin (10 μM) was pre-incubated for 3 min. **a** Representative recordings of ATP-induced mitochondrial energization for a control and biapigenin-treated mitochondria. *Arrows* indicate addition of oligomycin (1 μg/ml). **b** Biapigenin (10 μM) significantly increased ATP-induced energization (from 142 to 185 mV; $P < 0.01$, when compared with control). Atractyloside (40 μM), bongkrekic acid (16 μM), and oligomycin (1 μg/ml) significantly inhibited ATP-induced energization. **c** ATPase activity (assessed in disrupted mitochondria) was slightly, but significantly, decreased in the presence of biapigenin (10 μM). Oligomycin was used as a control. Values are presented as mean ± SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (comparatively to control); $^{\#\#}$ $P < 0.01$

(e.g., oligomycin) or inhibitors of the ANT (bongkrekic acid or atractyloside) also significantly inhibited ADP-induced depolarization.

Oligomycin, a specific inhibitor of proton flow through the F_O subunit of the ATP synthase (Zanotti et al. 1992), was unable to block the hyperpolarizing effect of biapigenin, which suggests that the ATP synthase is not a major player on the effect caused by biapigenin on ADP-induced depolarization and repolarization.

Atractyloside (40 μM) and bongkrekic acid (16 μM), two specific inhibitors of the ANT (Bruni et al. 1964; Henderson and Lardy 1970; Dahout-Gonzalez et al. 2005), were able to inhibit the hyperpolarizing effect of biapigenin in the presence of ADP (Fig. 2F and H, respectively). Atractyloside blocks the ANT in a pore-forming conformation, termed *c*-conformation (*c*, for cytosolic side), whereas bongkrekic acid blocks the ANT in a non-pore forming conformation, termed *m*-conformation (*m*, for

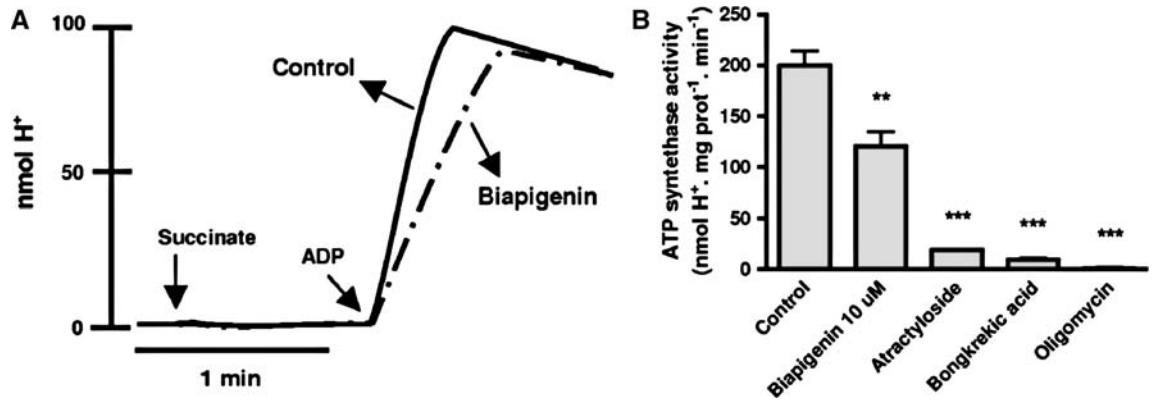

Fig. 6 Effect of biapigenin in the ATP synthase activity in rat brain mitochondria (0.8 mg/ml). ATP-synthase activity was assessed by monitoring pH variations in energized mitochondria after addition of 125 μM ADP. At the end of each experiment, calibration was performed by adding 50 nmol NaOH and counter-tittered with 50 nmol HCl. **a** Representative recordings of ATP-synthase activity for control and biapigenin-treated mitochondria. **b** ATP-synthase activity was decreased after incubation with biapigenin (10 μM) and robustly inhibited in the presence of atractyloside (40 μM), bongkrekic acid (16 μM), or oligomycin (1 μg/ml). Values are presented as mean ± SEM from three independent experiments. ** $P < 0.01$, *** $P < 0.001$ (comparatively to control)

Table 2 Mitochondrial adenylate nucleotides (pmol/mg per protein) measured 1 min after addition of ADP (125 μM) to mitochondria energized with 8 mM succinate

	ATP		ADP		AMP	
	−biapigenin	+biapigenin	−biapigenin	+biapigenin	−biapigenin	+biapigenin
Control	29739 ± 667	5411 ± 755***	1396 ± 198	24211 ± 2650***	1357 ± 112	1881 ± 68**
Atractyloside	5012 ± 590***	2973 ± 295	24086 ± 2157***	28545 ± 1142	1785 ± 157**	1496 ± 30
Bongkrekic acid	1439 ± 147***	1588 ± 48	26786 ± 901***	30456 ± 1332	1833 ± 74**	1574 ± 92
Oligomycin	1914 ± 56***	1934 ± 253	29085 ± 1662***	31171 ± 758	2112 ± 69***	1844 ± 201

Biapigenin (10 μM) was incubated for 3 min alone or co-incubated with atractyloside (40 μM), bongkrekic acid (16 μM), or oligomycin (1 μg/ml). Values are presented as mean ± SEM from three independent experiments

** $P < 0.01$, *** $P < 0.001$ (comparatively to the control)

matrix side) (Dahout-Gonzalez et al. 2005). The inhibitory effect of atractyloside and bongkrekic acid on the hyperpolarization induced by biapigenin, after ADP addition, suggests that the effects of biapigenin occur at the ANT level.

