Biochimica et Biophysica Acta 1797 (2010) 1245–1250

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

# The dopamine-D2-receptor agonist ropinirole dose-dependently blocks the $Ca^{2+}$-triggered permeability transition of mitochondria

Suhel Parvez [a,1], Kirstin Winkler-Stuck [a], Silvia Hertel [a], Peter Schönfeld [b], Detlef Siemen [a,*]

[a] Dept. of Neurology, Otto-von-Guericke-University, Leipziger Str. 44, D-39120 Magdeburg, Germany
[b] Institute of Biochemistry and Cell Bioloy, Otto-von-Guericke-University, Leipziger Str. 44, D-39120 Magdeburg, Germany

ARTICLE INFO

*Article history:*
Received 6 October 2009
Received in revised form 22 January 2010
Accepted 1 February 2010
Available online 6 February 2010

*Keywords:*
Parkinson's disease
Permeability transition
Apoptosis
Mitochondria
Ropinirole
Neuroprotection

ABSTRACT

Ropinirole, an agonist of the post-synaptic dopamine D2-receptor, exerts neuroprotective activity. The mechanism is still under discussion. Assuming that this neuroprotection might be associated with inhibition of the apoptotic cascade underlying cell death, we examined a possible effect of ropinirole on the permeability transition pore (mtPTP) in the mitochondrial inner membrane. Using isolated rat liver mitochondria, the effect of ropinirole was studied on $Ca^{2+}$-triggered large amplitude swelling, membrane depolarization and cytochrome *c* release. In addition, the effect of ropinirole on oxidation of added, membrane-impermeable NADH was investigated. The results revealed doubtlessly, that ropinirole can inhibit permeability transition. In patch-clamp experiments on mitoplasts, we show directly that ropinirole interacts with the mtPTP. Thus, ropinirole reversibly inhibits the opening of mtPTP with an $IC_{50}$ of 3.4 μM and a Hill coefficient of 1.3. In both systems (i.e. energized mitochondria and mitoplasts) the inhibitory effect on permeability transition was attenuated by increasing concentrations of inorganic phosphate. In addition, we showed with antimycin A-treated mitochondria that ropinirole failed to suppress respiratory chain-linked reactive oxygen species release. In conclusion, our data suggest that the neuroprotective activity of ropinirole is due to the blockade of the $Ca^{2+}$-triggered permeability transition.



## 1. Introduction

The characteristic feature of Parkinson's disease (PD), a neurodegenerative disorder, is the progressive loss of dopaminergic neurons of the substantia nigra, resulting in a deficiency of dopamine in the corpus striatum. Ropinirole, a non-ergoline dopamine-D2-agonist which is strongly active at $D_2/D_3$ receptor sites, but has negligible activity at $D_1$ receptor sites [1], is an effective drug for the treatment of PD symptoms [2]. In addition, ropinirole binds to the dopamine type 2 autoreceptors, thereby reducing dopamine release. This activity of ropinirole is important because oxidative dopamine inactivation by the monoaminooxidase is a source of reactive oxygen species (ROS), which are likely to damage oxidatively nigrostriatal tissue [3]. Interestingly, it has been discussed that ropinirole could scavenge ROS directly, as well as, indirectly via activation of enzymes of the antioxidative cellular defense system, such as the glutathione peroxidase, the catalase, and the superoxide dismutase [4].

Recent attention has focused on the possible role of dopamine agonists in neuroprotection, probably by inhibition of the apoptotic cell death. Mitochondria are playing a critical role in apoptotic cell death, by releasing several apoptotic factors (such as cytochrome *c*, Cyt *c*). It has been proposed that opening of the mitochondrial permeability transition pore (mtPTP) is somehow related to the release of these apoptotic factors [5]. The underlying permeabilization of the inner mitochondrial membrane to non-permeable solutes abolished the electrochemical proton gradient, thereby impairing energy-dependent mitochondrial functions, e.g. ATP generation or calcium sequestration (for review see [6]). Various effectors, mostly $Ca^{2+}$, inorganic phosphate (Pi), protonophoric uncouplers or oxidants of critical SH-groups initiate opening of the mtPTP [7–9]. The molecular identity of the mtPTP is still unknown [10–12]. It appears to operate as a $Ca^{2+}$-, Pi-, voltage-, pH-, and redox-gated channel [12–15].

In two previous studies it was shown that pramipexole, also a dopamine-D2-agonist, can inhibit opening of the mtPTP [16,17]. This is a surprising observation suggesting that D2-receptor agonists (chemical structures of pramipexole and ropinirole are given in Fig. 1) could desensitize mtPTP against opening. To further substantiate this hypothesis, we examined the effect of ropinirole on the mtPTP by

*Abbreviations:* CsA, cyclosporin A; Cyt *c*, cytochrome *c*; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; $IC_{50}$, inhibitor concentration causing 50% inhibition; mtPTP, mitochondrial permeability transition pore; PD, Parkinson's disease; RBM, rat brain mitochondria; RLM, rat liver mitochondria; ROS, reactive oxygen species; Pi, inorganic phosphate; Po, open probability; $\Delta\psi_m$, mitochondrial membrane potential

* Corresponding author. Tel.: +49 391 67 14415; fax: +49 391 67 290418.
*E-mail address:* detlef.siemen@medizin.uni-magdeburg.de (D. Siemen).
[1] Present address: Department of Neurophysiology, Leibniz-Institute for Neurobiology, Brenneckestrasse 6, 39118 Magdeburg, Germany.

