

STILBENE DERIVATIVES INHIBIT THE ACTIVITY OF THE INNER MITOCHONDRIAL MEMBRANE CHLORIDE CHANNELS

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Abstract: Ion channels selective for chloride ions are present in all biological membranes, where they regulate the cell volume or membrane potential. Various chloride channels from mitochondrial membranes have been described in recent years. The aim of our study was to characterize the effect of stilbene derivatives on single-chloride channel activity in the inner mitochondrial membrane. The measurements were performed after the reconstitution into a planar lipid bilayer of the inner mitochondrial membranes from rat skeletal muscle (SMM), rat brain (BM) and heart (HM) mitochondria. After incorporation in a symmetric 450/450 mM KCl solution (*cis/trans*), the chloride channels were recorded with a mean conductance of 155 ± 5 pS (rat skeletal muscle) and 120 ± 16 pS (rat brain). The conductances of the chloride channels from the rat heart mitochondria in 250/50

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Abbreviations used: BLM – black lipid membrane; BM – brain mitochondria; CLICs – chloride intracellular channels; DIDS – 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DTNB – 6,6'-dinitro-3,3'-dithiodibenzoic acid; HM – heart mitochondria; ITS – 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid; IMAC – mitochondrial inner membrane anion channel; SITS – 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid; SMM – skeletal muscle mitochondria; SMP – submitochondrial particles; TNF α – tumor necrosis factor α ; UPCs – uncoupling proteins

mM KCl (*cis/trans*) gradient solutions were within the 70-130 pS range. The chloride channels were inhibited by these two stilbene derivatives: 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS). The skeletal muscle mitochondrial chloride channel was blocked after the addition of 1 mM DIDS or SITS, whereas the brain mitochondrial channel was blocked by 300 μ M DIDS or SITS. The chloride channel from the rat heart mitochondria was inhibited by 50-100 μ M DIDS. The inhibitory effect of DIDS was irreversible. Our results confirm the presence of chloride channels sensitive to stilbene derivatives in the inner mitochondrial membrane from rat skeletal muscle, brain and heart cells.

Key words: Mitochondria, Chloride channel, Stilbene derivatives, Black lipid membrane

INTRODUCTION

The ion channels in the mitochondrial membranes are a popular subject of research due to their involvement in the regulation of cellular life/death events. The potassium channels in the inner mitochondrial membrane are thought to be involved in cytoprotection. Their activation by potassium channel openers lowered the incidence of cell death under various conditions, such as oxidative stress or ischemia [1]. It was recently shown that an increase in the level of chloride channel expression leads to apoptosis. Interestingly, chloride channel inhibition by stilbene derivatives, such as DIDS, rescued the cells from apoptotic death when applied during reperfusion [2]. There is still very limited knowledge about the inner mitochondrial membrane chloride channels. One of the better characterized anion channels from the inner mitochondrial membrane is IMAC (the mitochondrial inner membrane anion channel) [3, 4], which is regulated by Mg^{2+} ions and the pH in the mitochondrial matrix. A decrease in matrix Mg^{2+} concentration or matrix alkalization enhances the permeability of the inner membrane to Cl^- [3, 5]. The molecular structure of the IMAC channel has yet to be defined. At the end of the 1980s, Sorgato *et al.* [6], using the patch-clamp technique, described an inner mitochondrial membrane anion channel with a conductance of 108 pS. Subsequently, this 108-pS channel was located in patch-clamp experiments conducted on mitoplasts in the liver [7], heart [7], brain [8] and brown adipose tissue [9]. The properties of IMAC and the 108-pS channel seemed to be similar [10]. Two distinct anion channels were identified in reconstituted cardiac mitoplasts [11].

Among the chloride channels observed in the inner mitochondrial membrane, there is only one – CLIC4 (also called mtCLIC or p64H1) – that has been cloned [12]. CLIC4 belongs to the protein family of chloride intracellular channels (CLIC), including protein p64 [13], CLIC1 through CLIC6 [12, 14-18] and panchorin [19]. The CLIC proteins are expressed in almost all mammalian cells: CLIC4 is highly expressed in the skin, kidneys, liver and brain. Furthermore, this chloride channel has been found in the endoplasmic reticulum, Golgi and

large dense core vesicles in the brain, and in the cytoplasm and inner mitochondrial membrane in human and mouse keratinocytes [12, 20]. It was observed that CLIC4 gene expression was regulated by apoptotic factors, such as p53 and tumor necrosis factor α (TNF α) [21]. Overexpression of CLIC4 results in apoptosis, which is preceded by a loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation [21, 22]. As described by Singh and Ashley [23], CLIC4 forms a channel (conductance \sim 15 pS) that is poorly selective for chloride ions (with a mean Cl⁻:K⁺ selectivity of 0.54 ± 0.09). The activity of the CLIC4 channel was blocked after the addition of 0.2 mM DTNB. In this study, we examined the effect of Cl⁻ channel blockers, namely the stilbene derivatives 4,4'-diisothiocyano-2,2'-stilbenedisulfonate (DIDS) and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), on the activity of inner mitochondrial membrane chloride channels from rat skeletal muscle (SMM), rat brain (BM) and rat heart (HM) cells. Using black lipid membrane measurements, we found new evidence that a chloride channel from the inner mitochondrial membrane from rat skeletal muscle, rat brain and rat heart cells, is sensitive to DIDS and SITS.

MATERIALS AND METHODS

Chemicals

L- α -phosphatidyl-choline (asolectin) and n-decane were obtained from Sigma-Aldrich, Germany. All the chemicals used were of the highest purity available commercially.

Isolation of rat skeletal muscle mitochondria and preparation of submitochondrial particles (SMP)

Rat skeletal muscle mitochondria were isolated at 4°C as previously described [24, 25]. Briefly, the mitochondria were suspended in the isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH₂PO₄, 5 mM MOPS, 0.1% BSA, pH 7.15) and centrifuged at 14,000 x g for 10 min. Then the mitochondrial pellet was again suspended in the isolation buffer. The suspension was loaded on top of a Percoll solution (30% Percoll, 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4) and centrifuged at 35,000 x g for 30 min. The mitochondrial fraction was then collected and washed twice with the isolation buffer and resuspended at 10-20 mg of protein/ml.