As expected, increased oxygen consumption due to ADP addition (state 3 respiration) to energize mitochondria was reduced in the presence of the inhibitors of the ANT, atractyloside and bongkrekic acid. Interestingly, the effect of bongkrekic acid on state 3 respiration was not affected by biapigenin (data not shown), whereas the inhibitory effect of atractyloside on state 3 respiration was prevented by biapigenin (Fig. 3c). Taken together, these results further support the notion that the effects of biapigenin occur at the ANT level, and also indicate that they are conformational dependent.

Uncoupled respiration induced by FCCP was significantly inhibited in the presence of both biapigenin and atractyloside, whereas bongkrekic acid had no significant effect (Fig. 4). Several authors suggested that FCCP mechanism does not involve membrane translocation with the participation of the ANT (Brustovetsky et al. 1990; Andreyev et al. 2005); however, we found that FCCP-induced uncoupled respiration was inhibited in the presence of atractyloside. Based on the present data, we cannot discard a possible involvement of the ANT in the uncoupling effects mediated by FCCP in brain. The majority of these previous studies were performed using liver mitochondria, where the major ANT isoform found is the ANT2, whereas in brain, the most common isoform is ANT1 (Dorner et al. 1999). It is possible that differences in tissue expression of a specific isoform may account for observed differences in the effect of several molecules including FCCP, ANT inhibitors, or even biapigenin. The results also suggest that biapigenin may also inhibit the respiratory chain. Interestingly, the ANT has been suggested to act as a proton channel (Brustovetsky and Klingenberg 1994; Shabalina et al. 2006). It has been proposed that the ANT1 and ANT2 are responsible for different aspects of proton conductance: the ANT1 is more associated with aspects of basal proton conductance, whereas the ANT2 seems to play a significant role in uncoupled proton