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.02.001

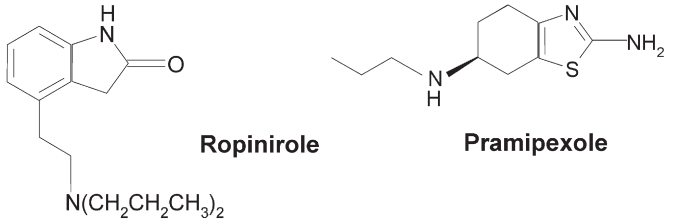


**Fig. 1.** Chemical structures of pramipexole and ropinirole.

using both, isolated mitochondria and mitoplasts. To compare patch-clamp-studies at a single mitochondrium, with studies done with mitochondrial incubations (measurement of swelling, membrane polarisation, etc.), we used mostly rat liver mitochondria for practical reasons. We report here that a dose-dependent block of the mtPTP by ropinirole has been found, an activity which might contribute to its clinically observed neuroprotective effect.

## 2. Materials and methods

### 2.1. Preparation of mitochondria

Liver mitochondria were prepared from adult Wistar rats (RLM) by differential centrifugation. Briefly, the liver was homogenized in an ice-cold isolation buffer containing 0.25 M sucrose, 1 mM EDTA adjusted by Tris to pH 7.4, and centrifuged at 800 *g* for 5 min. The supernatant was centrifuged at 5100 *g* for 4 min. Thereafter, the obtained pellet was resuspended again in a 0.25 M sucrose medium adjusted by Tris to pH 7.4, and centrifuged at 12,300 *g* for 2 min. Finally, the pellet was resuspended in a 0.25 M sucrose medium adjusted by Tris to pH 7.4, centrifuged at 12,300 *g* for 10 min, and resuspended in a buffer containing 0.25 M sucrose, 0.5 mM EDTA adjusted by Tris to pH 7.4. The protein concentration of the stock suspension was 20–35 mg/mL as determined using the biuret method. Mitochondria from rat brain (RBM) were prepared as described in [18].

### 2.2. Monitoring of the $Ca^{2+}$-triggered permeability transition

#### 2.2.1. Swelling measurements

Aliquots of the mitochondrial stock suspension (RLM, 1 mg of protein/mL) were added to an incubation medium containing 125 mM KCl, 20 mM Trizma base, 1 mM $MgCl_2$, 1 μM EGTA, 5 mM glutamate, and 5 mM malate (pH 7.2). Permeability transition was induced by addition of $CaCl_2$ and monitored by measuring the decrease of light scattering due to the large amplitude swelling of mitochondria at 540 nm using a Varian Cary 3 spectrophotometer at 30 °C. Swelling was recorded during a period of 10 min and is expressed as a percentage of initial absorbance.

#### 2.2.2. Monitoring of the mitochondrial membrane potential ($\Delta\psi_m$)

Depolarization of the inner mitochondrial membrane was monitored with the membrane-permeable cation safranine O using the Perkin-Elmer Luminescence Spectrophotometer LS 50B (excitation 495 nm, emission 586 nm) as first described in [19]. Aliquots of the mitochondrial stock suspension (RLM or RBM, 1 mg of protein/mL) were suspended in incubation medium (see Section 2.2.1). Further additions were 5 μM safranine O and different concentrations of Pi (0.1, 2.0 mM; 0 mM Pi as a control).

### 2.3. Measurement of $H_2O_2$ release

Generation of $O_2^{\bullet-}$ was measured as the release of $H_2O_2$ from mitochondria using the Amplex Red/horseradish peroxidase (HRP) system. The non-fluorescent Amplex Red becomes oxidized by $H_2O_2$ to the fluorescent resorufin. Briefly, mitochondria (0.2 mg of mitochondrial protein/mL) were incubated at 25 °C in incubation medium (see Section 2.2.1) supplemented with 5 μM Amplex Red, 2 U/mL HRP and, in addition, 2 U/mL Cu,Zn-superoxide dismutase, for quantitative conversion of $O_2^{\bullet-}$ into $H_2O_2$. The increase in the fluorescence was monitored using a Perkin-Elmer LS-50B fluorescence spectrometer (excitation at 560 nm, emission at 590 nm). Fluorescence signals were calibrated by additions of known quantities of $H_2O_2$.

### 2.4. Respiration measurements

The oxygen consumption of RLM was measured at 30 °C using the high-resolution Oroboros oxygraph (Anton Paar, Graz). The measurements were performed in incubation medium, supplemented either with 0.1 mM or 10 mM Pi. For the measurement of the $Ca^{2+}$-dependent respiration, free calcium concentrations were calculated by means of the BAD3-program which is based on the equations reported in [20].

### 2.5. Cytochrome c release

Mitochondria (1 mg of protein) were incubated in 1 mL of incubation medium, supplemented with 2 mM Pi. Effectors were applied for 5 min at 30 °C in the same way as given in the legend of Fig. 3. The suspension was then centrifuged at 12,300 × *g* for 3 min. An aliquot of the supernatant (0.5 mL) was carefully removed, and the protein therein precipitated with acetone-deoxycholate. The precipitated protein or mitochondrial pellet was dissolved in 0.5 mL buffer composed of 66 mM Tris, 10% glycerol, 2% SDS, 0.003% bromphenol blue and 5 mg/mL of dithiothreitol, pH 6.8. For immunoblots, 50 μL aliquots of dissolved supernatant protein or 50 μL aliquots of dissolved pellet protein (1:10 diluted) were run on 15% polyacrylamide gels. Thereafter, the separated proteins were electrotransferred to PVDF membranes (transfer buffer: 25 mM Tris–HCl pH 8.3; 20% methanol) that were rinsed with transfer buffer and blocked overnight in blocking buffer (137 mM NaCl, 10 mM Tris, pH 7.3) supplemented with 5% milk powder. Cyt *c* was immunostained with mouse anti-cytochrome *c* antibody [7H8] (1:500) from PharMingen (BD Biosciences, Erembodgem, Belgium) plus secondary anti-mouse Ig bound to horseradish peroxidase (1:5000) from Dianova (Hamburg, Germany). Peroxidase activity was detected using the enhanced chemiluminescence detection kit (Amersham, Ismaning, Germany) and X-ray film.

