J Bioenerg Biomembr (2207) 39:301–311
DOI 10.1007/s10863-007-9090-1

ORIGINAL PAPER

Inhibition of anion channels derived from mitochondrial membranes of the rat heart by stilbene disulfonate—DIDS

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Received: 19 September 2006 / Accepted: 1 May 2007 / Published online: 25 September 2007

Abstract The objective of this work was to characterize in more detail the inhibition effect of diisothiocyanatostilbene-2′,2-disulfonic acid (DIDS) on anion channels isolated from the rat heart mitochondria. The channels reconstituted into a planar lipid membrane displayed limited powers of discrimination between anions and cations and the ion conductance measured under asymmetric (250/50 mM KCl, *cis*/*trans*) and symmetric (150 mM KCl) conditions was ~100 pS. DIDS caused a dramatic decrease in the channel activity (IC_{50}=11.7±3.1 μM) only when it was added to the *cis* side of a planar lipid membrane. The inhibition was accompanied by the significant prolongation of closings and the shortening of openings within the burst as well as gaps between bursts were prolonged and durations of bursts were reduced. The blockade was complete and irreversible when concentration of DIDS was increased up to 200 μM. Our data indicate that DIDS is an allosteric blocker of mitochondrial anion channels and this specific effect could be used as a tool for reliable identification of anion channels on the functional level.

Keywords Mitochondria · Anion channel · DIDS · Planar lipid bilayer · Channel gating kinetics

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Introduction

Anion channels are pore-forming membrane proteins that allow the passive transport of anions across biological membranes. It is widely believed that Cl^- is the predominant permeating species in all organisms, therefore these channels are often referred to as Cl^- channels. They are ubiquitously expressed in almost all eukaryotic cells and are localized to plasma membranes and membranes of intracellular organelles (Nilius and Droogmans 2003).

In the plasma membrane, Cl^- channels are involved in the regulation of cell volume, the transepithelial transport of salt and water as well as in the modulation of the electrical excitability in neurons. Intracellular Cl^- channels participate in many housekeeping processes, such as volume and pH regulation in organelles. At present, the precise biological functions of Cl^- channels are still poorly understood. Although, in the past decade, there has been remarkable progress in understanding the roles of Cl^- channels in the development of a broad spectrum of diseases such as dystrophia myotonica (Franke et al. 1990; Mankodi et al. 2002), cystic fibrosis (Riordan et al. 1989), epilepsy (Baulac et al. 2001), cataract (Young et al. 2000) and ostopetrosis (Kornak et al. 2001; Cleiren et al. 2001). Thus, Cl^- channels should be considered as potential therapeutic targets for novel pharmacological treatment of these diseases (Suh and Yuspa 2005). In this regard, Cl^- channels have been nominated as a drug target for cancer therapy (Kwang and Yuspa 2005), despite the lack of a direct connection of Cl^- channels to cancer pathogenesis. A universal characteristic of cancer cells is their ability to escape from apoptosis—a highly regulated process that enables an organism to eliminate unwanted cells (Hanahan and Weinberg 2000).

One of the main pathways of apoptosis is referred as mitochondria-dependent. Thus, mitochondria are not only required for ATP production, but are the key organelles that integrate apoptotic signals in damaged cells (Danial and Korsmeyer 2004). Recently, a mitochondrial Cl^- channel (CLIC4) was recognized as a new specific effector protein of apoptosis that is capable of altering mitochondrial function, leading to cell death (Fernandez-Salas et al. 2002). Expression of this protein is directly regulated by p53—a tumor suppressor protein (Fernandez-Salas et al. 1999) that is mutated in half of all human cancers (Lane 1992).

From the functional point of view, various anion channels have been identified in both the inner and outer mitochondrial membranes by the application of electrophysiological techniques. The major component of the outer mitochondrial membrane is VDAC (voltage-dependent, anion-selective channel) regulating metabolite flux across the membrane (Schein et al. 1976; Bathori et al. 1998; Xu et al. 1999). When reconstituted in a planar lipid membrane, the VDAC commonly appears as a large channel (~4 nS in 1 M KCl; De Pinto et al. 1987). A unique feature of this channel is its voltage dependent transitions from anion-selective state (open state) to states of equal or smaller conductance that are cation-selective (closed states). Although, both closed and open states exhibit ion selectivity, it is only weak (Schein et al. 1976; Pavlov et al. 2005).