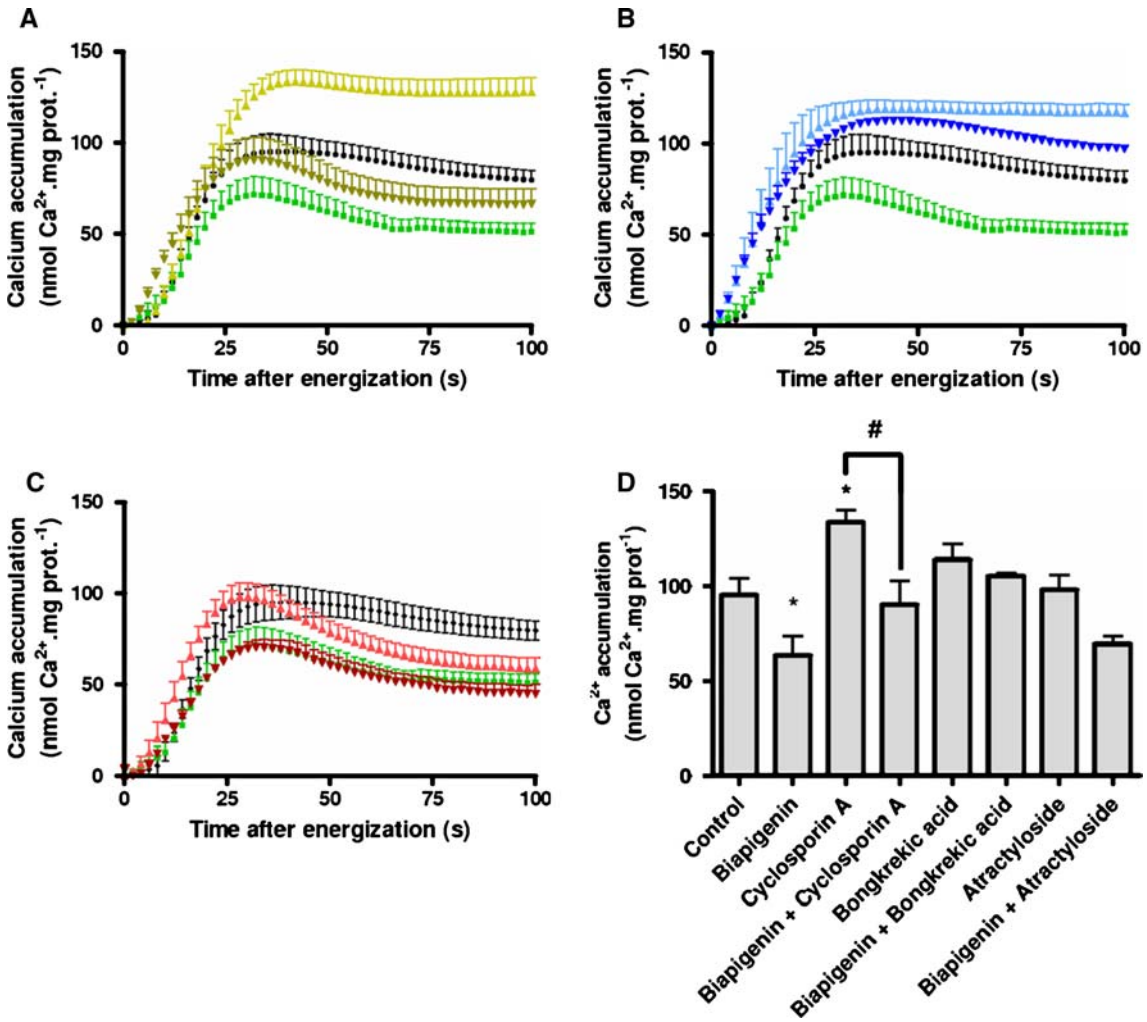

Fig. 7 Mitochondrial calcium uptake. Mitochondrial calcium uptake and retention were evaluated in isolated rat brain mitochondria (0.2 mg/ml) after energization with 8 mM succinate (energization driven calcium uptake, ● control). Biapigenin (10 μM, ■) was incubated for 3 min. Mitochondria were also incubated with: **a** cyclosporin A alone (0.6 μM, ▲) or co-incubated with biapigenin (▼); **b** bongkrekic acid alone (16 μM, ▲) or co-incubated with biapigenin (▼); **c** atractyloside alone (40 μM, ▲), or co-incubated with biapigenin (▼). Values are presented as mean + SEM from three to eight independent experiments. **d** Maximal mitochondrial calcium accumulation. Values are presented as mean ± SEM from three to eight independent experiments. * $P < 0.05$ (comparatively to control); # $P < 0.05$

conductance (Shabalina et al. 2006). It is, therefore, tentative to speculate that the inhibition of the ANT by specific molecules, such as atractyloside and bongkrekic acid or even biapigenin, may interfere with mitochondrial membrane proton conductance.

ADP/ATP Translocation to Mitochondrial Matrix Is Not Blocked by Biapigenin

The inhibitory effects in ADP-induced depolarization suggest that biapigenin can decrease the entry of ADP into the mitochondrial matrix through the ANT, although effects on the ATP synthase activity cannot be excluded at this point. Interaction of biapigenin with the phosphorylation system agrees with the observation that biapigenin decreases the ADP/O ratio. Interestingly, state 3 respiration is not entirely inhibited by biapigenin (Fig. 3a), suggesting that biapigenin does not completely inhibit ADP influx as opposed to ANT inhibition with atractyloside or bongkrekic acid.

The ATP synthase activity was significantly reduced by biapigenin (Fig. 6b). Moreover, inhibition of ATP synthesis by biapigenin (as measured by HPLC) by atractyloside or by bongkrekic acid or oligomycin (Table 2) was similar. Since the two methods used in this study to determine the ATP-synthase activity require functionally intact mitochondria with active ANT participation, an ANT-related effect of biapigenin is still a good explanation. The effect of biapigenin in ATP-synthase activity (assessed by pH variations) can be associated with the inhibition of the ANT, reducing ADP entry (or ATP