### 2.6. Patch-clamp measurements

Single-channel currents were recorded from mitoplasts using the patch-clamp technique [17]. Mitochondria were subjected to a hypotonic treatment (1 min with a solution composed of 5 mM K-HEPES and 1 mM $CaCl_2$, pH 7.2) in order to break the outer membrane (OM). Thereafter, isotonicity was restored by adding a solution composed of 750 mM KCl, 80 mM K-HEPES, and 1 mM $CaCl_2$ (pH 7.2). This procedure resulted in swollen mitochondria (mitoplasts, 5–20 μm diameters), which had lost their OM. Electrodes were made from borosilicate-glass (Harvard, Edenbridge, UK) with resistances of 10–20 MΩ. Current was recorded by means of an EPC-7 patch-clamp amplifier (HEKA electronics, Lambrecht, Germany). Free-floating mitoplasts were approached by means of an electrically driven micromanipulator and attached to the tip of the pipette by gentle suction. Standard pipette and bath solutions consisted of 150 mM KCl, 20 mM K-HEPES, and 0.1 mM $CaCl_2$ (pH 7.2). Test solutions were added through the glass capillaries of a peristaltic-pump driven flow system at room temperature. Currents were low-pass filtered at a frequency of 0.5 kHz and sampled at 2.5 kHz. Analysis was done using

the Pclamp 9.2 software (Axon instruments, Foster City, CA). The open probability ($P_o$) was determined as described previously [17,21].

### 2.7. Materials

ADP, CsA, horseradish peroxidase, Cu,Zn-superoxide dismutase, cytochrome *c*, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, antimycin A, NADH, safranine O, and $CaCl_2$ were purchased from Sigma (Deisenhofen, Germany). Amplex Red was from Invitrogen (Karlsruhe, Germany), ropinirole (4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one monohydrochloride) was obtained from GlaxoSmithKline (Brentford, Middlesex, UK).

## 3. Results

### 3.1. Effect of ropinirole on $Ca^{2+}$ triggered permeability transition

The effects of various concentrations ropinirole on the opening of the mtPTP by $Ca^{2+}$ is shown with energized rat liver mitochondria in Fig. 2. Large amplitude swelling was obtained with 50 nmol $Ca^{2+}$/mg of protein. This swelling was completely prevented in the presence of 1 μM of the mtPTP-inhibitor CsA. Ropinirole inhibited the $Ca^{2+}$-triggered permeability transition completely and dose-dependently with a half-maximal inhibitor concentration ($IC_{50}$) of 50 μM. Furthermore, the $Ca^{2+}$-triggered permeability transition was monitored by the depolarization of the inner mitochondrial membrane using the cationic $\Delta\psi_m$-probe safranine O. The increase of the safranine fluorescence after addition of $Ca^{2+}$ (50 nmol/mg of protein) indicates onset of permeability transition (Fig. 3A, C, E). The protonophore FCCP (50 nM) was used to induce complete depolarization. In order to examine the effect of ropinirole on the $Ca^{2+}$-triggered depolarization, mitochondria were preincubated with 50 μM ropinirole (Fig. 3B, D, F). Ropinirole partially inhibited the depolarization in the absence as well as in the presence of low Pi concentrations (≤μL 0.1 mM) (Fig. 3B, D). However, ropinirole failed to prevent the depolarization at 2 mM Pi (Fig. 3F). The $Ca^{2+}$-triggered permeability transition was further substantiated as the release of Cyt *c* from the intermembrane space. Similar to CsA, ropinirole prevented the $Ca^{2+}$-induced release of Cyt *c* (not shown).

### 3.2. Effect of ropinirole on $Ca^{2+}$-induced oxidation of external NADH

The intact inner mitochondrial membrane is impermeable to NADH. Therefore, rat liver mitochondria are unable to use external

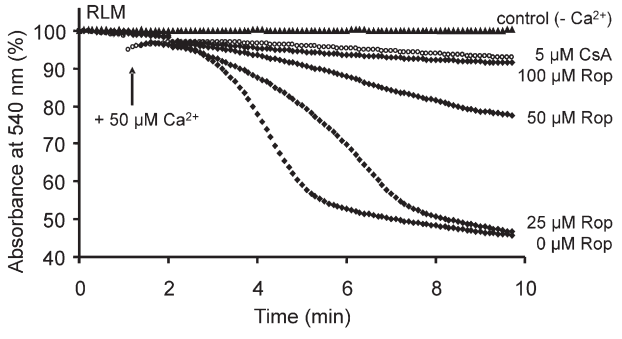


**Fig. 2.** Ropinirole inhibits $Ca^{2+}$-triggered mitochondrial large amplitude swelling. Mitochondria (RLM; 1 mg of protein/mL) were energized with glutamate plus malate (5 mM/5 mM). Opening of the mtPTP was initiated by addition of $CaCl_2$ (final concentration 50 μM, corresponding to 50 nmol $Ca^{2+}$/mg mitochondrial protein). Large amplitude swelling after addition of $Ca^{2+}$ indicates opening of the mtPTP (permeability transition). CsA (5 μM) completely suppressed $Ca^{2+}$ swelling. RLM were pretreated (4 min) with the indicated concentrations of ropinirole. Representative data from three independent experiments are shown.