It has been shown that the inner mitochondrial membrane contains an anion uniport pathway characterized by flux measurements in intact mitochondria. Thus, the existence of the inner membrane anion channel or IMAC it has been postulated (Garlid and Beavis 1986; Beavis 1992). The most physiologically important inhibitors of IMAC are H^+ and Mg^{2+} which inhibit from the matrix (inner) side of the inner membrane (Beavis and Powers 1989; Beavis and Garlid 1987). Characterization of IMAC relied almost exclusively on light scattering (matrix swelling) studies. The protein or gene for this anion pathway has not yet been identified. It is not known, in fact, whether IMAC is a channel at all. Undoubtedly, more insight into permeation properties of the inner mitochondrial membrane has been obtained by tackling this membrane with electrophysiological tools. The 108 pS channel or centum picosiemens channel, mCS, was the first anion channel to be discovered in the inner membrane of mitochondria using the patch-clamp technique in the presence of symmetric 150 mM KCl (Sorgato et al. 1987). This channel is only slightly anion selective (P_{Cl}^-/P_K^+=4.5), voltage sensitive and regulated by pH (Borecky et al. 1997). Comparing substrate specificity, inhibitor specificity, and pH dependence of the 108 pS channel with IMAC, it has been suggested that these channels might be identical (Borecky et al. 1997). The other high conductance anion channel, also referred to as mitochondrial megachannel, MCC, has been identified in the inner membrane of mitochondria (Kinnally et al. 1989). One of the unique characteristics is rapid fluctuations between a minimum of nine conductance levels ranging from 40 pS to over 1,000 pS in symmetrical 150 mM KCl (Zorov et al. 1992; Kinnally et al. 1996). On the basis of voltage and Ca^{2+}-dependent activation, it has been hypothesized that MCC may be associated with the adenine nucleotide translocator (ANT; Zoratti and Szabó 1995). However, Lohret et al. (1996) brought strong evidence using ANT-deficient *Saccharomyces cerevisiae* that MCC activity is independent of ANT. The uncoupling protein (UCP) is the other candidate for a mitochondrial anion channel (Huang and Klingenberg 1996). The channel exhibits 150 or 75 pS conductance in symmetrical 100 mM KCl indicating that it is either a dimer of two monomeric channels or a monomeric channel with 50% subconductance state. Furthermore, it has been shown that UCP is voltage sensitive and strongly discriminates against cations (P_{Cl}^-/P_K^+~17). Importantly, pH and divalent cations (Mg^{2+} and Ca^{2+}) from the matrix side do not influence channel activity of this protein (Huang and Klingenberg 1996). In addition, a low conductance channel of about 45 pS in 150 mM KCl and with weak anion selectivity (P_{Cl}^-/P_K^+~2) referred to as alkaline-induced anion-selective activity, AAA, appeared after exposure of patch-clamped mitoplasts (mitochondria with the outer membrane removed) to alkaline pH (pH=8.2) (Antonenko et al. 1994).

Toxins provide the most powerful pharmacological tools to help to unravel channel functions. Stilbene disulfonates are one of the popular classes of anion channel inhibitors, often used in cell studies. Among the most frequently tested potential inhibitors of anion channels is diisothiocyanatostilbene-2′,2-disulfonic acid (DIDS). This compound is known to inhibit anion channels by reversible and nonreversible mechanisms (Bridges et al. 1989). The nonreversible effect is presumably the result of covalent bonds formed between isothiocyanate groups of DIDS and a variety of amino acid residues of the channel. Mitochondrial anion channels were also tested for the inhibition by DIDS. Malonate transport via IMAC was reversibly blocked by DIDS with an IC_{50} of 26 μM (Beavis and Davatol-Hag 1996). Strong block effect of 20 μM DIDS was reported also for UCP channel (Huang and Klingenberg 1996). Considering the fact that IMAC and 108 pS channel were suggested to be identical proteins, it is surprising that the 108 pS anion channel incorporated into liposomes was not blocked by DIDS (Sorgato et al. 1989). The reason for this discrepancy could be in the orientation of the isolated channel in the liposomes. It has been shown for the UCP channel that DIDS blocks the channel only from one side (Bridges et al. 1989; Huang and Klingenberg 1996).

Hence, the aim of our work was to examine in more detail the effect of DIDS on anion channels derived from mitochondrial membranes, using the method of reconsti-

tution of an ion channel into planar lipid membrane. To contribute to a deeper understanding of the blockage mechanism, we performed a detailed analysis of the channel gating kinetics and examined whether there is some kind of specificity in the inhibitory effect of DIDS on mitochondrial anion channels that could be used as a tool for anion channel identification. The possible relation to already characterized mitochondrial anion channels is also discussed.