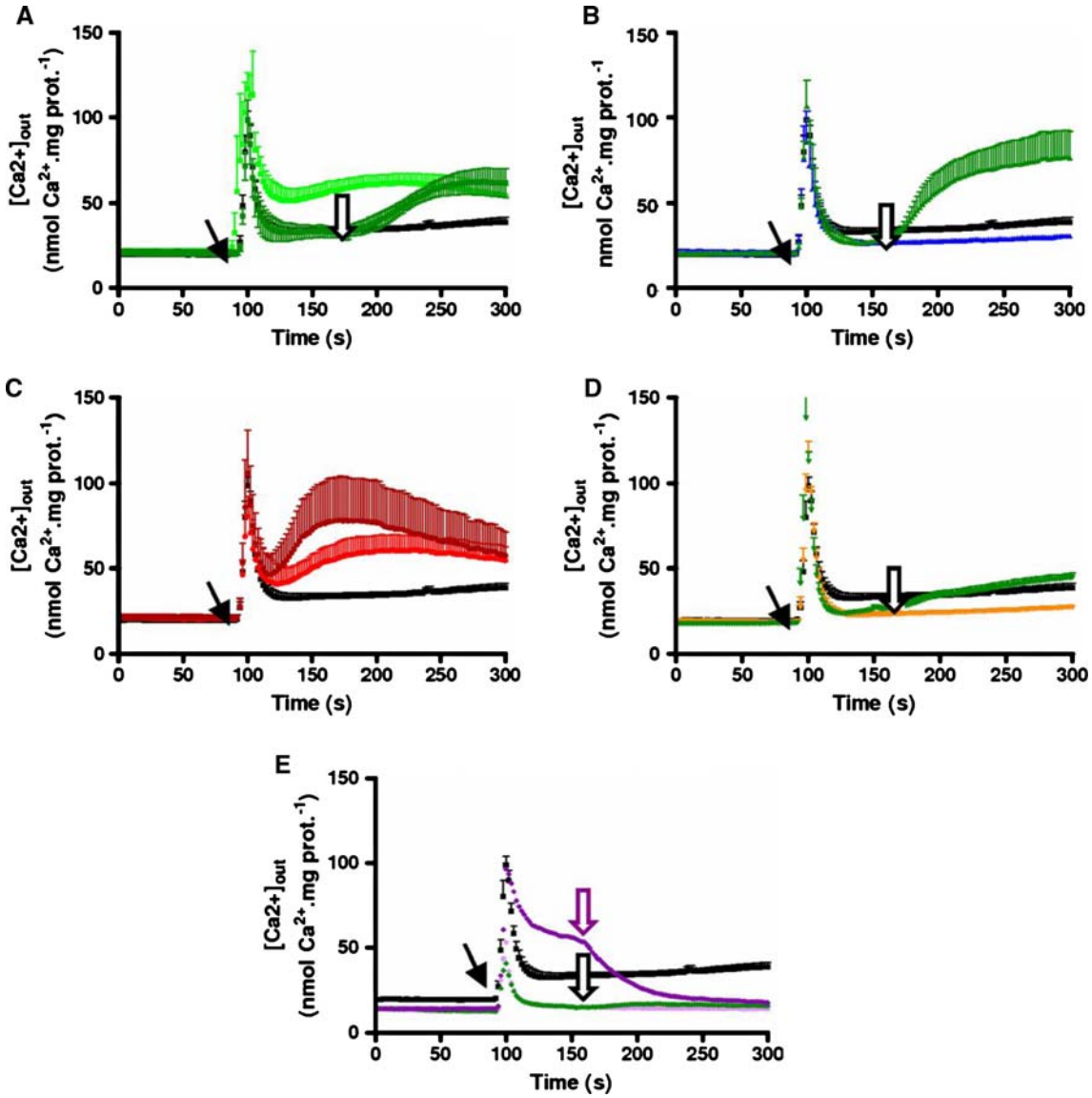

Fig. 8 Biapigenin reduces mitochondrial calcium accumulation and increases calcium efflux. Mitochondrial calcium retention was evaluated in energized mitochondria (0.2 mg/ml) upon addition of a calcium pulse (40 μM). Calcium present in the reaction medium ($[Ca^{2+}]_{out}$) was assessed by monitoring Calcium Green 5-N (100 nM) fluorescence; decrease in fluorescence corresponds to calcium accumulation by mitochondria, whereas increased fluorescence corresponds to mitochondrial calcium efflux into the reaction medium (■, control). **a** Pre-incubation of mitochondria with biapigenin (10 μM), for 3 min, decreased maximal calcium accumulation (■); addition of a pulse of biapigenin (indicated by *white arrow*, ■) induced mitochondrial calcium efflux. **b** Bongkrekic acid (16 μM) increased mitochondrial calcium accumulation (▲), but did not prevent biapigenin-induced calcium efflux (▲). **c** Pre-incubation with atractyloside (40 μM)-induced calcium efflux (●), and this effect was additive with biapigenin (●). **d** Cyclosporin A (0.6 μM) increased calcium accumulation (▼) and partially blocked the effect of biapigenin in calcium efflux (▼). **e** ADP (125 μM) plus oligomycin (1 μg/ml) increased mitochondrial calcium accumulation (◆). ADP plus oligomycin completely blocked biapigenin-induced decrease in calcium accumulation and biapigenin-mediated calcium efflux (◆). Following pre-incubation with biapigenin, addition of a pulse of 125 μM ADP (*purple arrow*) increased calcium accumulation (◆). Values are presented as mean ± SEM from two to three independent experiments

efflux). However, interaction of biapigenin on the ATP synthase cannot be excluded; in fact, induction of decoupling of proton flow through the F_O subunit is known to affect ADP phosphorylation (Pietrobon et al. 1987; Bravo et al. 2001). Also, the concept of synthasome has recently been proposed. The groups of Ko and Chen showed a strong association between the ATP synthase complex and the ANT (Ko et al. 2003; Chen et al. 2004), which raises the idea that drugs targeting either of the proteins involved in mitochondrial phosphorylation could also be able to exert indirect effects in the proteins present in this functional complex.

The results in this study suggest that biapigenin does not inhibit ATP translocation into the mitochondrial matrix. Moreover, the hyperpolarizing effects of biapigenin on ATP-induced energization (Fig. 5a) seem to be related with a decrease in proton back flow into the matrix. Interestingly, this effect of biapigenin is also likely ANT-conformation sensitive, since bongkrekic acid-induced conformational state of ANT was not affected in the presence of biapigenin, whereas ANT in atractyloside-induced conformation was inhibited by biapigenin (Fig. 5b).

Atractyloside and bongkrekic acid bind to mutually exclusive sites (Dahout-Gonzalez et al. 2006). Atractyloside is a non-permeant inhibitor of ADP binding to the ANT (Bruni et al. 1965), whereas bongkrekic acid must cross the mitochondrial inner membrane to exert its inhibitory effects in ADP-binding sites in the matrix side. Consequently, in the presence of bongkrekic acid plus biapigenin, a decrease in ADP export/ATP import from/into the matrix would occur and, therefore, may lead to a decrease in ATP availability for further $\Delta\Psi_m$ generation. In summary, it seems that biapigenin and atractyloside share a common target in the ANT. Figure 9 proposes a model for the functional action of biapigenin that may help to explain the effect of biapigenin in the ANT function, and how this can result in implications for mitochondrial calcium homeostasis (Halestrap and Brenner 2003).

Biapigenin Induces Calcium-Dependent mPTP Opening

The opening of the mPTP is modulated by several factors such as high $\Delta\Psi_m$, low matrix pH, or the presence of adenine nucleotides. Moreover, under conditions that maintain the reducing status of the mitochondrial matrix (NADH or NADPH, antioxidants), the opening of the mPTP is inhibited (Vieira et al. 2000; Brookes et al. 2004). On the other hand, calcium and ROS, among other factors, are the known inducers of pore opening. Calcium accumulation by mitochondria occurs at the expense of $\Delta\Psi_m$ and excessive calcium uptake dissipates $\Delta\Psi_m$ to a level at which mPTP opening is not inhibited, inducing ROS generation, which ends up by causing damage to mitochondrial membrane proteins, resulting in further dissipation of $\Delta\Psi_m$ in a vicious cycle (Brookes et al. 2004).