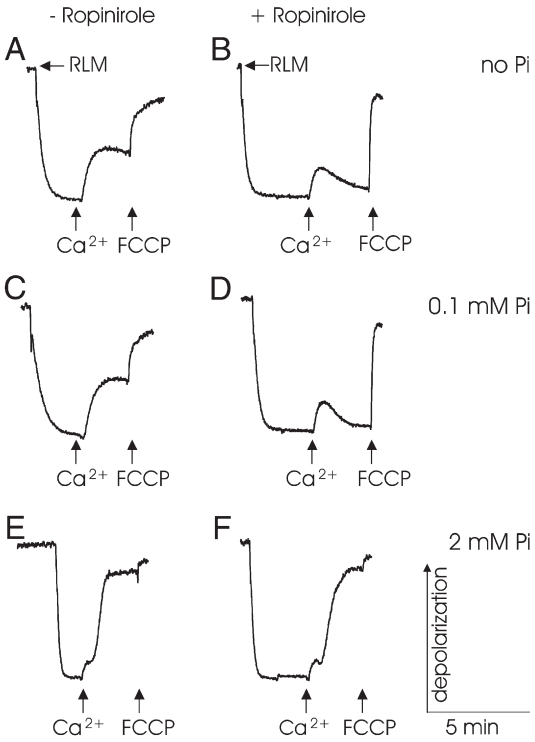


**Fig. 3.** Phosphate attenuates ropinirole-induced inhibition of the permeability transition. RLM (1 mg of protein/mL) were added to incubation buffer supplemented with 10 μM safranine O. In control incubations (traces A, C, E) mitochondria were not pretreated with ropinirole. In traces B, D, F, mitochondria were pretreated with 100 μM ropinirole. To initiate the opening of mtPTP, $Ca^{2+}$ was added as in Fig. 2. Pi concentrations were added as indicated. Increase in safranine fluorescence indicates depolarization of the inner mitochondrial membrane. Complete depolarization was obtained with FCCP (50 nM).

NADH as fuel for establishing the electrochemical proton gradient. However, after opening of the mtPTP by $Ca^{2+}$, external NADH can enter the matrix compartment where it is oxidized by complex I of the respiratory chain. To examine if ropinirole can protect the mtPTP from opening, we measured the mitochondrial respiration with external NADH after inducing opening of the mtPTP by $Ca^{2+}$ [22].

A typical experiment demonstrating the changes in respiration is shown in Fig. 4 (panels A—C). RLM exhibited slow basal respiration in the absence of $Ca^{2+}$. Addition of $Ca^{2+}$ (65 μM) induced a transient increase of oxygen uptake due to the electrophoretic $Ca^{2+}$-uptake into the mitochondrial matrix compartment. This addition of $Ca^{2+}$ initiated opening of the mtPTP, which slightly enhanced basal respiration due to uncoupling of the mitochondria (Fig. 4A). Fully uncoupled respiration was not seen since matrix-NAD, as a coenzyme of complex I, became strongly diluted under this condition. However, when NADH (1 mM) was subsequently added the oxygen uptake was enhanced dramatically indicating access of the added NADH to complex I. In contrast, no change of the oxygen uptake occurred after NADH addition, when opening of the mtPTP was blocked by CsA (Fig. 4B). A similar result was obtained with ropinirole (≥μL 100 μM, Fig. 4C).

### 3.3. Brain mitochondria and possible side effects of ropinirole

To account for the possible significance of the ropinirole-linked inhibition of the permeability transition in neuroprotection, we applied ropinirole to energized rat brain mitochondria (RBM). Firstly, the effect of ropinirole (100 μM) was studied by monitoring FCCP-triggered opening of the mtPTP, as shown in Fig. 5. To sensitize RBM for undergoing FCCP-triggered permeability transition, mitochondria were first loaded with an incremental amount of $Ca^{2+}$ (Fig. 5B).