Freshly prepared mitochondria were sonicated for 8 x 15 s and centrifuged at 14,000 x g for 15 min to pellet the unbroken mitochondria. The supernatant was again centrifuged at 140,000 x g for 35 min, and the SMP were resuspended in the isolation buffer without BSA at 5 mg of protein/ml.

Isolation of rat brain mitochondria and preparation of submitochondrial particles (SMP)

Wistar rats (60-90 days old) were used. The brains minus the cerebella were used for the inner mitochondrial membrane preparation. The mitochondria were

isolated at ice-cold conditions according to the protocol described by Kudin *et al.* [26]. Each rat was anesthetized with chloroform and killed by decapitation. The brain was minced, 10 ml of MSE-nagarase solution (0.05% nagarase in MSE solution) was added, and the mixture was homogenized at 600 revs/s using a Potter homogenizer. Then, 20 ml of MSE solution (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml BSA, pH 7.4) was added and the diluted homogenate was centrifuged at $2,000 \times g$ for 4 min. Afterwards, the supernatant was passed through a cheesecloth and centrifuged at $12,000 \times g$ for 9 min. To permeabilize the synaptosomes, the pellet was dissolved in 10 ml of MSE-digtonin (0.02% digtonin in MSE solution) and homogenized 8-10 times. Finally, the suspension was centrifuged at $12,000 \times g$ 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 for 3×45 s and ultracentrifuged [27]. The submitochondrial particles were resuspended at a final concentration of about 4 mg protein per ml.

Isolation of rat heart mitochondrial inner membrane vesicles

Mitochondria from the hearts of male Wistar rats were isolated as previously described [28]. The details of the isolation procedure were given in [29].

Black lipid membrane (BLM) measurements

Measurements of the channel activity in the brain and skeletal muscle mitochondria were performed essentially as previously described [30-33]. Briefly, BLMs were formed in a 250 μ m diameter hole drilled in a Delrin cup (Warner Instrument, CT) which separated two chambers (*cis* and *trans*, each of 1 ml internal volume). The chambers contained a 450/50 or 450/450 mM KCl (*cis/trans*) and 20 mM Tris-HCl solution, pH 7.2. The outline of the aperture was coated with a lipid solution and N₂-dried prior to bilayer formation to improve membrane stability. The BLMs were painted using asolectin in n-decane at a final concentration of 25 mg lipid/ml. Rat skeletal muscle SMP (5 mg of protein/ml, 1-5 μ l) was added to the *cis* compartment. All the measurements were carried out at room temperature (25°C). The formation and thinning of the bilayer was monitored by capacitance measurements and optical observations. The final accepted capacitance values ranged from 110 to 180 pF. Electrical connections were made by Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Voltage was applied to the *trans* compartment of the chamber and the *cis* compartment was grounded (Fig. 1). The current was measured using a Bilayer Membrane Amplifier (BLM-120, BioLogic). Single-channel data was filtered at 100 Hz. The current was digitized at a sampling rate of 100 kHz (A/D converter PowerLab 2/20, ADInstruments) and transferred to a PC for off-line analysis by Chart v5.2.2 (PowerLab ADInstruments) and pCLAMP9.0 (Axon Instruments). The pCLAMP9.0 software package was used for data processing. The channel recordings illustrated are representative of the conductance most frequently

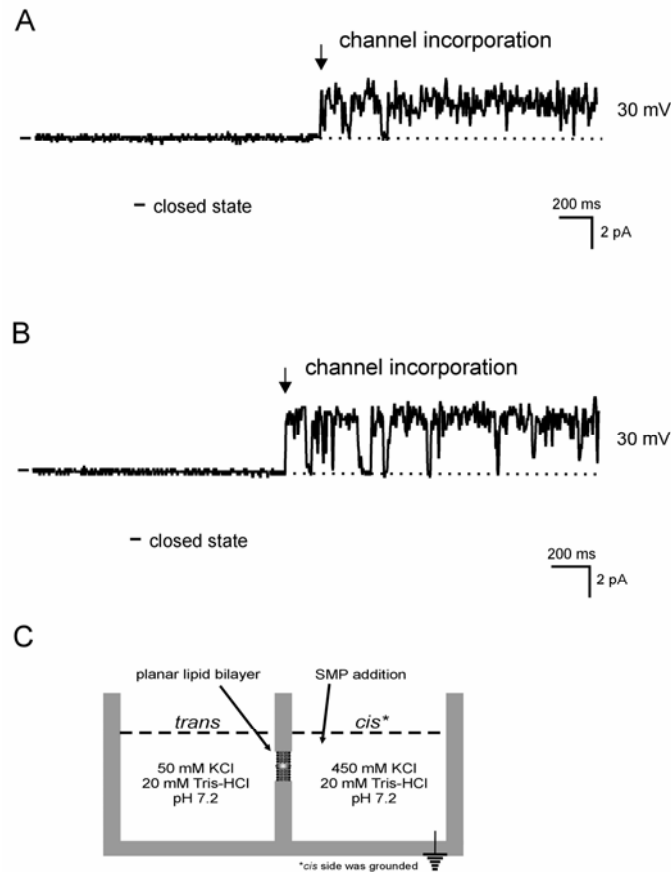


Fig. 1. Single-channel recordings of mitoCl channels from rat skeletal muscle and brain mitochondria in planar lipid bilayers. A – Recordings of the mitoCl channel from rat skeletal muscle mitochondria and B – from rat brain mitochondria in a 450/50 mM KCl gradient solution before and after the incorporation of SMP vesicles (arrow) at 30 mV. C – Configuration of the *cis* and *trans* compartments used in the experiments. Reconstitution of the inner mitochondrial membranes into a planar lipid bilayer was performed as described in the Materials and Methods section.

observed under the given conditions. The conductance was calculated from the current-voltage relationship. Data from three or more independent experiments is reported as the mean values \pm SD (standard deviation).