The data here presented describe that mitochondrial calcium uptake capacity was reduced in the presence of biapigenin. The observation that the addition of a pulse of biapigenin to calcium-loaded energized mitochondria induces calcium efflux suggests that, in the presence of calcium, biapigenin can induce mPTP opening. This effect would cause calcium efflux resulting in a decrease in mitochondrial calcium accumulation, as observed (Fig. 8d). When incubated with atractyloside, which induces a pore-forming conformation, the effect of biapigenin was additive, i.e., a contribution toward decreased calcium accumulation. These results seem to be in accordance with data from TPP^+ uptake and mitochondrial respiration, suggesting that opening of the mPTP can occur in the presence of biapigenin and atractyloside or, in this case, of biapigenin and calcium. ADP plus oligomycin or cyclosporin A, two inhibitors of mPTP opening in brain mitochondria (Brustovetsky and Dubinsky 2000; Halestrap 2006), efficiently inhibited the effects of biapigenin. The observation suggests an involvement of the mPTP in biapigenin-induced calcium efflux, possibly by modulating ANT function, which may include increased cyclophilin D binding to the ANT. In fact, it has been proposed that cyclophilin D modulates ANT function, as binding of cyclophilin D to the matrix surface of the ANT favors calcium-triggered ANT-conformational change to a non-specific pore (He and Lemasters 2002; Halestrap and Brenner 2003).

Taken together, the results in this study suggest that biapigenin increases calcium efflux from mitochondria, possible by inducing transient mPTP opening in such a way that allows the release of excessive calcium and relief of mitochondrial burden. The functional effect of biapigenin in mitochondrial calcium reported in this article is closely similar to the reported effect of minocycline in decreasing calcium uptake in brain mitochondria (Fernandez-Gomez et al. 2005; Mansson et al. 2007). Decreased mitochondrial calcium uptake may contribute to preserve mitochondrial functions, especially under stressful conditions, and protect neurons from excitotoxic cell death (Stout et al. 1998; Urushitani et al. 2001; Duchen 2004; Dubinsky et al. 2004; Fernandez-Gomez et al. 2005). A tentative mechanism for the action of biapigenin is depicted in Fig. 9.

Conclusions

In conclusion, this study indicates that the major mitochondrial target of biapigenin is the ANT. We propose that the interaction of biapigenin with its targets contributes to the protection of mitochondrial function, by inducing transient mPTP opening and a decrease in mitochondrial calcium retention during excitotoxic events.

In fact, modulation of mitochondrial physiology, namely at the level of mitochondrial calcium-loading capacity, is likely to play an important role in the protection of mitochondrial function. This may be especially relevant under excitotoxic events, where high amounts of calcium taken up by energized mitochondria lead to irreversible mitochondrial depolarization and, consequently, to mitochondrial dysfunction. It seems plausible that biapigenin, by