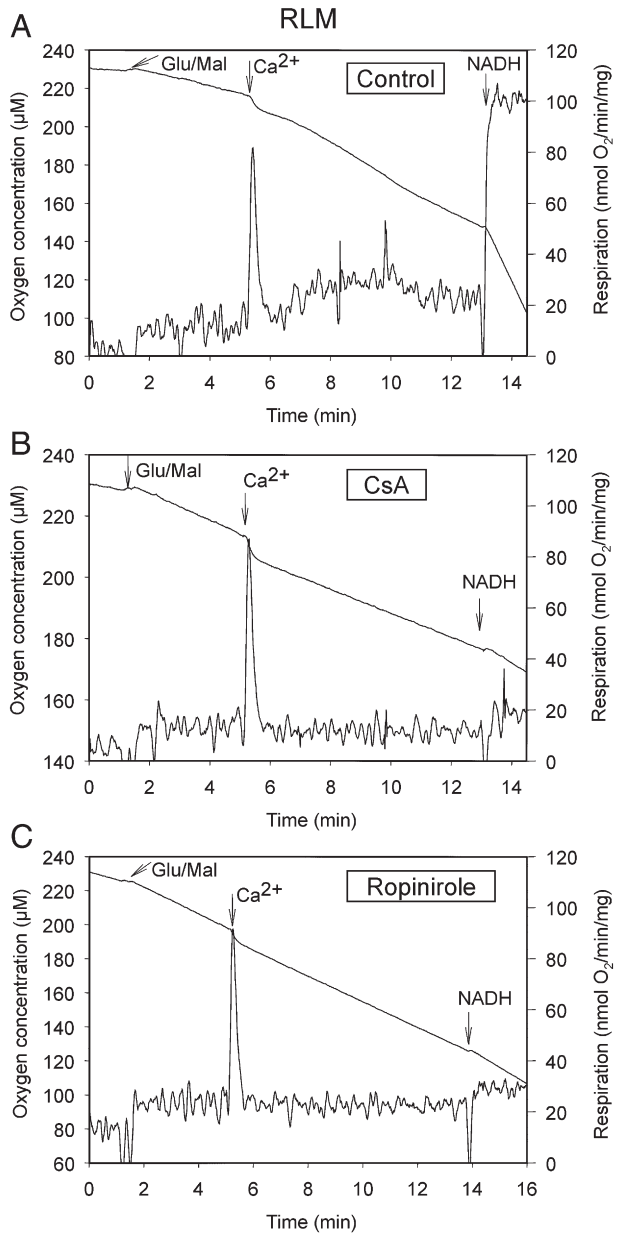


**Fig. 4.** Ropinirole prevents oxidation of external NADH by $Ca^{2+}$-treated mitochondria. Changes in medium-oxygen concentration and that of its first-derivative (rate of respiration) of the control incubation are shown. RLM (0.33 mg of protein/mL) were added to substrate-free incubation medium. Glutamate plus malate (Glu/Mal; 5 mM/5 mM), $Ca^{2+}$ (50 μM), and NADH (1 mM) were added as indicated. A: Opening of the mtPTP by $Ca^{2+}$ enables mitochondria to oxidize the NADH added, resulting in an increased rate of respiration. B: In the presence of CsA (2 μM) oxidation of added NADH is blocked. C: The rate of respiration of RLM pretreated with ropinirole (1 mM) is inhibited, as well. The data shown are representative of three experiments.

Subsequently, a very low amount of FCCP (0.05 μM) was added. As can be seen, ropinirole clearly inhibited the permeability transition-linked depolarization.

Furthermore, we examined a possible effect of ropinirole on the respiration of resting and of phosphorylating mitochondria. It was found that ropinirole affects respiration only slightly, even when it was applied up to 2 mM (Fig. 5A). Consequently, ropinirole does not act as an uncoupler or as an inhibitor of the oxidative phosphorylation.

Finally, we examined a possible antioxidative activity of ropinirole on the respiratory chain-generated ROS release by the mitochondria. For this purpose, the mitochondrial ROS production was stimulated by impairing the respiratory chain with the complex III-inhibitor antimycin A. ROS production was estimated by monitoring the $H_2O_2$ release using the Amplex Red/HRP system. Panel C of Fig. 5 shows clearly that ropinirole applied within the concentration range of 0.1 to 1 mM does not diminish $H_2O_2$ release, thereby excluding a direct $H_2O_2$ scavenging activity of ropinirole.

### 3.4. Effect of ropinirole on mtPTP in mitoplasts

In order to show the blocking effect of ropinirole directly upon the mtPTP, patch-clamp techniques were applied for measuring single-channel currents in liver mitoplasts. The recordings from patches with sufficient seal resistance showed a $Ca^{2+}$-induced channel conductance of more than 1 nS and a large variety of subconductances, which are typical features of the mtPTP. A complete block by ropinirole was

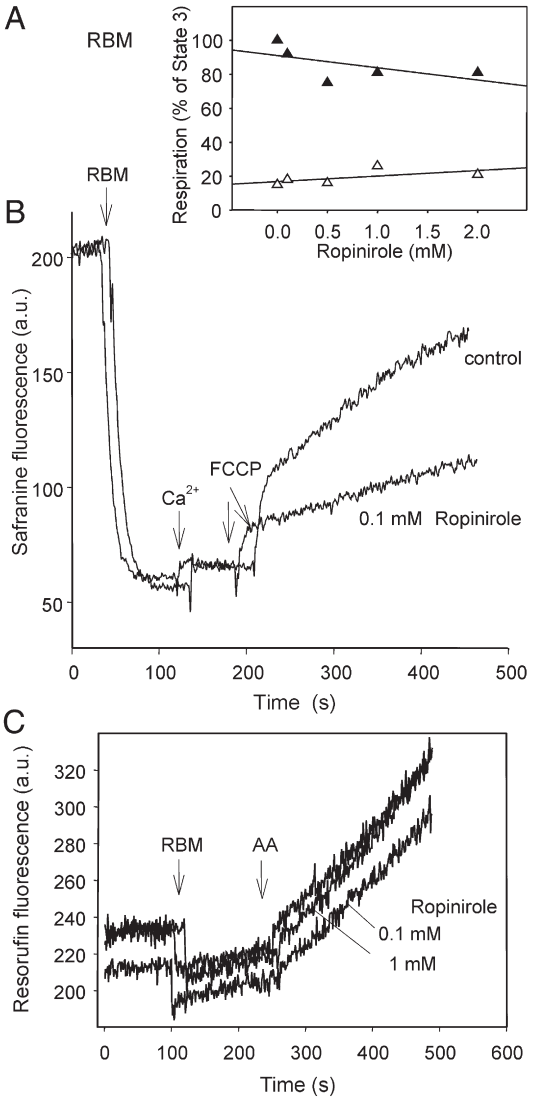


**Fig. 5.** Effect of ropinirole on respiration, permeability transition, and ROS using brain mitochondria. A: The effects of four increasing ropinirole concentrations (plus control) on the resting (open triangles) and ADP-induced respiration (State 3 respiration, filled triangles) of mitochondria from one rat brain are shown. State-3 respiration (41 nmol $O_2$/min/mg of protein) was stimulated by 2 mM of ADP. B: The effect of ropinirole on FCCP-triggered permeability transition is shown. RBM (1 mg/mL) were preloaded with $Ca^{2+}$ (50 nmol/mg protein), the final concentration of FCCP was 0.05 μM. C: The effect of ropinirole (0.1 or 1 mM) on the release of ROS by antimycin A-inhibited mitochondria is shown. Final concentration of antimycin A was 5 μM. All experiments were done in incubation medium supplemented with 0.1 mM Pi.

observed at a concentration of 5 µM (Fig. 6, traces 2 and 3). At this concentration, the effect was reversible upon washout in the control solution (Fig. 6, trace 4). The channel events could be dose-dependently and reversibly blocked by the mtPTP-inhibitor CsA (Fig. 6, traces 5 and 6), as was described previously for other inhibitors [13,16,21]. The analysis of the patch-clamp experiments showed that ropinirole inhibited the open probability ($P_o$) of the mtPTP at concentrations ranging from 30 nM to 10 µM. The best fit of the concentration–response curve was obtained with an $IC_{50}$ of 3.4 µM and a Hill coefficient of 1.3 (Fig. 7).