Measurements of single-channel activity from cardiac mitochondria were performed under the optimal conditions for cardiac inner mitochondrial membranes. BLM were formed across an aperture (diameter \sim 0.1 mm) separating the *cis* and *trans* chambers using a mixture of dioleoyl-glycero-phosphatidylcholine, dioleoyl-glycero-phosphatidylserine, and dioleoyl-glycero-phosphoethanolamine at a molar ratio of 3:2:1 in n-decane (20 mg/ml), in

a manner similar to the method used in a previous study [29]. The composition of the solutions was: in the *trans* chamber, 50 mM KCl, 2 mM MgCl₂, 0.4 mM CaCl₂, 1 mM EGTA, 2 mM Na₂ATP, 10/5 mM Hepes/Tris, 7.4 pH; and in the *cis* chamber, 250 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 2 mM Na₂ATP, 10/5 mM Hepes/Tris, 7.4 pH. The mitochondrial membrane vesicles were added to the *cis* chamber and fused by increasing the KCl concentration. After the fusion of the vesicles, the increased concentration of KCl was mitigated and the excessive proteins were removed by perfusion of the *cis* chamber. All the voltages reported refer to the *trans* side, and the *cis* side was grounded. Under these conditions, positive current amplitude that increases on the application of positive voltages means a flux of chloride anions from the *cis* to the *trans* side. All the procedures were carried out at room temperature (20-22°C). Single-channel chloride currents were measured with the bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT, USA). The data was recorded at frequencies from 0 Hz to 1000 Hz, meaning that the low pass filter was 0 Hz and the high pass filter was set to 1000 Hz. The data for Figs 7A,B and 8A,B were filtered using the low pass filter at 0 Hz and the high pass filter at 250 Hz. Open and close dwell times were fitted by second-order exponential functions, giving the open dwell times τ_1 and τ_2 and the close dwell times τ_1 and τ_2 .

Protein concentration assay

The protein concentrations of the SMP particle preparations were measured with the Bio-Rad Protein Assay kit. The bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Reconstitution of the inner mitochondrial membrane into a lipid bilayer

In our study, we used the black lipid membrane technique to measure the activity of the inner mitochondrial membrane channels. Purified mitochondria were obtained by differential centrifugation (see the Materials and Methods section). Reconstitutions of SMP were performed in gradient solutions of 450/50 mM (*cis/trans*) KCl (Fig. 1) or 250/50 mM KCl (*cis/trans*), or in a symmetric solution of 450/450 mM KCl (*cis/trans*). We observed three types of ion channels differing in their single-channel conductance and selectivity: the large conductance Ca²⁺-activated potassium channel (mitoBK_{Ca} channel), the ATP-regulated potassium channel (mitoK_{ATP} channel), and the chloride channels. Only the single-channel recordings of the chloride channels are analyzed here.

The single-channel properties of the mitochondrial chloride channel

Submitochondrial particles from rat skeletal muscle mitochondria (SMM) (n = 47) and rat brain mitochondria (BM) (n = 25) were reconstituted into BLM, and the current changes for the ion channels were observed. In our experiments at 0 mV in 450/50 mM KCl (*cis/trans*) solutions, when the current amplitude was directed upwards (above the line indicating the current at the closed state), it designated an anion-selective channel, and when directed downwards, it

designated a cation-selective one. Incorporation of the mitoCl channels into the BLM was usually noticed within 30 minutes of the addition of SMP to the *cis* compartment. The range of amplitudes for the mitoCl channel from SMM at 30 mV was from 1.6 pA to 3.4 pA, with the average amplitude being about 2.2 ± 0.6 pA, while the range of amplitudes for the mitoCl channel from BM was from 3.3 to 4.7 pA (average 3.9 ± 0.5 pA). Single-channel recordings of the mitoCl channels from SMM and BM were done at different voltages in a 450/50 mM KCl gradient (*cis/trans*) (Figs 2A and 3A). The activity of the mitoCl channels from SMM at negative voltages was very low (Fig. 2A).

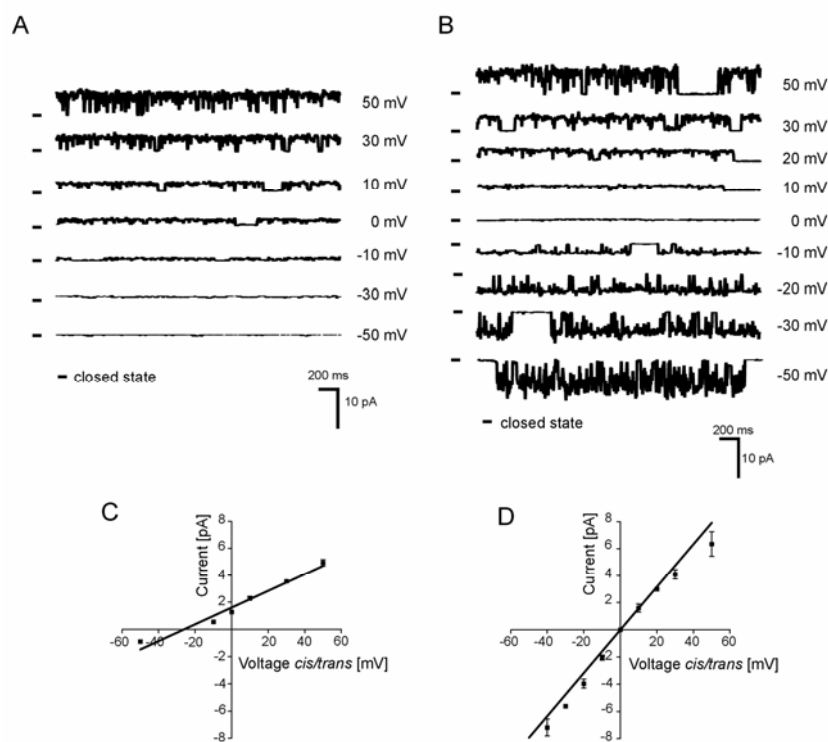


Fig. 2. Single-channel recordings of the mitoCl channel from SMM in planar lipid bilayers. A – Single-channel recordings in a 450/50 mM KCl gradient (*cis/trans*) solution and B – in a symmetric 450/450 mM KCl (*cis/trans*) solution at different voltages. C – Current-voltage characteristics of single channel events in a 450/50 mM KCl gradient (*cis/trans*) solution ($n = 9$), D – in a symmetric 450/450 mM KCl (*cis/trans*) solution ($n = 3$). “-” indicates current at the closed state of the channel. Recordings were low-pass filtered at 100 Hz.