Materials and methods

Isolation of submitochondrial particles (SMP)

SMP were isolated as described earlier (Malekova et al. 2007). In brief, hearts were removed into an ice-cold isolation buffer containing: 50 mM sucrose, 200 mM MANNITOL, 5 mM KH_2PO_4, 1 mM EGTA and 5 mM MOPS (pH 7.30 adjusted with KOH), with 0.2% bovine serum albumin (BSA). Atria were excised, and ventricles cut into 1 mm^3 pieces. Two 10 s-long homogenization cycles were performed using Tissue-tearor homogenizer, followed by a 15 min centrifugation at 750×*g*. The supernatant was stored on ice, and the pellet was resuspended in isolation buffer, and homogenized using a Potter–Elvehjem tissue grinder with a Teflon pestle to release the remaining mitochondria. The homogenate was then centrifuged at 750×*g*, and the supernatant combined with that obtained from the previous step and further centrifuged at 7,000×*g* for 10 min. The obtained pellet was suspended in isolation buffer containing no EGTA or BSA, and immediately used for preparation of SMP. The mitochondrial suspension was sonicated 8×15 s at 35 kHz on ice and then centrifuged at 10,000×*g* for 10 min. The obtained supernatant was again centrifuged at 150,000×*g* for 30 min. The final membrane pellet of SMP was resuspended in EGTA- and BSA-free isolation buffer, frozen in liquid nitrogen and stored at −70 °C until used. All procedures were performed at 4 °C.

Dot blot hybridization

One microliter of SMP diluted with HBS (10 mM HEPES, 150 mM NaCl, 20 mM NaN_3, 1.2 mM $CaCl_2$; pH=7.35) to achieve required concentrations were blotted onto a nitrocellulose membrane. All incubations were done at room temperature. After air-drying, the membrane was blocked by PB [5% bovine serum albumin (BSA) in HBS] for 30 min. The membrane was rinsed with HBS and incubated with mouse anti ATP synthase β (CHEMICON International, Temecula, CA, USA) diluted 1:1,000 in PB for 3 h. After washing with HBS, the membrane was incubated with goat anti-mouse IgG-FITC (Sigma-Aldrich, Munich, Germany) at a dilution of 1:100 in PB for 50 min. The membrane was washed again with HBS and after mounting in a solution of 90% glycerol/HBS supplemented with 1,4-diazabicyclo[2.2.2]octane (DABCO) and sandwiched between glass plates. The signal was detected using the TYPHOON system (Amersham Pharmacia Biotech, Sunnyvale, CA, USA). A clearly defined spot at the site where SMP was spotted was considered as a positive result. A trace reaction or the absence of any reaction was considered as a negative result.

Lipid bilayer measurement

Anion channels derived from mitochondrial membranes were incorporated into a planar lipid bilayer and single channel currents were recorded under voltage-clamp conditions. Bilayers were composed of a 3:2:1 mixture of DOPC / DOPE / DOPS (Avanti Polar Lipids, Inc., Alabaster, AL) dissolved in decane at a final concentration of 25 mg/ml. SMP were added to the *cis* chamber near the bilayer painted across a 50 μm aperture in the wall of a polystyrene cup. The *cis* chamber was filled with 1 ml of 250 mM KCl or choline chloride, 10 mM HEPES, 5 mM Tris, 2 mM $MgCl_2$, 100 μM $CaCl_2$ (pH=7.4), and the *trans* chamber with 1 ml of 50 mM KCl or choline chloride, 10 mM HEPES, 5 mM Tris, 2 mM $MgCl_2$, 400 μM $CaCl_2$, 1 mM EGTA (pH=7.4). Under symmetric conditions, concentration of KCl or choline chloride in both chambers was equal to 150 mM. Free Ca^{2+} concentration was calculated by WinMaxc32 version 2.50 (http://www.stanford.edu/~cpatton/maxc.html). DIDS (MP Biomedicals) was prepared as a concentrated stock in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. This solution was further diluted to less concentrated stock solutions with distilled water. Thus, DMSO in the bath solutions did not exceed 1%. The *trans* chamber was connected to the head-stage input of Warner BC-525C amplifier (Warner Instruments, Inc., Hamden, CT, USA) and the *cis* chamber was held at ground. Electrical signals were filtered at 1 kHz with an analog filter and digitized at 4 kHz. Before analysis, signals were further digitally filtered at 500 Hz with a Gaussian filter. Data acquisition and analysis were performed with a commercially available software package (pCLAMP 5.5, Axon Instruments, Burlingame, CA, USA) using an IBM-compatible Pentium computer and A/D-D/A converter (Digidata 1200, Axon Instruments Inc., Forster City, CA, USA). The current amplitude of channel openings was determined from the differences of medians of the best-fit Gaussians for the baseline and the open-channel current level. Ion conductance was calculated from linear regression of the points in the current-voltage relationship between −20 mV and +25 mV. Open probability (P_o) was