As the $IC_{50}$ is approximately an order of magnitude lower than in the swelling experiments we hypothesized that the missing Pi could explain this observation. Therefore, additional experiments were performed to investigate the role of increased Pi concentrations for the blocking effect of ropinirole. 10 µM ropinirole in combination with 10 mM Pi did not exhibit a similar blocking effect as 10 µM ropinirole alone, and a similar trend was observed in the case of 1 µM CsA alone and in combination with 10 mM Pi, where high Pi concentration attenuated the blocking effect of CsA (Fig. 8).

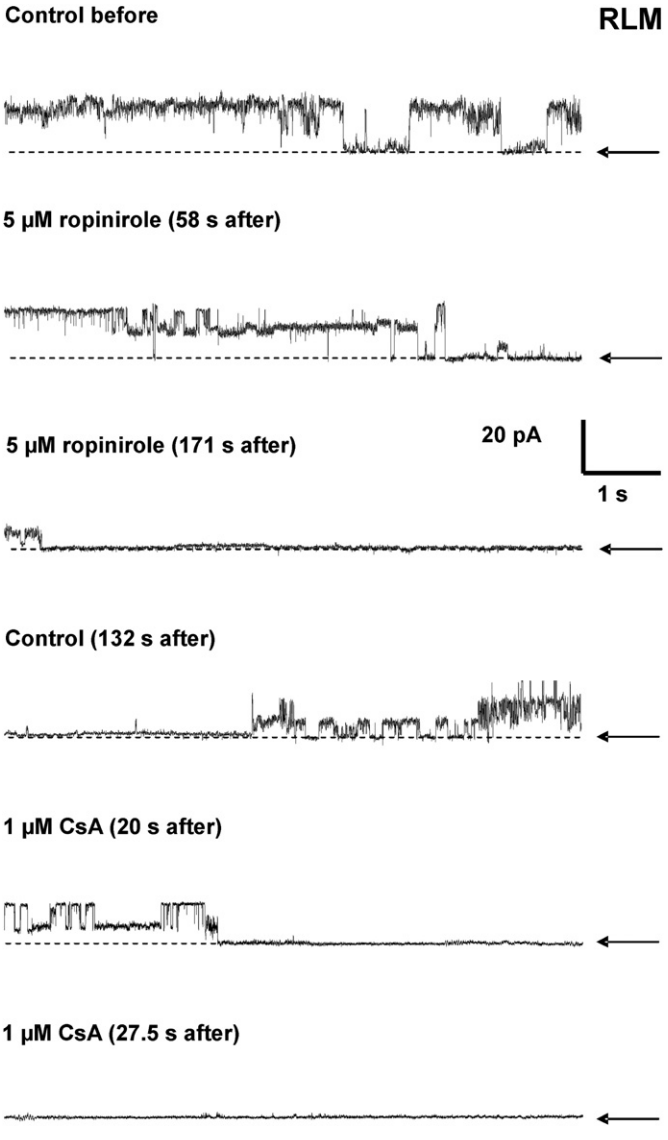


**Fig. 6.** Representative current traces of mtPTP conductivity before, during, and after application of ropinirole or CsA. Baseline PTP activity (control, 1st trace) was blocked by 5 µM ropinirole (2nd and 3 rd trace, at 58 and 171 s, respectively, after switching to ropinirole). Reversibility (4th trace, after switching to control). A different experiment shows vanishing single-channel activity 20 s (5th trace) and 27.5 s (6th trace) after 1 µM CsA as a control for the identity of the mtPTP. Dashed lines and arrows indicate the fully closed state. Holding potential ($E_h$): +20 mV.

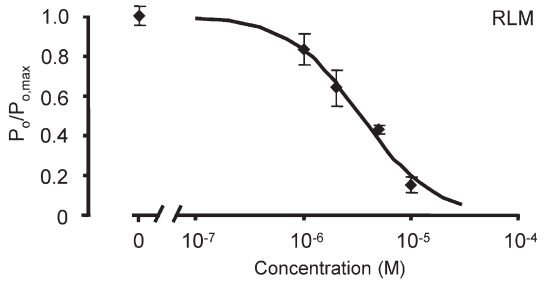


**Fig. 7.** Concentration–response curve for the normalised open probability ($P_o$) under the influence of ropinirole at $E_h = +20$ mV. $P_o$ was estimated by all point analysis of single-channel data in 3 to 5 1-min segments before ropinirole application and in 3 to 5 1-min segments after addition of ropinirole. Mean $P_o$ was calculated from up to 4 experiments at each concentration. Continuous curve was calculated by means of the Hill equation. Data are presented as the mean ± SE.

## 4. Discussion

Mitochondria exert a key function in apoptotic cell death (for review, [23]). This view has stimulated the search for drugs, which are able to protect mitochondria against permeability transition. In this study, we examined a possible inhibitory activity of ropinirole, a non-ergoline dopamine-D2-receptor agonist, on the opening of the mtPTP. The effect of ropinirole on mtPTP opening was demonstrated with liver mitochondria by mitochondrial swelling, membrane depolarization, Cyt *c* release and the oxidation of external NADH after treatment with $Ca^{2+}$. In our second approach, we examined the effect of ropinirole on the mtPTP-based conductance using mitoplasts. Despite mitoplasts being energetically impaired they offer the advantage to reveal a possible interaction of ropinirole with the mtPTP more directly. We have shown that ropinirole is able to block the $Ca^{2+}$-triggered opening of mtPTP in isolated, energized liver mitochondria (Figs. 2 and 3). This finding was further substantiated by demonstrating an inhibitory activity of ropinirole on mtPTP mediated membrane-conductivity in mitoplasts (Figs. 6–8). Importantly, ropinirole lowered the open probability ($P_o$) of the mtPTP at pharmacological low concentrations, ranging from 30 nM to 10 µM. Keeping in mind, that ropinirole is a doubled-charged cation at physiological pH [24], there is reason to assume that it can bind to negatively-charged