The mean reversal potential calculated after curve fitting to the experimental data was -25 mV (Fig. 2C), which translated into a calculated conductance of the examined channel of 60 ± 3 pS. No activity of mitoCl from BM was observed at

negative voltages (Fig. 3A and B). The open state of the channels from BM was up at 0 mV. In Fig. 3C, the points at which we did not observe any mitoCl channel activity are marked with triangles (-30 mV and -50 mV). Figs 2D and 3D show the current-voltage relationships for a single-channel opening for mitoCl channels from SMM and BM at different voltages under symmetric conditions. The conductance value estimated in a symmetric 450/450 (*cis/trans*) mM KCl solution for the mitoCl channel from SMM was 155 pS, while the conductance for the mitoCl channel from BM only at positive potentials was about 120 ± 16 pS.

Submitochondrial particles from rat heart mitochondria (HM) were reconstituted as described in the Materials and Methods section. The chloride channels from HM showed the classical, stable, constant opening and closing chloride current amplitude $\geq 99\%$ of the time (Fig. 8A). The conductances of the channels were within the range of 70-130 pS.

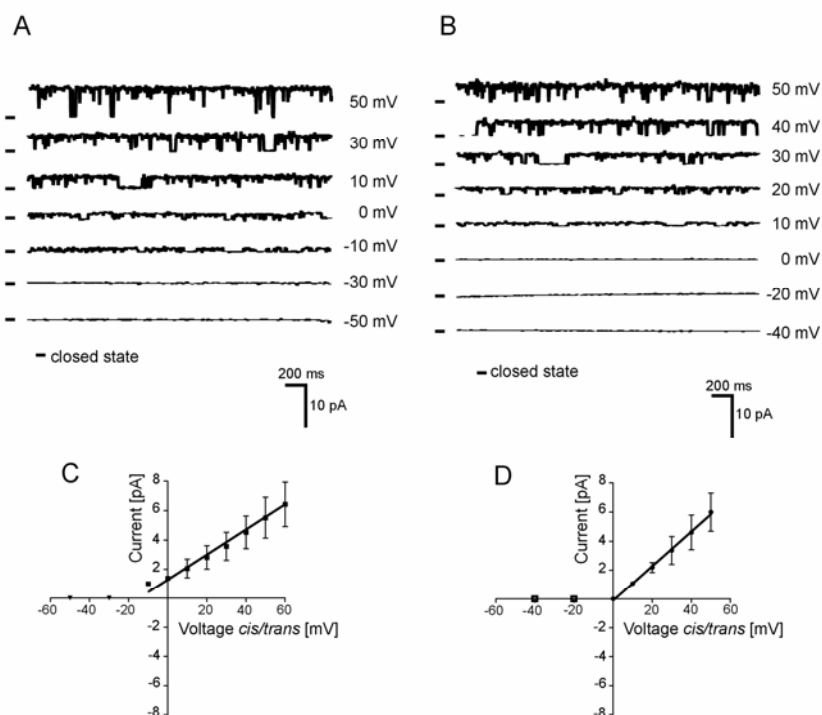


Fig. 3. Single-channel recordings of the mitoCl channel from BM in a planar lipid bilayer. A – Single-channel recordings of rat brain mitoCl channel in 450/50 mM KCl gradient (*cis/trans*) and B – in symmetric 450/450 mM KCl (*cis/trans*) solutions at different voltages. C – The current-voltage characteristics of single channel events in a 450/50 mM KCl gradient (*cis/trans*) solution and D – in a symmetric 450/450 mM KCl (*cis/trans*) solution. “-” indicates current at the closed state of the channel. Recordings were low-pass filtered at 100 Hz.

The effect of DIDS and SITS on the activity of mitoCl channels

It is known that stilbene derivatives, such as DIDS and SITS, modulate anion channel and transporter activity by both irreversible and reversible mechanisms. These compounds contain at least one isothiocyanate group, which allows them to react with the ϵ -amino group of a lysine residue on the transporter or anion channel [34]. IC_{50} values ranging from 5 μ M to 2 mM have been postulated for DIDS and SITS [35]. In our study, we observed that under gradient conditions, DIDS and SITS inhibited the mitoCl channel activity from SMM and BM immediately after being added to the *cis* or the *trans* compartment (Figs 4A, 4B and 5A, B).