calculated from the current recordings with duration >3 min. Relative anion–cation permeability ratios were calculated from zero-current reversal potential (E_{rev}) using the Planck equation (Barry 2006):

$$E_{rev} = \frac{RT}{F} \frac{\left[\left(\frac{P_{Cl^-}}{P_{X^+}}\right) - 1\right]}{\left[1 + \left(\frac{P_{Cl^-}}{P_{X^+}}\right)\right]} \ln\left[\frac{\alpha_{XCl}^{trans}}{\alpha_{XCl}^{cis}}\right], \quad (1)$$

where R, T and F are the gas constant, temperature in K and Faraday constant, respectively; a_{XCl} is the mean activity of salt. Species X^+ is K^+ or choline$^+$. Mean ion activities were used instead of ion concentrations, with mean activity coefficients computed using the Davies equation (Davies 1938):

$$\log f = -0.5\left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right), \quad (2)$$

where I is ionic strength of the solution.

Dwell time distributions were fitted by the maximum likelihood estimation method. The critical shut time for definition of bursts was estimated according to Colquhoun and Sakmann (1985). This approach chooses critical shut time so that equal proportions of short and long intervals are misclassified. The dose response relationship for inhibition by DIDS was fitted with the Hill equation for inactivation (SigmaPlot 8.02, Systat Software Inc., Richmond, CA) and the resulting IC_{50} values from individual experiments were averaged. Results are reported as means±SD. Significance of differences were analyzed by the Student t test with Welch approximation and are regarded as statistically significant at $p<0.05$.

Results

Purity of isolated SMP

The SMP fraction used in our study was isolated according to Malekova et al. (2007). They showed negligible contamination of this fraction by sarcolemma and by sarcoplasmic reticulum. Furthermore, Western blot analysis revealed no detectable contamination by Golgi and nuclear membranes. Because no method for separation of outer and inner mitochondrial membranes was employed (e.g. French press; Decker and Greenawalt 1977), the presence of both membranes in the SMP fraction was expected. In fact, Malekova et al. (2007) revealed a significant amount of VDAC in the SMP fraction indicating that the outer mitochondrial membrane was present. In our study, we performed a dot blot assay in order to test whether the SMP fraction isolated according to Malekova et al. (2007) contains a detectable amount of ATP synthase as a marker of the inner mitochondrial membrane.

Fig. 1 ATP synthase dot blot assay on the SMP fraction. Four concentrations (610 ng, 61 ng, 6.1 ng, 0.61 ng) of the SMP fraction were spotted on a nitrocellulose membrane and revealed using mouse anti ATP synthase β

Figure 1 shows clear signal at the site where SMP was spotted and was obtained using primary anti ATP synthase β and secondary goat anti-mouse IgG-FITC

General properties of anion channels derived from mitochondrial membranes

After incorporation of SMP isolated from the rat heart into a planar lipid membrane we observed ion channels predominantly conductive for Cl^- ions in KCl solutions. Figure 2a shows typical current traces recorded in asymmetric (250/50 mM) KCl solutions. All channels were highly active under control conditions with open probability (P_o) of 0.77±0.13 (n=14). Anion channels dwelled predominantly in open states with frequent visits to short-lived closed states. The channel behavior appeared to be burst-like because longer periods of silence separating periods of activity also occurred. Application of burst analysis to the channel activity revealed two exponential distributions for openings as well as gaps within a burst. This demonstrates that the channel occupies at least two different open states with the mean open times of 1.05±0.82 ms and 5.1±3.1 ms (n=5) and two different closed states with the mean closed times of 0.37±0.11 ms and 1.55±0.26 ms (n=5). These parameters reflect intraburst transitions. Furthermore, parameters characterizing burst properties were calculated. Open and closed time distributions were well fitted with one exponential curves with the mean burst duration of 117±59 ms and the mean between-burst duration of 17.4±5.5 ms (n=5).

Regulation of the channel activity by voltage has been shown to be a common property of most mitochondrial anion channels. The SMP fraction is likely to also contain K^+ channels (Garlid 1996), therefore we used as standard experimental conditions asymmetric (250/50 mM) KCl solutions because it was apparent directly from a direction of the ion current whether the reconstituted channel is primarily conductive for either Cl^- or K^+. However, so far, single channel properties of mitochondrial anion channels were studied in various symmetric KCl solutions (Sorgato et al. 1987; Zorov et al. 1992; Kinnally et al. 1996), therefore we examined the voltage response under both asymmetric (250/50 mM) and symmetric (150 mM) KCl