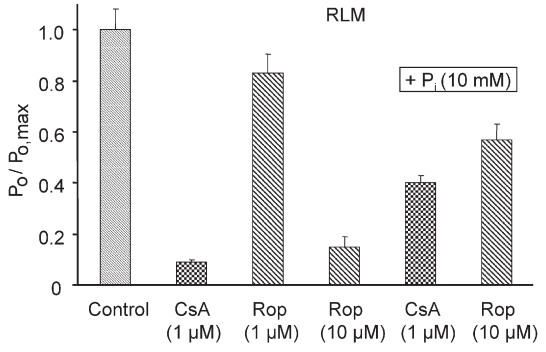


**Fig. 8.** Phosphate attenuates the inhibitory activity of ropinirole on mtPTP-based conductivity of mitoplasts. Mean $P_o$-values (± SE) of experiments with 1 µM ropinirole (0.83, $n = 3$) or 10 µM ropinirole (0.15, $n = 4$) alone or with ropinirole (10 µM) plus Pi (10 mM) in flow system only (0.57, $n = 4$), as compared with mean $P_o$ before adding test substances. Reduction was significant in these three measurements on the 5%, 1% and on the 5% level, respectively. Experiments were also performed with CsA (1 µM) alone (0.09, $n = 3$) or with CsA (1 µM) plus Pi (10 mM) in flow system only (0.40, $n = 3$) as a positive control. Reduction was significant on the 1% and on the 5% level, respectively.

groups of mtPTP (mainly, carboxylate groups). Furthermore, the high lipophilicity of ropinirole (log partition coefficient (octanol/phosphate buffer) = 3.31) suggests that it can permeate biological membranes easily [24]. Taken together, from both, lipophilicity and cationic nature of ropinirole, it is likely that ropinirole becomes enriched in the mitochondrial matrix compartment driven by $\Delta\psi_m$. Thus, ropinirole could bind to the cytosolic-side and/or to the matrix-side of the mtPTP complex.

The inhibitory activity of ropinirole on the mtPTP opening is concentration-dependently antagonized by Pi (Fig. 3). When mitochondria were suspended in 1 mM Pi-containing medium, the inhibitory activity of ropinirole was significantly reduced. With mitoplasts, a similar Pi-sensitivity of the inhibitory effect of ropinirole on the $Ca^{2+}$-induced mtPTP opening was found. Attenuation of the ropinirole-linked inhibition of the opening of the mPTP by Pi fits well with the fact, that Pi is generally referred to act as a PTP inducer [25]. In addition, a direct interaction of the negatively-charged Pi with the mtPTP-bound ropinirole or with the free ropinirole itself could contribute to this attenuating effect of Pi. In the first case, it is likely that Pi removed ropinirole from the mtPTP, whereas in the second case Pi decreased the effective ropinirole concentration in the medium.

Moreover, it became evident recently that the relationship between Pi and mtPTP opening is much more complex than previously thought (see for a recent review [26]). Due to these results Pi is necessary for the CsA inhibition of the mtPTP [14]. In this case, the 'co-inhibitory activity' of Pi might be attributed to a distinct interaction of Pi with the mtPTP itself or with the mtPTP-inhibitor complex. In this context it is important that addition of Pi to mitochondria suspended in Pi-free medium decreases the matrix-pH (Pi is co-transported with $H^+$ or in exchange to $OH^-$), an observation paralled by an increase in $\Delta\psi_m$ [27]. This decrease of matrix-pH is likely to change the protonation of critical histidyl residues of the mtPTP thus preventing the inhibitor complex from binding to the mtPTP [28].

It has been discussed also that neuroprotection by ropinerole could be partly attributed to direct ROS scavenging and/or an activation of the cellular enzymatic antioxidative defense system [4]. Our results do not support such activity of ropinirole (Fig. 5C).

Finally, a crucial issue is: could ropinirole inhibit opening of the mtPTP in human brain tissue at concentrations used therapeutically? There are several arguments for an inhibitory effect of ropinirole on the mtPTP under these conditions. First, a blockade of the $Ca^{2+}$-triggered permeability transition is also found in experiments with mitochondria isolated from rat brain (Fig. 5B). Secondly, as mentioned above, ropinirole is a highly lipophilic compound, suggesting that it permeates biological membrane easily. Thirdly, when patients are treated with so-called "tid-dosing" (4 mg), the serum concentration of ropinirole could be increased up to 100 nM [29]. Moreover, due to the existing membrane potentials at the plasma membrane (about −60 mV) and the inner mitochondrial membrane (about −150 mV), a $10^2$- and $10^7$-fold accumulation of the double-positive-charged ropinirole is calculated for the cytosolic compartment and the matrix compartment, respectively. However, the concentration of free ropinirole in both compartments should be much lower, since it is likely that it binds to anionic binding-sites of membrane phospholipids and proteins. In conclusion, this study suggests that there is good reason to assume that ropinirole is active as an inhibitor of the mtPTP under *in vivo* conditions.

## Acknowledgements

Ropinirole was a gift from GlaxoSmithKline (Brentford, Middlesex, UK). We are indebted to Dr. J. Lindquist for reading the manuscript. Financial support from the BMFT, the State of Sachsen-Anhalt, and by the 'Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE)' is gratefully acknowledged.