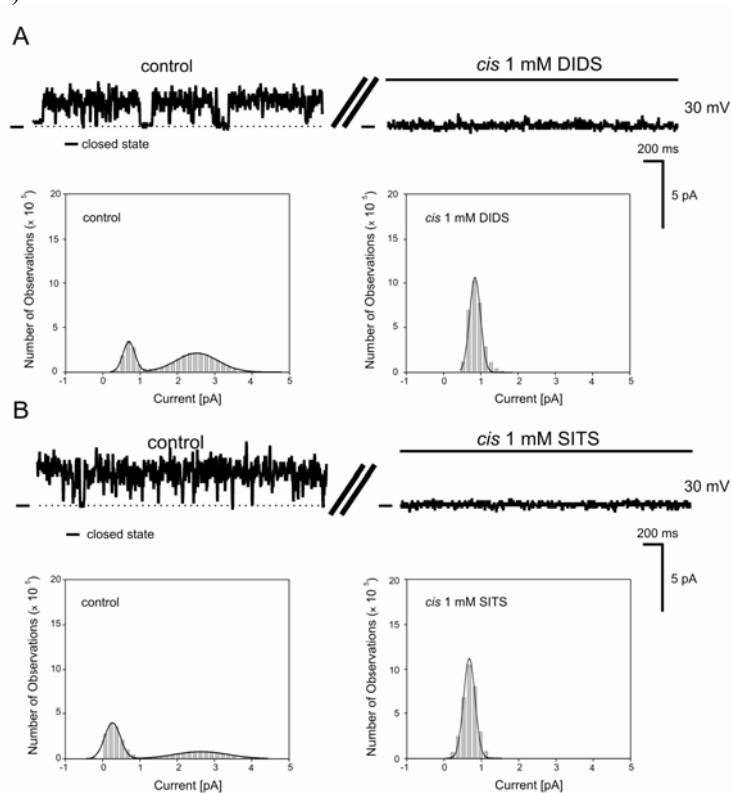


Fig. 4. The effects of stilbene derivatives (DIDS and SITS) on the activity of the mitoCl channel from skeletal muscle mitochondria. A – The mitoCl channel activity in a 450/50 mM KCl gradient (*cis/trans*) solution at 30 mV with amplitude histograms fitted with superimposed Gaussian curves under control conditions and after the addition of 1 mM DIDS to the *cis* compartment. B – Single-channel recordings of the mitoCl channel in a 450/50 mM KCl gradient (*cis/trans*) solution at 30 mV with amplitude histograms fitted with superimposed Gaussian curves under control conditions and after the addition of 1 mM SITS to the *cis* compartment. “-” indicates current at the closed state of the channel. Recordings were low-pass filtered at 100 Hz.

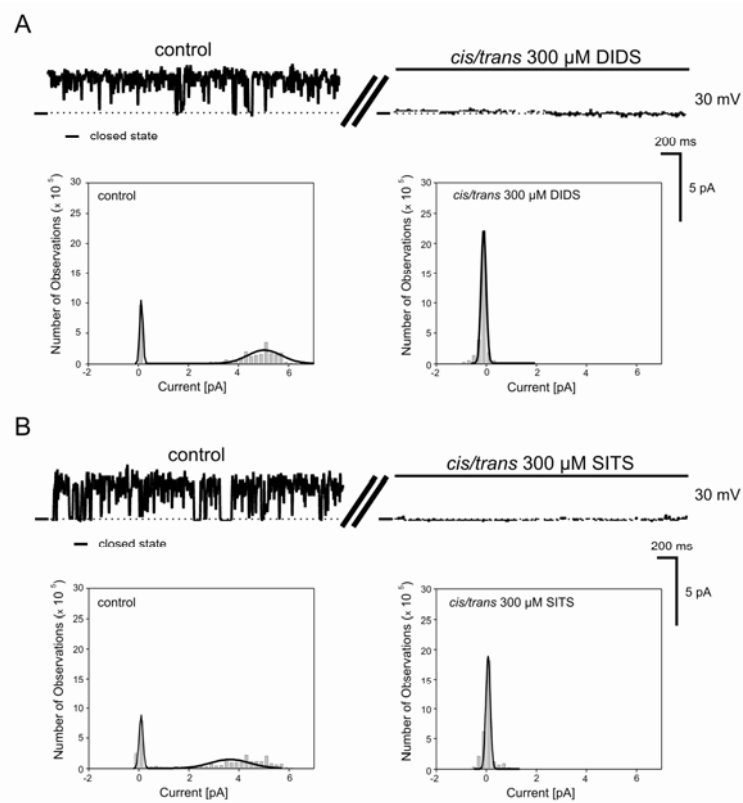


Fig. 5. The effect of DIDS and SITS on the activity of the mitoCl channel from rat brain mitochondria. Single-channel recordings of the mitoCl channel in a 450/50 mM KCl gradient (*cis/trans*) solution at 40 mV with amplitude histograms fitted with superimposed Gaussian curves under control conditions and after the addition of A – 300 μM DIDS or B – 300 μM SITS to the *cis* and *trans* compartments. “-” indicates the current at the closed state of the channel. Recordings were low-pass filtered at 100 Hz.

Our results suggest that the mitoCl channels from SMM and BM show different sensitivities to the stilbene derivatives. Skeletal muscle mitochondrial mitoCl channels were completely blocked after the addition of 0.8-1 mM DIDS (Fig. 6) or SITS to the *cis* compartment, whereas the brain mitochondrial mitoCl channels could be blocked with 300 μM of DIDS or SITS added to the *cis* and the *trans* compartments (Figs 4 A, B and 5 A, B). The dose dependence (Fig. 6) allows the EC_{50} to be estimated as ~0.2 mM for skeletal muscle mitochondrial chloride channels.

Adding DIDS or SITS to the *trans* compartment had no effect on the activity of the chloride channels from skeletal muscle mitochondria (Fig. 7A and B). We should point out that the DIDS or SITS concentrations needed for the inhibition of our mitoCl channels were higher than those sufficient to block the IMAC and UCP channels [36, 37].

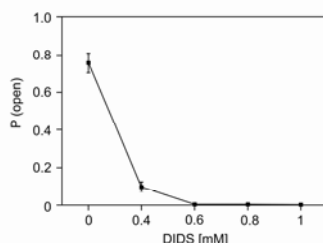


Fig. 6. The dose-response effect of DIDS on skeletal muscle mitochondrial chloride channels. Measurements of the DIDS inhibition of the channel activity were performed at concentrations of 0.4 mM and 0.6 mM (no channel activity was observed at 0.8 mM and 1 mM DIDS after addition to the *cis* side).