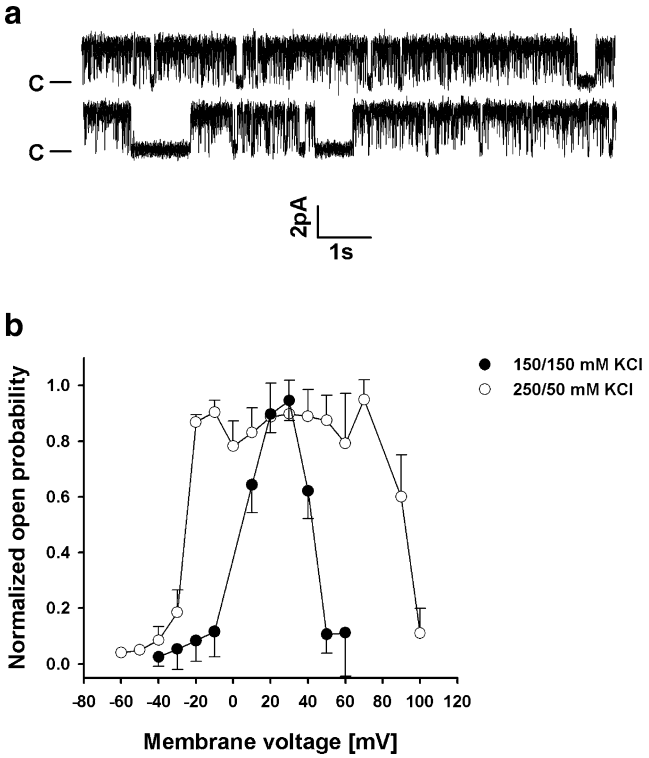

Fig. 2 Voltage dependence of mitochondrial anion channels. **a** Representative current traces of the anion channel recorded at 0 mV membrane potential and under asymmetric KCl conditions (250/50 mM, *cis*/*trans*). Channel openings are in the upward direction. *Dashes* at the *left* of the tracings indicate the closed state of the channel (*C*). All anion channels exhibited high activity under control conditions (P_o=0.77±0.13; n=14). The channel behavior appeared to be burst-like because longer periods of silence separating periods of activity also occurred. **b** Voltage dependence of normalized P_o under asymmetric (*open circle*) and symmetric (*filled circle*) KCl conditions (n=6)

conditions. In both cases, we showed a bell-shaped dependence of P_o on voltage ranging from -60 mV to +100 mV (Fig. 2B). Of note, under symmetric KCl conditions the voltage response is significantly narrower, centered at ~+20 to +30 mV. In asymmetric solutions, anion channels exhibited a P_o vs voltage curve with a broad plateau at voltages ranging from −20 to +70 mV. Voltage above +100 mV and below −100 mV substantially inhibited all observed anion channels. Furthermore, the maximal extent of the channel activation by voltage was not affected by experimental conditions. Absolute value for P_{omax} was 0.914±0.072 (n=6) for asymmetric and 0.851±0.084 (n=6) for symmetric KCl solutions.

Permeation properties of anion channels derived from mitochondrial membranes

A typical feature of the current–voltage characteristics observed under asymmetric KCl conditions was their asymmetry in the voltage range between −60 mV and +60 mV (Fig. 3a). The ion conductance determined from a linear fit to current-voltage relation was 43.6±4.5 pS (n=6) when voltage below −20 mV was applied. Whereas, the ion conductance was 99±13 pS (n=14) when voltage above −20 mV was used. The observed apparent asymmetry is likely attributed to a rectification of ion flux inside the pore of a channel. To get more insight into the mechanism responsible for rectification, we obtained current-voltage dependence also under symmetric KCl conditions. As shown on Fig. 3a, this dependence was well fitted by a linear curve over the whole range of applied voltage with conductance of 96.4±7.0 pS (n=4). These results demonstrated that revealed rectification of current flow through studied anion channels was associated only with asymmetric conditions pointing to Goldman–Hodgkin–Katz rectification (Hille 1992). Surprisingly, the similar results were obtained when substituting KCl with choline chloride (Fig. 3b). The current-voltage relation was linear under symmetric conditions with conductance of 92±14 pS (n=9). Under asymmetric conditions, two linear curves were used to describe the current-voltage characteristic due to asymmetry. Channels exhibited ion conductance of 44.8±4.2 pS (n=3) for voltages below −20 mV and 96.5±1.7 pS (n=3) for voltages above −20 mV. No significant differences were revealed for corresponding values of the ion conductance determined in KCl and choline chloride solutions. This indicates that the studied anion channels do not distinguish between KCl and choline chloride leading to an assumption that anion–cation permeability ratios for Cl^- K^+ and Cl^-–$choline^+$ could be similar. To test this hypothesis, we determined the anion-cation selectivity of studied anion channels by measuring changes in zero-current reversal potential (E_{rev}) when concentration of KCl in *trans* solution was decreased to 60 and 20% (Fig. 3c). The generated data were fitted with the Planck equation providing a P_{Cl}^-/P_{K}^+ value of 3.68±0.51. The same type of experiments and analysis were performed for choline chloride (Fig. 3d) and a $P_{Cl}^-/P_{choline}^+$ value of 3.00±0.90 was obtained. As expected, anion-cation permeability ratios were not significantly different.

