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Reconstitution of brain mitochondria inner membrane into planar lipid bilayer

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Short communication

Abstact. Ion channels are present in the inner mitochondrial membrane. They play an important role in cellular processes. Potassium and chloride channels are involved in regulation of mitochondrial volume, membrane potential and acidification. The mitochondrial potassium channels have been suggested as triggers and end effectors in cytoprotection. In our study we measured single channel activities after reconstitution of submitochondrial particles from rat brain mitochondria into planar lipid membranes. After incorporation, two different potassium selective currents were recorded with single channel conductance from 260 to 320 pS and from 70 to 90 pS in gradient (*cis/trans*) 50/450 and 50/150 mM KCl solutions, respectively. We also observed activity of the chloride ion channel. The measured single channel conductance was from 80 to 90 pS in gradient (*cis/trans*) 50/450 mM KCl solution. Our results suggest that various ion channels are present in the inner mitochondrial membrane of brain mitochondria.

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Ion channels specific for potassium or chloride ions are present in membranes of intracellular organelles such as sarcoplasmic (endoplasmic) reticulum (Zucchi and Ronca-Testoni 1997), mitochondria (Szewczyk and Wojtczak 2002), nucleus (Ashley 2003) and zymogen granules (Thevenod 2002). They probably play an important role in cellular processes such as compensation of electrical charges, formation of ΔpH or regulation of volume changes (Szewczyk 1998). The activity of intracellular ion channels can be analyzed by electrophysiological techniques such as patch-clamp or reconstitution into planar lipid bilayer.

In a mitochondrial inner membrane few types of ion channels were observed (for review see Szewczyk and Wojtczak 2002). In 1991, a mitochondrial ATP-regulated potassium channel (mitoK$_{ATP}$ channel) was described in liver mitochondria (Inoue et al. 1991). Later, a similar channel was observed in heart muscle (Paucek et al. 1992), brain (Bajgar et al. 2001, Debska et al. 2001), skeletal muscle (Debska et al. 2002) and human lymphocytes (Dahlem et al. 2004). It has been shown that activity of mitoK$_{ATP}$ channel is inhibited by Mg^{2+}, ATP/Mg^{2+} complex and quinine (Bednarczyk et al. 2004, 2005, Paucek et al. 1992), 5-hydroxydecanoic acid (Jaburek et al. 1998) and antidiabetic sulfonylureas such as glibenclamide (Paucek et al. 1992). The mitoK$_{ATP}$ channel is stimulated by GDP or GTP and potassium channel openers (KCOs) such as diazoxide (Garlid et al. 1996, Paucek et al. 1996).

Another K^+ selective ion channel known as big conductance potassium channel (mitoBK$_{Ca}$ channel) was described in inner mitochondrial membrane of the human glioma cell line LN229 (Siemen et al. 1999) and in heart ventricular cells (Xu et al. 2002). The mitoBK$_{Ca}$ channel is voltage dependent and can be stimulated at micromolar, physiological concentrations of calcium ions. The mitoBK$_{Ca}$ channel can be activated by KCOs, e.g., NS1619 (for review see Kicinska et al. 2004) and inhibited by charybdotoxin, iberiotoxin and paxilline (Sato et al. 2005, Xu et al. 2002).

Both the mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels play a key role in cytoprotection (Szewczyk and Marban 1999). Activation of mitoK$_{ATP}$ channel by KCOs in heart muscle and brain tissue can protect against cell death during ischemia-reperfusion injury (Garlid et al. 2003, Kicinska and Szewczyk 2003, Kis et al. 2004, Liu et al. 2002, O'Rourke 2004). Similarly, activation of the mitoBK$_{Ca}$ channel in heart muscle cells can be beneficial under stress conditions (Sato et al. 2005, Xu et al. 2002).

Using patch-clamp technique, the presence of chloride ion channels has been reported in mitochondria from brown adipose tissue (Klitsch and Siemen 1991), liver (Antonenko et al. 1994) and heart muscle (Hayman et al. 1993). A chloride ion channel known as mtCLIC/CLIC4 has been identified in mitochondria from mouse keratinocytes, lung brain, liver and skin (Fernandez-Salas et al. 1999). This channel is also present in inner mitochondrial membrane of human keratinocytes (Fernandez-Salas et al. 2002). It was shown that the expression of mtCLIC can be regulated by p53 or tumor necrosis factor α (Fernandez-Salas et al. 1999). Chloride channels are involved in several crucial cell processes such as regulating cell volume and stabilization of membrane potential (Jentsch and Gunther 1997).

The aim of the present study was to identify the profile of ion channels present in rat brain inner mitochondrial membrane. The characterization was performed on the level of single channel recordings with the application of black lipid membranes (BLM) technique. Measuring single channel activity with the BLM technique gives us a unique opportunity to study transport of ions from both sides of artificial membrane.