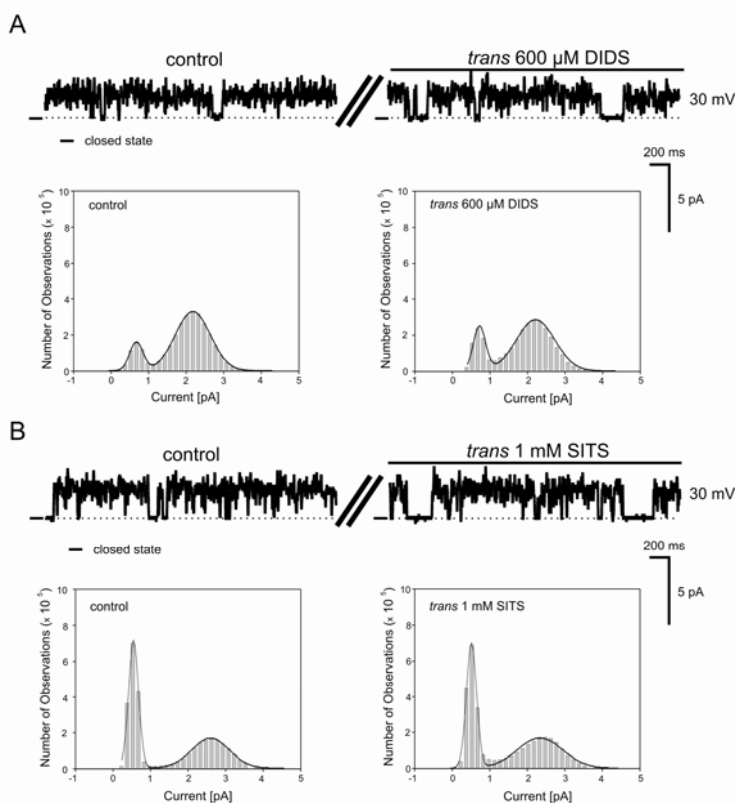


Fig. 7. The effects of stilbene derivatives (DIDS and SITS) on the activity of mitoCl channels from skeletal muscle mitochondria. A – Single-channel recordings of mitoCl channels in a 450/50 mM KCl gradient (*cis/trans*) solution at 30 mV with amplitude histograms fitted with superimposed Gaussian curves under control conditions and after the addition of 600 μ M DIDS to the *trans* compartment. B – The mitoCl channel activity in a 450/50 mM KCl gradient (*cis/trans*) solution at 30 mV with amplitude histograms fitted with superimposed Gaussian curves under control conditions and after the addition of 1 mM SITS to the *trans* compartment. “-” indicates current at the closed state of the channel. Recordings were low-pass filtered at 100 Hz.

The use of DIDS or SITS helps in determining the orientation of the mitoCl channels in the BLM, as described by Huang and Klingenberg [37]. The inhibitory effect of DIDS was observed from the matrix side of the uncoupling protein from the brown adipose tissue. In our experiments, both DIDS and SITS blocked the SMM mitoCl channels from the *cis* side of the BLM, suggesting that this may correspond to the matrix side of mitochondria.

The activity of chloride channels from rat heart mitochondria was also inhibited by DIDS (50-100 μM) ($n = 5$). This inhibition was observed only when DIDS was added to the *cis* side. As can be seen in the single-channel current (Fig. 8A), DIDS perturbed the channel activity, blocking it completely by inducing channel closure. The effect of DIDS was time-dependent, needing about 3 minutes to fully develop (Fig. 8B). The inhibition was nearly complete and irreversible at 100 μM (Fig. 8A).

DIDS did not have a significant effect on the single-channel amplitude, but it did decrease the open dwell time (Fig. 9A) and increase the close dwell time of the channels (Fig. 9B), indicating that it could affect the gating mechanism of the chloride channels. We did not observe a sudden block of the chloride channels, but rather a time-dependent inhibition, which may indicate that DIDS had to penetrate into a membrane target and accumulate at the target site on the channel, causing irreversible inhibition during a long application. We observed that after a short application of DIDS (< 1 min), the inhibition effect was reversible.

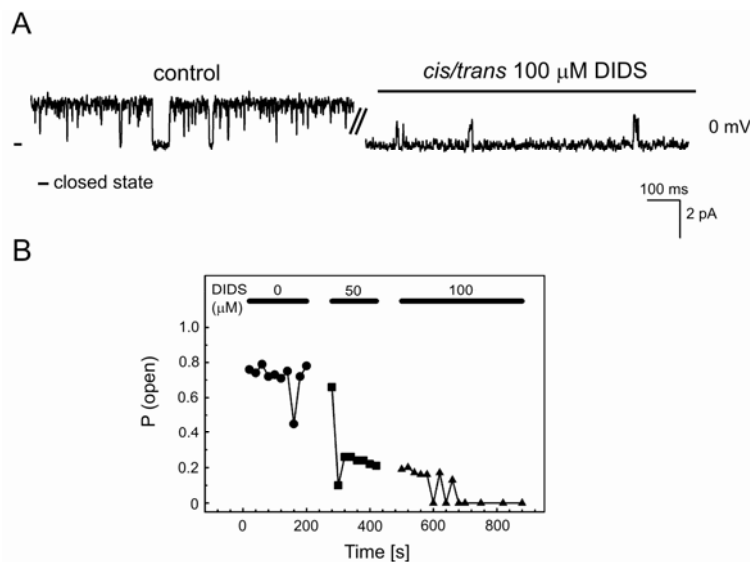


Fig. 8. The effect of DIDS on the single chloride channel current from rat heart mitochondria. A – A single-channel recording of chloride channels in a 250/50 mM KCl gradient (*cis/trans*) solution at 0 mV under control conditions and after the addition of 100 μM DIDS. B – The concentration-dependent effect of DIDS on the chloride channel open probability (representative experiment, $n = 5$).