Inhibition by DIDS

A non-specific anion channel inhibitor—DIDS was used as a modulator of mitochondrial anion channels. The inhibitor applied to both sides of a planar lipid membrane exhibited inhibition effect on the channel activity only from the *cis* side. Figure 4b shows representative single-channel traces of the anion channel in the presence of different concentrations of DIDS. For each dose response, P_o was plotted against DIDS concentration. Individual curves were fitted with the Hill equation for inactivation and the yielded

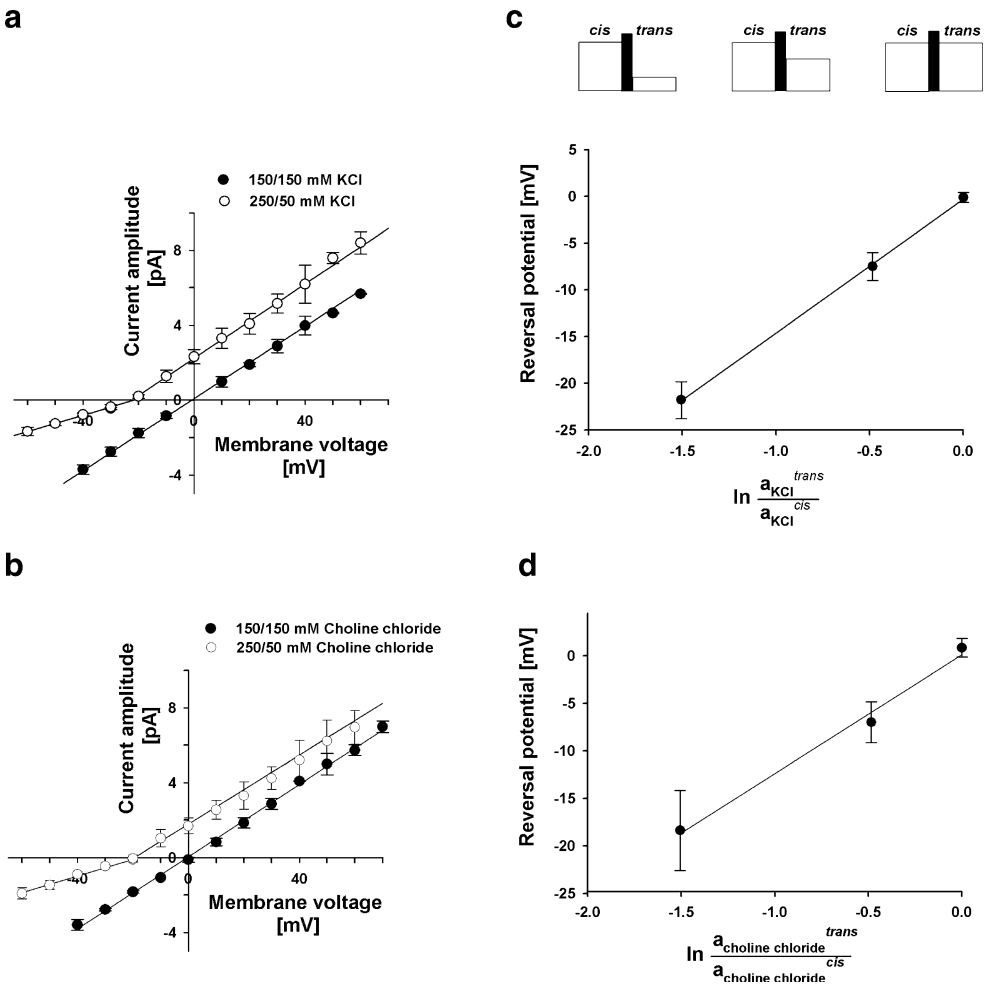

Fig. 3 Permeation properties of mitochondrial anion channels. **a** Current–voltage relationship obtained under asymmetric KCl (*open circle*, 250/50 mM, *cis*/*trans*) and symmetrical KCl conditions (*filled circle*, 150 mM). Under asymmetric conditions, the ion conductance determined from a linear fit was 43.6±4.5 pS (n=6) when voltage below −20 mV was applied, whereas, the ion conductance was 99± 13 pS (n=14) when the voltage above −20 mV was used. Under symmetric conditions, the ion conductance was 96.4±7.0 pS (n=4). **b** The current–voltage relationship obtained under asymmetric (*open circle*, 250/50 mM *cis/trans*) and symmetric choline chloride conditions (*filled circle*, 150 mM). Under asymmetric conditions the ion conductance determined from a linear fit was 44.8±4.2 pS (n=3) when voltage below −20 mV was applied, whereas, the ion conductance was 96.5±1.7 pS (n=3) when the voltage above −20 mV was used. Under symmetric conditions, the ion conductance was 92±14 pS (n=9). **c** and **d** Dilution potential experiments used to determine relative anion–cation permeability ratios from reversal potential data. The KCl (**c**) and choline chloride (**d**) activity gradients for 60 and 20% dilution of the *trans* solution. The fitting of these data by the Planck equation yielded P_{Cl^-}/P_{K^+}=3.68±0.51 (**c**) and $P_{Cl^-}/P_{choline^+}$=3.00±0.90 (**d**)