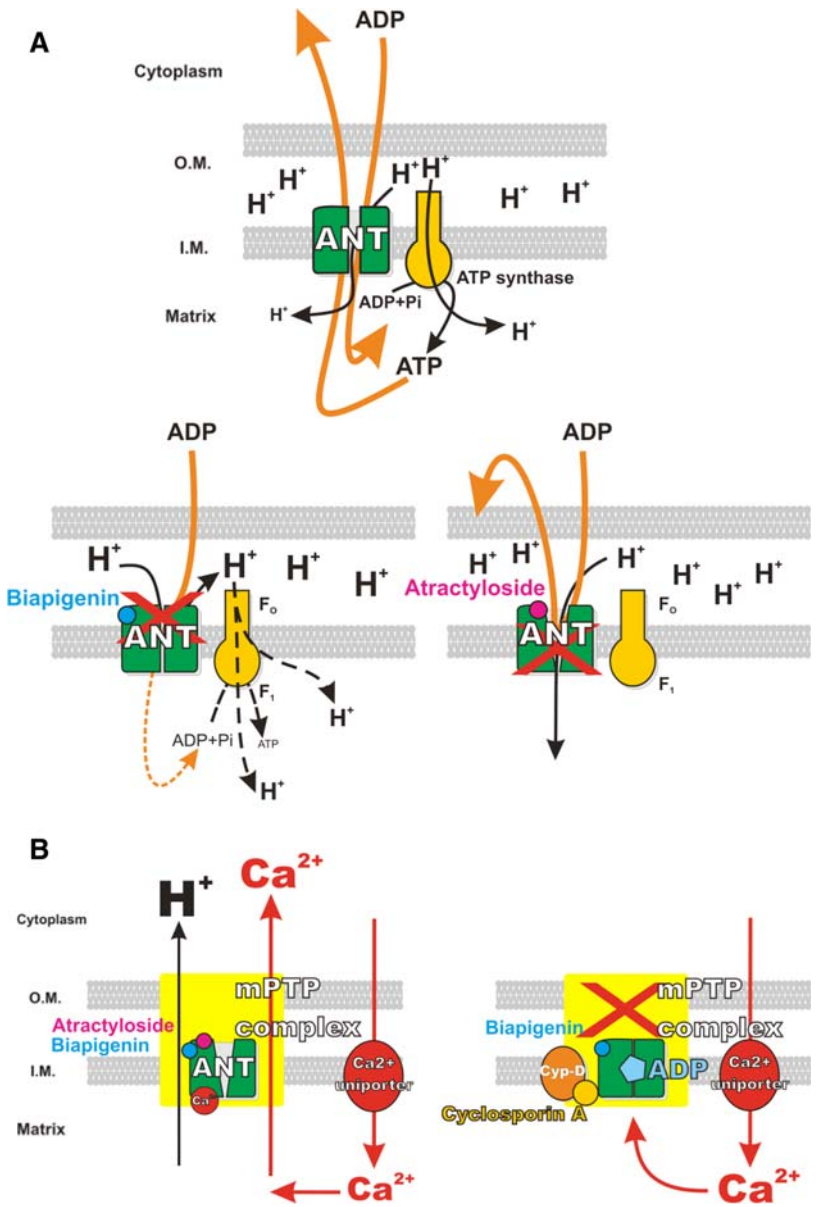

Fig. 9 Integrative scheme highlighting possible mitochondrial targets of biapigenin. **a** Biapigenin modulates ANT function. Under basal conditions (*top*), ATP synthesis is dependent on the import of ADP through the ANT, and of ADP phosphorylation (which occurs at the expense of the $\Delta\Psi_m$, established by the respiratory chain). One molecule of synthesized ATP is then translocated to the intermembrane space through the ANT, by exchange with one molecule of ADP. In the presence of biapigenin (10 μM, *bottom left*), proton flow through the ANT is blocked resulting in hyperpolarization, although state 3 respiration is still observable. The results suggest that biapigenin does not completely block ADP import to mitochondrial matrix. However, ATP synthesis is significantly inhibited by biapigenin. It is plausible to conceive that mitochondrial phosphorylation could also be inhibited due to a decoupling effect of biapigenin, i.e., proton slippage between the F_O and F_1 subunits of the ATP-synthase complex in the presence of biapigenin. The hyperpolarizing effect of biapigenin is blocked by bongkrekic acid and by atractyloside, two ANT inhibitors. Atractyloside (*bottom, right*) induces a pore-forming conformation of the ANT, blocking ADP translocation to the mitochondrial matrix, thereby indirectly inhibiting ATP synthesis. **b** Biapigenin induces calcium release, through the mPTP complex. The interaction of biapigenin with the ANT in the presence of calcium or atractyloside, leads to mPTP opening with consequent dissipation of the $\Delta\Psi_m$. Addition of biapigenin to calcium-loaded energized mitochondria results in mitochondrial calcium efflux, which was blocked in the presence of cyclosporin A and ADP plus oligomycin. Cyclosporin A and ADP plus oligomycin (to avoid ADP phosphorylation into ATP) inhibit mPTP opening, thereby increasing mitochondrial calcium uptake capacity (reduced efflux). The target of cyclosporin A is cyclophlin D, which is known to interact with the ANT inducing a pore-forming conformation. When cyclosporin A is present, cyclophlin D is unable to interact with the ANT, thereby increasing calcium accumulation capacity. These results suggest that the effects of biapigenin in mitochondrial calcium handling are due to interactions with a component of the mPTP complex, probably the ANT. *ANT* adenine nucleotide translocator, *Cyp-D* cyclophylin D, *mPTP* mitochondrial permeability transition pore, *O.M.* outer mitochondrial membrane, *I.M.* inner mitochondrial membrane

inducing transient mPTP opening and decreasing mitochondrial calcium retention, would be able to relieve mitochondria from the calcium burden, therefore contributing for maintenance of mitochondrial function under stressful conditions. Ultimately, this would translate into a neuroprotective action that may help neurons in dealing with calcium overload under excitotoxic events.

Acknowledgment We acknowledge the Portuguese Foundation for Science and Technology and FEDER for support, PhD grant SFRH/BD/13488/2003, and research projects POCI/SAU-NEU/58492/2004.

References

Andreyev AY, Kushnareva YE, Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry 70:200–214

Belisle E, Kowaltowski AJ (2002) Opening of mitochondrial K+ channels increases ischemic ATP levels by preventing hydrolysis. J Bioenerg Biomembr 34:285–298

Bravo C, Vargas-Suárez M, Rodríguez-Enríquez S, Lova-Tavera H, Moreno-Sánchez R (2001) Metabolic changes induced by cold stress in rat liver mitochondria. J Bioenerg Biomembr 33:289–301

Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 287:C817–C833

Bruni A, Luciani S, Contessa AR (1964) Inhibition by atractyloside of the binding of adenine-nucleotides to rat-liver mitochondria. Nature 201:1219–1220

Bruni A, Luciani S, Bortignon C (1965) Competitive reversal by adenine nucleotides of atractyloside effect on mitochondrial energy transfer. Biochim Biophys Acta 97:434–441

Brustovetsky N, Dubinsky JM (2000) Dual responses of CNS mitochondria to elevated calcium. J Neurosci 20:103–113

Brustovetsky N, Klingenberg M (1994) The reconstituted ADP/ATP carrier can mediate H+ transport by free fatty acids, which is further stimulated by mersalyl. J Biol Chem 269:27329–27336