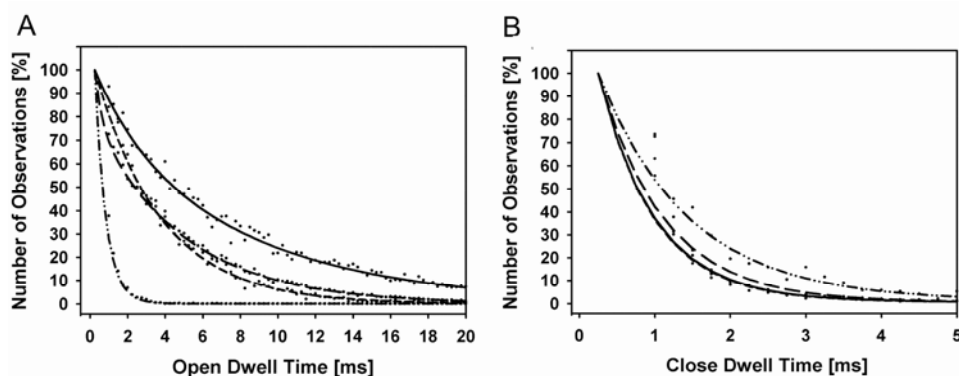


Fig. 9. Open dwell time and close dwell time distribution and fitted curves for the single chloride channels from rat heart mitochondria. A – Open dwell time distribution and fitted curves for the single chloride channels. Solid line - Control: $\tau_1 = 2.40$ ms, proportion (P) = 0.09; $\tau_2 = 8.25$ ms, P = 0.91. Long dashed line - 50 μ M DIDS: $\tau_1 = 0.38$ ms, P = 0.04; $\tau_2 = 4.75$ ms, P = 0.96. Short dashed line - 100 μ M DIDS (0-30 s after application): $\tau_1 = 3.52$ ms, P = 0.64, $\tau_2 = 3.52$ ms, P = 0.04. Dashed-dotted-dotted line - 100 μ M DIDS (2-4 min after application): $\tau_1 = 0.59$ ms, P = 0.76, $\tau_2 = 463$ ms, P = 0.24 (representative experiment). B – Close dwell time distribution and fitted curves for the single chloride channels. Solid line - Control: $\tau_1 = 0.74$ ms, P = 0.92; $\tau_2 = 5.37$ ms, P = 0.08. Long dashed line - 50 μ M DIDS: $\tau_1 = 0.93$ ms, P = 0.93; $\tau_2 = 7.43$ ms, P = 0.07. Short dashed line - 100 μ M DIDS (0-30 s after application): $\tau_1 = 0.73$ ms, P = 0.91, $\tau_2 = 7.16$ ms, P = 0.09. Dashed-dotted-dotted line - 100 μ M DIDS (2-4 min after application): $\tau_1 = 1.19$ ms, P = 0.70, $\tau_2 = 42.2$ ms, P = 0.30 (representative experiment).

In conclusion, the results of our investigations show that the chloride channel activity present in the inner mitochondrial membrane (from skeletal muscle, brain and heart mitochondria) can be blocked by stilbene derivatives. The sensitivity of the channels from the three tissues to these compounds decreases in the order: heart > brain > skeletal muscle. This variability is probably due to the structural differences in the chloride channels in the mitochondrial inner membrane. As the effect of stilbene derivatives was observed only from the one side of the BLM system, it is likely that the regulatory site of the mitoCl channel exhibits polarity, i.e. it faces the matrix or “cytosolic” side.

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REFERENCES

1. Szewczyk, A., Skalska, J., Glab, M., Kulawiak, B., Malinska, D., Koszela-Piotrowska, I. and Kunz, W.S. Mitochondrial potassium channels: From pharmacology to function. **Biochim. Biophys. Acta** 1757 (2006) 715-720.
2. Wang, X., Takahashi, N., Uramoto, H. and Okada, Y. Chloride channel inhibition prevents ROS-dependent apoptosis induced by ischemia-reperfusion in mouse cardiomyocytes. **Cell. Physiol. Biochem.** 16 (2005) 147-154.
3. Beavis, A.D. and Garlid, K.D. The mitochondrial inner membrane anion channel. Regulation by divalent cations and protons. **J. Biol. Chem.** 262 (1987) 15085-15093.
4. Beavis, A.D. Properties of the inner membrane anion channel in intact mitochondria. **J. Bioenerg. Biomembr.** 24 (1992) 77-90.
5. Schönfeld, P., Sayeed, I., Bohnensack, R. and Siemen, D. Fatty acids induce chloride permeation in rat liver mitochondria by activation of the inner membrane anion channel (IMAC). **J. Bioenerg. Biomembr.** 36 (2004) 241-248.
6. Sorgato, M.C., Keller, B.U. and Stuhmer, W. Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. **Nature** 330 (1987) 498-500.
7. Sorgato, M.C., Moran, O., De Pinto, V., Keller, B.U. and Stuehmer, W. Further investigation on the high-conductance ion channel of the inner membrane of mitochondria. **J. Bioenerg. Biomembr.** 21 (1989) 485-496.
8. Moran, O., Sandri, G., Panfili, E., Stuhmer, W. and Sorgato, M.C. Electrophysiological characterization of contact sites in brain mitochondria. **J. Biol. Chem.** 265 (1990) 908-913.
9. Klitsch, T. and Siemen, D. Inner mitochondrial membrane anion channel is present in brown adipocytes but is not identical with the uncoupling protein. **J. Membr. Biol.** 122 (1991) 69-75.
10. Borecky, J., Jezek, P. and Siemen, D. 108-pS channel in brown fat mitochondria might be identical to the inner membrane anion channel. **J. Biol. Chem.** 272 (1997) 19282-19289.
11. Hayman, K.A., Spurway, T.D. and Ashley, R.H. Single anion channels reconstituted from cardiac mitoplasts. **J. Membrane Biol.** 136 (1993) 181-190.
12. Fernandez-Salas, E., Sagar, M., Cheng, C., Yuspa, S.H. and Weinberg, W.C. p53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein. **J. Biol. Chem.** 274 (1999) 36488-36497.
13. Landry, D., Sullivan, S., Nicolaidis, M., Redhead, C., Edelman, A., Field, M., al-Awqati, Q. and Edwards, J. Molecular cloning and characterization of p64, a chloride channel protein from kidney microsomes. **J. Biol. Chem.** 268 (1993) 14948-14955.