values of IC_{50} were further averaged (IC_{50}=11.7±3.1 μM, n=5). When the concentration of DIDS was increased up to 200 μM, a complete irreversible inhibition of the channel activity was observed within seconds in all experiments (n= 5). The burst behavior of anion channels, less recognizable under control conditions, was clearly evident in the presence of DIDS (Fig. 4b). Periods of inactivity were prolonged and the duration of burst was shortened when the concentration of DIDS was gradually elevated. Simple outcomes of the visual inspection were further confirmed by detail analysis of the gating kinetics. First, we analyzed DIDS concentration dependence of the intraburst kinetics. Open and closed time distributions exhibited two exponential components. Figure 5b displays that both the mean open times were remarkably reduced in a similar way, however, the proportion of open events in each exponential shifted significantly towards the shorter component (τ_{O1}) when concentration of DIDS was gradually elevated to 100 μM. A different situation was revealed for closed time distributions. Inhibition by DIDS was associated with an increase in the longer mean closed time (τ_{C2}) while the shorter one (τ_{C1}) was not significantly affected. In addition, occupancy of this state (W_{C1}) was reduced. In contrast, DIDS modified anion channels preferred to dwell in the closed state with the longer mean closed time (τ_{C2}). This indicates that DIDS caused the gradual prolongation of closed time intervals

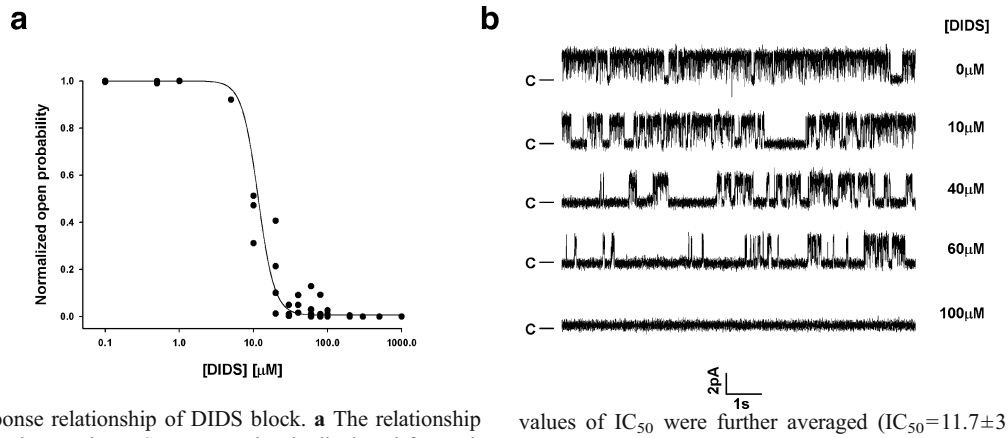

Fig. 4 Dose-response relationship of DIDS block. **a** The relationship between normalized P_o and DIDS concentration is displayed for each analyzed experiment. Single-channel activities were determined at 0 mV membrane potential under asymmetric KCl conditions (250/50 mM, *cis*/*trans*). Data were fitted with the Hill curve of inactivation and yielded values of IC_{50} were further averaged (IC_{50}=11.7±3.1 μM; n=5). **b** Representative current traces of the anion channel from mitochondrial membranes recorded in the presence of DIDS ranging from 10 to 100 μM. Channel openings are in the upward direction. *Dashes* at the *left* of the tracings indicate closed state of the channel (*C*)