Five adult Wistar rats (60–90 days old) were used in the study. The brains without cerebella were used for inner mitochondrial membrane preparation. Five independent preparations were obtained. The mitochondria were isolated at ice-cold conditions according to the protocol described by Kudin and coauthors 2004. Rats were anesthetized with chloroform and killed by decapitation. The brain was minced, 10 ml of MSE-nagarase solution was added, and all was homogenized at 600 units/s using a potter homogenizer. Thereafter, 20 ml of MSE solution was added and centrifuged at 2 000 × g for 4 min. After, the supernatant was passed through cheesecloth and centrifuged at 12 000 × g for 9 min. To permeabilize synaptosomes the pellet was dissolved with 10 ml of MSE-digitonin and homogenized 8–10 times. Finally, the suspension was centrifuged at 12 000 × g for 11 min. The pellet was dissolved in MSE solution to obtain about 20 mg protein per ml. Next, the rat brain mitochondria were thawed sonicated 3 × 15 s and ultracentrifuged (Cino and Del Maestro 1989). The submitochondrial particles (SMP) were resuspended at final concentration of about 4 mg per ml. All chemicals were of the highest purity available commercially. Solutions include the following: MSE solution (225 mM mannitol, 75 mM

sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml BSA, pH 7.4), MSE-nagarase solution (0.05% nagarase in MSE solution), MSE-digitonin solution (0.02% digitonin in MSE solution).

Experiments were performed by using black lipid membrane technique as previously described (Bednarczyk et al. 2004, 2005, Hordejuk et al. 2004). In brief, BLMs were formed in a 250 μm diameter hole drilled in a Delrin cup (Warner Instrument Corp., Hamden, CT USA), which separated two chambers (*cis/trans*). The chambers contained 50/150 or 50/450 mM KCl (*cis/trans*), 20 mM Tris-HCl, pH 7.2 solutions. The outline of the aperture was coated with a lipid solution and N_2 dried prior to bilayer formation to improve membrane stability. BLMs were painted using asolectin in *n*-decane at a final concentration of 25 mg lipid/ml. Rat brain SMP (4 mg of protein/ml, 1–3 μl) was added to the *trans* compartment. Formation and thinning of the bilayer was monitored by capacitance measurements and optical observations. Electrical connections were made by Ag/AgCl electrodes and agar salt bridges (3M KCl) to minimize liquid junction potentials. Voltage was applied to the *cis* compartment of the chamber and the *trans* compartment was grounded. The current was measured using a Bilayer Membrane Amplifier (BLM-120, BioLogic).

Signals were filtered at 500 Hz. The current was digitized at a sampling rate of 100 kHz (A/D converter PowerLab 2/25, ADInstruments) and transferred to PC for off-line analysis by Chart v5.2 (PowerLab ADInstruments) and pCLAMP8.1 (Axon Laboratory). The channel recordings illustrated are representative of the most frequently observed conductances under the given conditions. The conductance was calculated from the current-voltage relationship. Single channel currents were recorded at different voltages. The reversal potential was calculated from fitted curve (polynomial second order) and measured in experimental conditions. Data from the experiments were reported as mean value ± SD (standard deviation). The standard deviation was calculated using Chart v5.2 program for statistical analysis.

The preparation of inner mitochondrial membrane (submitochondrial particles) from rat brain was reconstituted into black lipid membrane and the currents selective for potassium and chloride channel were observed. The fusion of SMP particles to the bilayer was usually observed within 15–30 min after addition

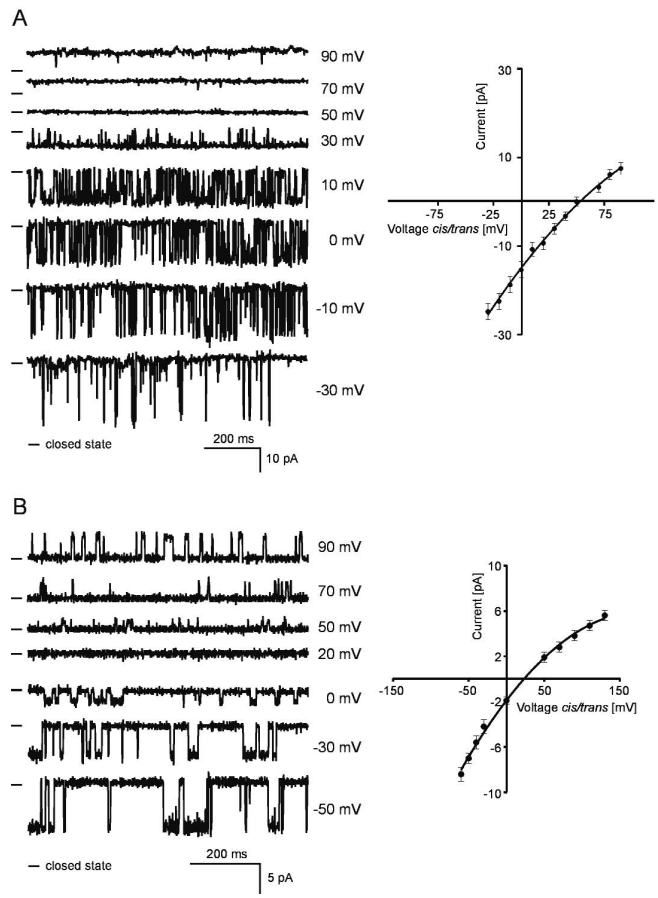

Fig. 1. Single channel recordings of potassium channel from rat brain mitochondria. (A) Single channel recordings and current-voltage characteristics of single channel events in (cis/trans) 50/450 mM KCl gradient conditions at different voltages. (–) Indicates the closed state of the channel. Recordings were low pass filtered at 500 Hz. (B) Single channel recordings and current-voltage characteristics of single channel events in (cis/trans) 50/150 mM KCl gradient conditions at different voltages. (–) Indicates the closed state of the channel. Recordings were low pass filtered at 500 Hz.