14. Tonini, R., Ferroni, A., Valenzuela, S.M., Warton, K., Campbell, T.J., Breit, S.N. and Mazzanti, M. Functional characterization of the NCC27 nuclear protein in stable transfected CHO-K1 cells. **FASEB J.** 14 (2000) 1171-1178.
15. Dulhunty, A.F., Pouliquin, P., Coggan, M., Gage, P.W. and Board, P.G. A recently identified member of the glutathione transferase structural family modifies cardiac RyR2 substate activity, coupled gating and activation by Ca^{2+} and ATP. **Biochem. J.** 390 (2005) 333-343.
16. Qian, Z., Okuhara, D., Abe, M.K. and Rosner, M.R. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. **J. Biol. Chem.** 274 (1999) 1621-1627.
17. Berryman, M., Bruno, J., Price, J. and Edwards, J.C. CLIC-5A functions as a chloride channel *in vitro* and associates with the cortical actin cytoskeleton *in vitro* and *in vivo*. **J. Biol. Chem.** 279 (2004) 34794-34801.
18. Friedli, M., Guipponi, M., Bertrand, S., Bertrand, D., Neerman-Arbez, M., Scott, H.S., Antonarakis, S.E. and Reymond, A. Identification of a novel member of the CLIC family, CLIC6, mapping to 21q22.12. **Gene** 320 (2003) 31-40.
19. Mizukawa, Y., Nishizawa, T., Nagao, T., Kitamura, K. and Urushidani, T. Cellular distribution of parchorin, a chloride intracellular channel-related protein, in various tissues. **Am. J. Physiol. Cell Physiol.** 282 (2002) C786-795.
20. Suh, K.S., Mutoh, M., Gerdes, M. and Yuspa, S.H. CLIC4, an intracellular chloride channel protein, is a novel molecular target for cancer therapy. **J. Investig. Dermatol. Symp. Proc.** 10 (2005) 105-109.
21. Fernandez-Salas, E., Suh, K.S., Speransky, V.V., Bowers, W.L., Levy, J.M., Adams, T., Pathak, K.R., Edwards, L.E., Hayes, D.D., Cheng, C., Steven, A.C., Weinberg, W.C. and Yuspa, S.H. mtCLIC/CLIC4, an organellar chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53. **Mol. Cell Biol.** 22 (2002) 3610-3620.
22. Suh, K.S., Mutoh, M., Nagashima, K., Fernandez-Salas, E., Edwards, L.E., Hayes, D.D., Crutchley, J.M., Marin, K.G., Dumont, R.A., Levy, J.M., Cheng, C., Garfield, S. and Yuspa, S.H. The organellar chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. **J. Biol. Chem.** 279 (2004) 4632-4641.
23. Singh, H. and Ashley, R.H. CLIC4 (p64H1) and its putative transmembrane domain form poorly selective, redox-regulated ion channels. **Mol. Membr. Biol.** 24 (2007) 41-52.
24. Wiśniewski, E., Kunz, W.S. and Gellerich, F.N. Phosphate affects the distribution of flux control among the enzymes of oxidative phosphorylation in rat skeletal muscle mitochondria. **J. Biol. Chem.** 268 (1993) 9343-9346.
25. Dębska, G., Kicińska, A., Skalska, J., Szewczyk, A., May, R., Elger, C.E. and Kunz, W.S. Opening of potassium channels modulates mitochondrial function in rat skeletal muscle. **Biochim. Biophys. Acta** 1556 (2002) 97-105.

26. Kudin, A, Bimpong-Buta, N.Y., Vielhaber, S., Elger, C.E. and Kunz, W.S. Characterization of superoxide-producing sites in isolated brain mitochondria. **J. Biol. Chem.** 279 (2004) 4127-4135.
27. Cino, M. and Del Maestro, R.F. Generation of hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapitative ischemia. **Arch. Biochem. Biophys.** 269 (1989) 623-638.
28. Holmuhamedov, E.L., Wang, L. and Terzic, A. ATP-sensitive K⁺ channel openers prevent Ca²⁺ overload in rat cardiac mitochondria. **J. Physiol.** 519 (1999) 347-360.
29. Malekova, L., Kominkova, A., Ferko, M., Stefanik, P., Krizanova, O., Ziegelhoffer, A., Szewczyk, A. and Ondrias, K. Bongkrekic acid and atractyloside inhibits chloride channels from mitochondrial membranes of rat heart. **Biochim. Biophys. Acta** 1767 (2007) 31-44.
30. Bednarczyk, P., Kicińska, A., Kominkova, V., Ondrias, K., Dołowy K. and Szewczyk, A. Quinine inhibits mitochondrial ATP-regulated potassium channel from bovine heart. **J. Membr. Biol.** 199 (2004) 63-72.
31. Bednarczyk, P., Dołowy, K. and Szewczyk, A. Matrix Mg²⁺ regulates mitochondrial ATP-dependent potassium channel from heart. **FEBS Lett.** 579 (2005) 1625-1632.
32. Kulawiak, B. and Bednarczyk, P. Reconstitution of brain mitochondria inner membrane into planar lipid bilayer **Acta Neurobiol. Exp. (Wars)**. 65 (2005) 271-276.
33. Hordejuk, R., Szewczyk, A. and Dołowy K. The heterogeneity of ion channels in chromaffin granule membranes. **Cell. Mol. Biol. Lett.** 11 (2006) 312-325.
34. Cabantchik, Z.I. and Greger, R. Chemical probes for anion transporters of mammalian cell membranes. **Am. J. Physiol.** 262 (1992) 803-827.
35. Jentsch, T.J., Stein, V., Weinreich, F. and Zdebik, A.A. Molecular structure and physiological function of chloride channels. **Physiol. Rev.** 82 (2002) 503-568.
36. Beavis, A.D. and Davatol-Hag, H. The mitochondrial inner membrane anion channel is inhibited by DIDS. **J. Bioenerg. Biomembr.** 28 (1996) 207-214.
37. Huang, S.G. and Klingenberg, M. Chloride channel properties of the uncoupling protein from brown adipose tissue mitochondria: a patch-clamp study. **Biochemistry** 35 (1996) 16806-16814.