## References

[1] C.G. Goetz, Dopaminergic agonists in the treatment of Parkinson's disease, Neurology 40 (1990) 54–57.
[2] C.E. Clarke, M. Guttman, Dopamine agonist monotherapy in Parkinson's disease, Lancet 360 (2002) 1767–1769.
[3] A.J. Carter, R.E. Muller, Pramipexole, a dopamine D2 autoreceptor agonist, decreases the extracellular concentration of dopamine in vivo, Eur. J. Pharmacol. 200 (1991) 65–72.
[4] M. Iida, I. Miyazaki, K. Tanaka, H. Kabuto, E. Iwata-Ichikawa, N. Ogawa, Dopamine D2 receptor-mediated antioxidant and neuroprotective effects of ropinirole, a dopamine agonist, Brain Res. 838 (1999) 51–59.
[5] G. van Loo, X. Saelens, M. van Gurp, M. MacFarlane, S.J. Martin, P. Vandenabeele, The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet, Cell Death Differ. 9 (2002) 1031–1042.
[6] A.P. Halestrap, G.P. McStay, S.J. Clarke, The permeability transition pore complex: another view, Biochimie 84 (2002) 153–166.
[7] K.M. Broekemeier, M.E. Dempsey, D.R. Pfeiffer, Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria, J Biol. Chem 264 (1989) 7826–7830.
[8] P. Bernardi, S. Vassanelli, P. Veronese, R. Colonna, I. Szabo, M. Zoratti, Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations, J. Biol. Chem. 267 (1992) 2934–2939.
[9] K.W. Kinnally, Y.N. Antonenko, D.B. Zorov, Modulation of inner mitochondrial membrane channel activity, J. Bioenerg. Biomembr. 24 (1992) 99–110.
[10] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139–176.
[11] Y. Cheng, G. Debska-Vielhaber, D. Siemen, Interaction of mitochondrial potassium channels with the permeability transition pore, FEBS Lett. (Dec 27. 2009) Electronic publication ahead of print.
[12] P. Bernardi, A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa, M.A. Forte, The mitochondrial permeability transition from in vitro artifact to disease target, FEBS J. 273 (2006) 2077–2099.
[13] C. Loupatatzis, G. Seitz, P. Schönfeld, F. Lang, D. Siemen, Single-channel currents of the permeability transition pore from the inner mitochondrial membrane of rat liver and of a human hepatoma cell line, Cell Physiol. Biochem. 12 (2002) 269–278.
[14] E. Basso, V. Petronilli, M.A. Forte, P. Bernardi, Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation, J. Biol. Chem. 283 (2008) 26307–26311.
[15] F. Di Lisa, M. Canton, R. Menabò, G. Dodoni, P. Bernardi, Mitochondria and reperfusion injury. The role of permeability transition, Basic Res. Cardiol. 98 (2003) 235–241.
[16] D.S. Cassarino, C.P. Fall, T.S. Smith, P.P. Bennett, Pramipexole reduces reactive oxygen species production in vivo and in vitro and inhibits the mitochondrial permeability transition produced by the Parkinsonian neurotoxin methylpyridinium ion, J. Neurochem. 71 (1998) 295–301.
[17] I. Sayeed, S. Parvez, K. Winkler-Stuck, G. Seitz, I. Trieu, C.-W. Wallesch, P. Schönfeld, D. Siemen, Patch clamp reveals powerful blockade of the mitochondrial permeability transition pore by the D2-receptor agonist pramipexole, FASEB J. 20 (2006) 556–558.
[18] Y. Liu, G. Fiskum, D. Schubert, Generation of reactive oxygen species by the mitochondrial electron transport chain, J. Neurochem. 80 (2002) 780–787.
[19] K.E.O. Åkerman, M.K.F. Wikström, Safranine as a probe of the mitochondrial membrane potential, FEBS Lett. 68 (1976) 191–197.
[20] S.P. Brooks, K.P. Storey, Bound and determined: a computer program for making buffers of defined ion concentrations, Anal. Biochem. 201 (1992) 119–126.
[21] S.A. Andrabi, I. Sayeed, D. Siemen, G. Wolf, T.F. Horn, Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin, FASEB J. 18 (2004) 869–871.
[22] E. Fontaine, O. Eriksson, F. Ichas, P. Bernardi, Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex I, J. Biol. Chem. 273 (1998) 12662–12668.
[23] D.R. Green, G. Kroemer, Pharmacological manipulation of cell death: clinical applications in sight? J. Clin. Invest. 115 (2005) 2610–2617.
[24] A. Luzardo-Alvarez, M.B. Delgado-Charro, J. Blanco-Mendez, Iontophoretic delivery of ropinirole hydrochloride: effect of current density and vehicle formulation, Pharm. Res. 18 (2001) 1714–1720.
[25] D.R. Hunter, R.A. Haworth, J.H. Southard, Relationship between configuration, function and permeability in calcium-treated mitochondria, J. Biol. Chem 251 (1976) 5069–5077.
[26] F. Di Lisa, P. Bernardi, A CaPful of mechanisms regulating the mitochondrial permeability transition, J. Mol. Cell. Cardiol. 46 (2009) 775–780.
[27] D.G. Nicholls, S.J. Ferguson, Bioenergetics 3, Academic Press, London, 2002.
[28] A. Nicolli, V. Petronilli, P. Bernardi, Modulation of the mitochondrial cyclosporine A-sensitive permeability transition pore by matrix pH. Evidence that the pore open-closed probability is regulated by reversible histidine protonation, Biochemistry 32 (1993) 4461–4465.
[29] J. Hubble, W.C. Koller, P. Atchison, A.C. Taylor, D.R. Citerone, B.D. Zussman, C.J. Friedman, N. Hawker, Linear pharmacokinetic behavior of ropinirole, J. Clin. Pharmacol. 40 (2000) 641–646.