to the *trans* compartment. We observed three types of ion channels with different single channel conductance and selectivity. First, original single channel recordings and current-voltage relationships for single channel opening in gradient 50/450 mM KCl (*cis/trans*) solution at different voltages was is shown (Fig. 1A) (*n*=21). The channel conductance is from 260 to 320 pS under gradient conditions. The reversal potential measured in the gradient 50/450 mM KCl (*cis/trans*) solution is equal to 50 mV and this proves that the examined channel is cation-selective. Mean reversal potential calculated after fitting curve to experimental data is equal

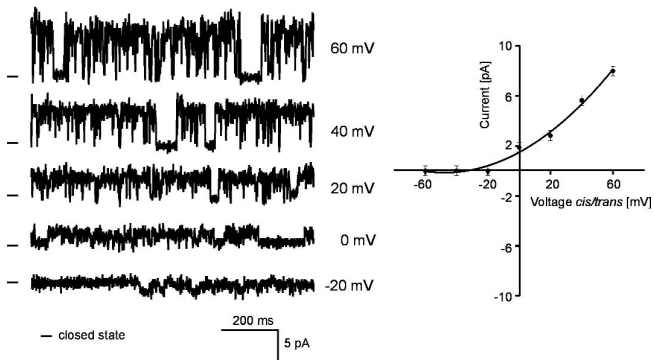

Fig. 2. Single channel recordings of anion-selective ion channel from rat brain mitochondria. Single channel recordings and current-voltage characteristics of single channel events in (cis/trans) 50/450 mM KCl gradient conditions at different voltages. (–) Indicates the closed state of the channel. Recordings were low pass filtered at 500 Hz.

to 53 mV. Additionally, we observed that the potassium channel activity was blocked by negative voltages, so this channel is voltage dependent. Our results indicate that observed activity was similar to the mitoBK$_{Ca}$ channel reported previously in glioma and cardiac mitochondria (Siemen et al. 1999, Xu et al. 2002). Second, Fig.1B presents single channel recordings and current-voltage relationships for single channel opening in gradient 50/150 mM KCl (*cis/trans*) solution at different voltages (*n*=4). The channel conductance is from 70 to 90 pS under gradient conditions. The reversal potential measured in the gradient 50/150 mM KCl (*cis/trans*) solution is equal to 20 mV and this proves that the examined channel is cation-selective. Mean reversal potential calculated after fitting curve to experimental data is equal to 23 mV. Previously similar channel from heart mitochondria was described as the mitoK$_{ATP}$ channel with conductance 18 pS, 56 pS and about 100 pS (Bednarczyk et al. 2004, Zhang et al. 2001). On the other hand, we observed activity of anion-selective ion channel (*n*=6). Figure 2 shows single channel recordings and current-voltage relationships for single channel opening in gradient 50/450 mM KCl (*cis/trans*) solution at different voltages. The channel conductance is from 80 to 90 pS under gradient conditions. The reversal potential measured in the gradient 50/450 mM KCl (*cis/trans*) solution is equal to -20 mV and this proves that the examined channel is anion-selective. Mean reversal potential calculated after fitting curve to experimental data is equal to 23 mV. Additionally, our data indicate that the channel is strongly voltage gated at negative voltages. Application of negative voltages below -20 mV caused closing of the channel. Probably, we have observed the voltage dependent chloride channel previously described in mitochondria from brown adipose and cardiac tissue (Hayman et al. 1993, Klitsch and Siemen 1991).

Summarizing this work, our results suggest that the rat brain inner mitochondrial membrane contains various types of potassium and chloride ion channels (Table I). We noticed the big conductance potassium channel similar to mitoBK$_{Ca}$ channel and small conductance potassium channels with properties similar to the mitoK$_{ATP}$ channel, previously described in heart mitochondria. We also reported the presence of chloride selective ion channel in the inner mitochondrial membrane.

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Table I

Electrophysiological properties of ion channels from rat brain inner mitochondrial membrane

	Conditions (mM KCl) (*cis/trans*)	Conductance (pS)	Fitting curve potential (mV)	Nernst potential (mV)
Big potassium channel	50/450	260-320	53	56.4
Small potassium channel	50/150	70-90	23	28.2
Chloride channel	50/450	80-90	-23	-56.4

For details see paragraph describing black lipid membrane technique. Nernst potential was calculated according to the method as described previously (Hille 2001).

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