Brustovetsky NN, Dedukhova VI, Egorova MV, Mokhova EN, Skulachev VP (1990) Inhibitors of the ATP/ADP antiporter suppress stimulation of mitochondrial respiration and H+ permeability by palmitate and anionic detergents. FEBS Lett 272:187–189

Chen C, Ko Y, Delannoy M, Ludtke SJ, Chiu W, Pedersen PL (2004) Mitochondrial ATP synthasome: three-dimensional structure by electron microscopy of the ATP synthase in complex formation with carriers for Pi and ADP/ATP. J Biol Chem 279:31761–31768

Cho J, Lee HK (2004) Wogonin inhibits excitotoxic and oxidative neuronal damage in primary cultured rat cortical cells. Eur J Pharmacol 485:105–110

Comelli M, Metelli G, Mavelli I (2007) Downmodulation of mitochondrial F0F1 ATP synthase by diazoxide in cardiac myoblasts: a dual effect of the drug. Am J Physiol Heart Circ Physiol 292:H820–H829

Dahout-Gonzalez C, Ramus C, Dassa EP, Dianoux AC, Brandolin G (2005) Conformation-dependent swinging of the matrix loop m2 of the mitochondrial *Saccharomyces cerevisiae* ADP/ATP carrier. Biochemistry 44:16310–16320

Dahout-Gonzalez C, Nury H, Trezeguet V, Lauquin GJ, Pebay-Peyroula E, Brandolin G (2006) Molecular, functional, and pathological aspects of the mitochondrial ADP/ATP carrier. Physiology 21:242–249

De Paola R, Muia C, Mazzon E, Genovese T, Crisafulli C, Menegazzi M, Caputi AP, Suzuki H, Cuzzocrea S (2005) Effect of *Hypericum perforatum* extract in a rat model of ischemia and reperfusion injury. Shock 24:255–263

Di Carlo G, Borrelli F, Ernst E, Izzo AA (2001) St John's wort: Prozac from the plant kingdom. Trends Pharmacol Sci 22:292–297

Dias ACP, Tomas-Barberan FA, Fernandes-Ferreira FA, Ferreres F (1998) Unusual flavonoids produced by callus of *Hypericum perforatum*. Phytochemistry 48:1165–1168

Dorner A, Olesch M, Giessen S, Pauschinger M, Schultheiss HP (1999) Transcription of the adenine nucleotide translocase isoforms in various types of tissues in the rat. Biochim Biophys Acta 1417:16–24

Dubinsky JM, Brustovetsky N, LaFrance R (2004) Protective roles of CNS mitochondria. J Bioenerg Biomembr 36:299–302

Duchen MR (2004) Mitochondria in health and disease: perspectives on a new mitochondrial biology. Mol Aspects Med 25:365–451

Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. Methods Enzymol 10:41–47

Fernandez-Gomez FJ, Galindo MF, Gomez-Lazaro M, González-Garcia C, Ceña V, Aguirre N, Jordán J (2005) Involvement of mitochondrial potential and calcium buffering capacity in minocycline cytoprotective actions. Neuroscience 133:959–967

Ferreira FM, Madeira VM, Moreno AJ (1997) Interactions of 2, 2-bis(p-chlorophenyl)-1, 1-dichloroethylene with mitochondrial oxidative phosphorylation. Biochem Pharmacol 53:299–308

Halestrap AP (2006) Calcium, mitochondria and reperfusion injury: a pore way to die. Biochem Soc Trans 34:232–237

Halestrap AP, Brenner C (2003) The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr Med Chem 10:1507–1525

Halestrap AP, McStay GP, Clarke SJ (2002) The permeability transition pore complex: another view. Biochimie 84:153–166

Halestrap AP, Clarke SJ, Khaliulin I (2007) The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 1767:1007–1031

He L, Lemasters JJ (2002) Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? FEBS Lett 512:1–7

Henderson PJ, Lardy HA (1970) Bongkrekic acid. An inhibitor of the adenine nucleotide translocase of mitochondria. J Biol Chem 245:1319–1326

Ichas F, Mazat JP (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1366:33–50

Ishige K, Schubert D, Sagara Y (2001) Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic Biol Med 30:433–446

Kamo N, Muratsugu M, Hongoh R, Kobatake Y (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 49:105–121

Ko YH, Delannoy M, Hullihen J, Chiu W, Pedersen PL (2003) Mitochondrial ATP synthasome. Cristae-enriched membranes and a multiwell detergent screening assay yield dispersed single complexes containing the ATP synthase and carriers for Pi and ADP/ATP. J Biol Chem 278:12305–12309

Kowaltowski AJ, Maciel EN, Fornazari M, Castilho RF (2006) Diazoxide protects against methylmalonate-induced neuronal toxicity. Exp Neurol 201:165–171

Kristal BS, Stavrovskaya IG, Narayanan MV, Krasnikov BF, Brown AM, Beal MF, Friedlander RM (2004) The mitochondrial

permeability transition as a target for neuroprotection. J Bioenerg Biomembr 36:309–312
Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87:99–163
Kumar V, Mdzinarishvili A, Kiewert C, Abbruscato T, Bickel U, van der Schyf CJ, Klein J (2006) NMDA receptor-antagonistic properties of hyperforin, a constituent of St. John's Wort. J Pharmacol Sci 102:47–54
Lee H, Bae JH, Lee SR (2004) Protective effect of green tea polyphenol EGCG against neuronal damage and brain edema after unilateral cerebral ischemia in gerbils. J Neurosci Res 77:892–900
Madeira VM, Antunes-Madeira MC, Carvalho AP (1974) Activation energies of the ATPase activity of sarcoplasmic reticulum. Biochem Biophys Res Commun 58:897–904
Mansson R, Hansson MJ, Morota S, Uchino H, Ekdhal CT, Elmér E (2007) Re-evaluation of mitochondrial permeability as a primary neuroprotective target of minocycline. Neurobiol Dis 25:198–205
Montero M, Lobaton CD, Hernandez-Sanmiguel E, Santodomingo J, Vay L, Moreno A, Alvarez J (2004) Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. Biochem J 384:19–24
Moreira PI, Santos MS, Sena C, Seica R, Oliveira CR (2005) Insulin protects against amyloid beta-peptide toxicity in brain mitochondria of diabetic rats. Neurobiol Dis 18:628–637
Murata M, Akao M, O'Rourke B, Marban E (2001) Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca(2+) overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. Circ Res 89:891–898
Oliveira PJ, Seica R, Coxito PM, Rolo AP, Palmeira CM, Santos MS, Moreno AJ (2003) Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats. FEBS Lett 554:511–514
Oliveira PJ, Bjork JÁ, Santos MS, Leino RL, Froberg MK, Moreno AJ, Wallace KB (2004) Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. Toxicol Appl Pharmacol 200:159–168
Parihar MS, Brewer GJ (2007) Mitoenergetic failure in Alzheimer disease. Am J Physiol Cell Physiol 292:C8–C23
Park C, So HS, Shin CH, Baek SH, Moon BS, Shin SH, Lee HS, Lee DW, Park R (2003) Quercetin protects the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. Biochem Pharmacol 66:1287–1295
Pietrobon D, Luvisetto S, Azzone GF (1987) Uncoupling of oxidative phosphorylation. Alternative mechanisms: intrinsic uncoupling or decoupling? Biochemistry 26:7339–7347
Rasola A, Bernardi P (2007) The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12:815–833
Rice-Evans C (2001) Flavonoid antioxidants. Curr Med Chem 8:797–807
Santos AC, Uyemura SA, Lopes JL, Bazon JN, Mingatto FE, Curti C (1998) Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. Free Radic Biol Med 24:1455–1461
Schroeter H, Williams RJ, Matin R, Iversen L, Rice-Evans CA (2000) Phenolic antioxidants attenuate neuronal cell death following uptake of oxidized low-density lipoprotein. Free Radic Biol Med 29:1222–1233
Shabalina IG, Kramarova TV, Nedergaard J, Cannon B (2006) Carboxyatractyloside effects on brown-fat mitochondria imply that the adenine nucleotide translocator isoforms ANT1 and ANT2 may be responsible for basal and fatty-acid-induced uncoupling respectively. Biochem J 399:405–414
Shin DH, Bae YC, Kim-Han JS, Lee JH, Choi IY, Son KH, Kang SS, Kim WK, Han BH (2006a) Polyphenol amentoflavone affords neuroprotection against neuronal hypoxic-ischemic brain damage via multiple mechanisms. J Neurochem 96:561–572
Shin WH, Park SJ, Kim EJ (2006b) Protective effects of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life Sci 79:286–296
Silva B, Oliveira PJ, Dias ACP, Malva JO (2008) Quercetin, kaempferol and biapigenin from *Hypericum perforatum* are neuroprotective against excitotoxic insults. Neurotoxicity Res 13:265–279
Stocchi V, Cucchiarini L, Magnani M, Chiarantini L, Palma P, Crescentini G (1985) Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. Anal Biochem 146:118–124
Stout AK, Raphael HM, Kanterewicz BI, Klann E, Reynolds IJ (1998) Glutamate-induced neuron death requires mitochondrial calcium uptake. Nat Neurosci 1:366–373
Teshima Y, Akao M, Li RA, Chong TH, Baumgartner WA, Johnston MV, Marbán E (2003) Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. Stroke 34:1796–1802
Urushitani M, Nakamizo T, Inoue R, Sawada H, Kihara T, Honda K, Akaike A, Shimohama S (2001) N-methyl-D-aspartate receptor-mediated mitochondrial Ca(2+) overload in acute excitotoxic motor neuron death: a mechanism distinct from chronic neurotoxicity after Ca(2+) influx. J Neurosci Res 63:377–387
Vieira HL, Haouzi D, El-Hamel C, Jacotot E, Belzacq AS, Brenner C, Kroemer G (2000) Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ 7:1146–1154
Wu L, Shen F, Lin L, Zhang X, Bruce IC, Xia Q (2006) The neuroprotection conferred by activating the mitochondrial ATP-sensitive K+ channel is mediated by inhibiting the mitochondrial permeability transition pore. Neurosci Lett 402:184–189
Yazawa K, Kihara T, Shen H, Shimmyo Y, Niidome T, Sugimoto H (2006) Distinct mechanisms underlie distinct polyphenol-induced neuroprotection. FEBS Lett 580:6623–6628
Zanotti F, Guerrieri F, Capozza G, Fiermonte M, Berden J, Papa S (1992) Role of F0 and F1 subunits in the gating and coupling function of mitochondrial H(+)-ATP synthase. The effect of dithiol reagents. Eur J Biochem 208:9–16