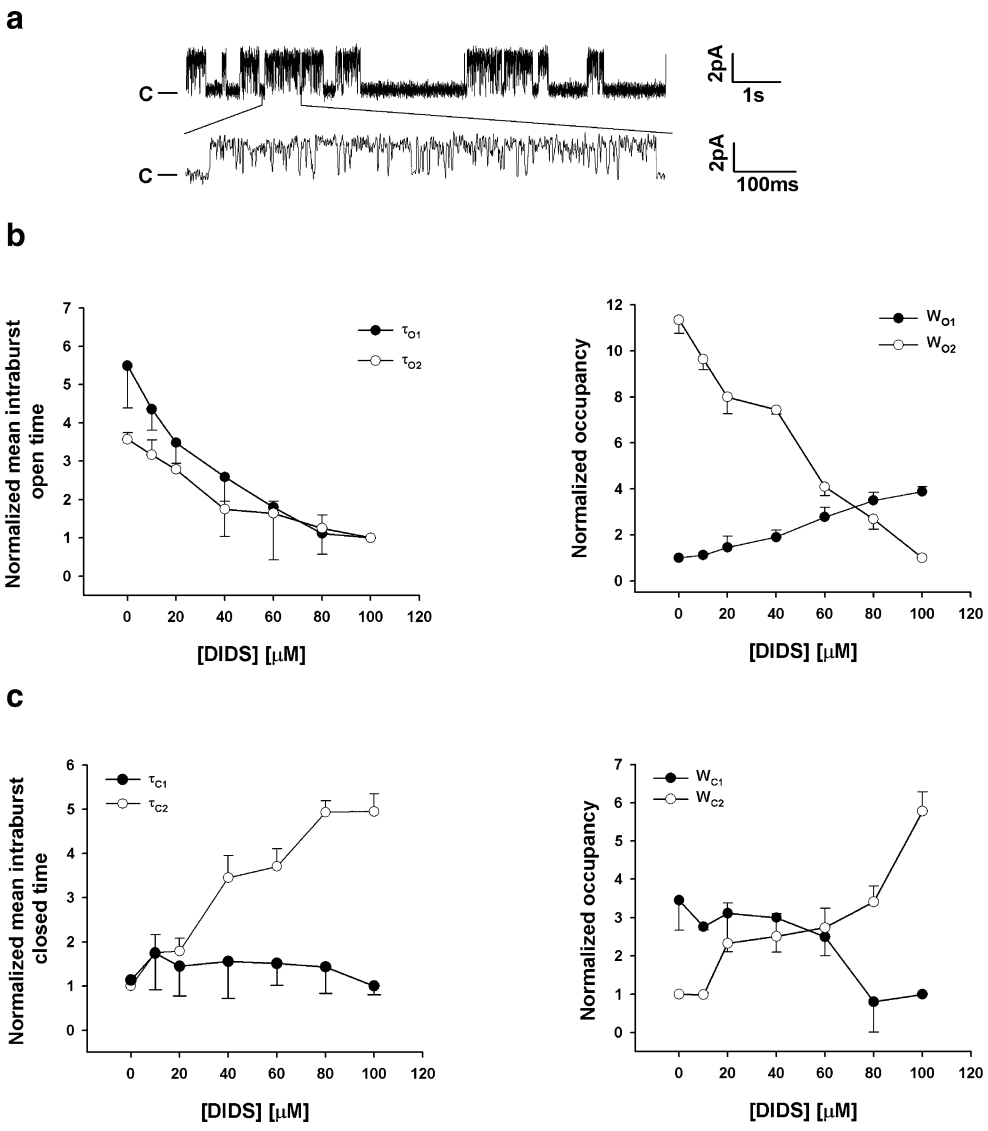

Fig. 5 The concentration-dependent effect of DIDS on intraburst kinetics of mitochondrial anion channels. **a** Representative current traces of the anion channel modified by 40 μM DIDS. The lower trace shows the detail of channel gating behavior inside the burst. Channel openings are in the upward direction. *Dashes* at the *left* of the tracings indicate the closed state of the anion channel (*C*). **b** Concentration dependences of the mean intraburst open times (τ_{O1}, τ_{O2}) and corresponding occupancies (W_{O1}, W_{O2}) derived from exponential fits to the open time histograms for DIDS inhibition (n=5). **c** Concentration dependences of the mean intraburst closed times (τ_{C1}, τ_{C2}) and corresponding occupancies (W_{C1}, W_{C2}) derived from exponential fits to the closed time histograms for DIDS inhibition (n=5)

and the gradual shortening of open time intervals within the burst what contributed to global reduction of P_o. Figure 6 summarizes the effect of DIDS on burst parameters. As we expected, the mean burst duration was dramatically reduced by approximately twenty times and the mean between-burst duration was prolonged by about six times as a result of the channel inhibition.

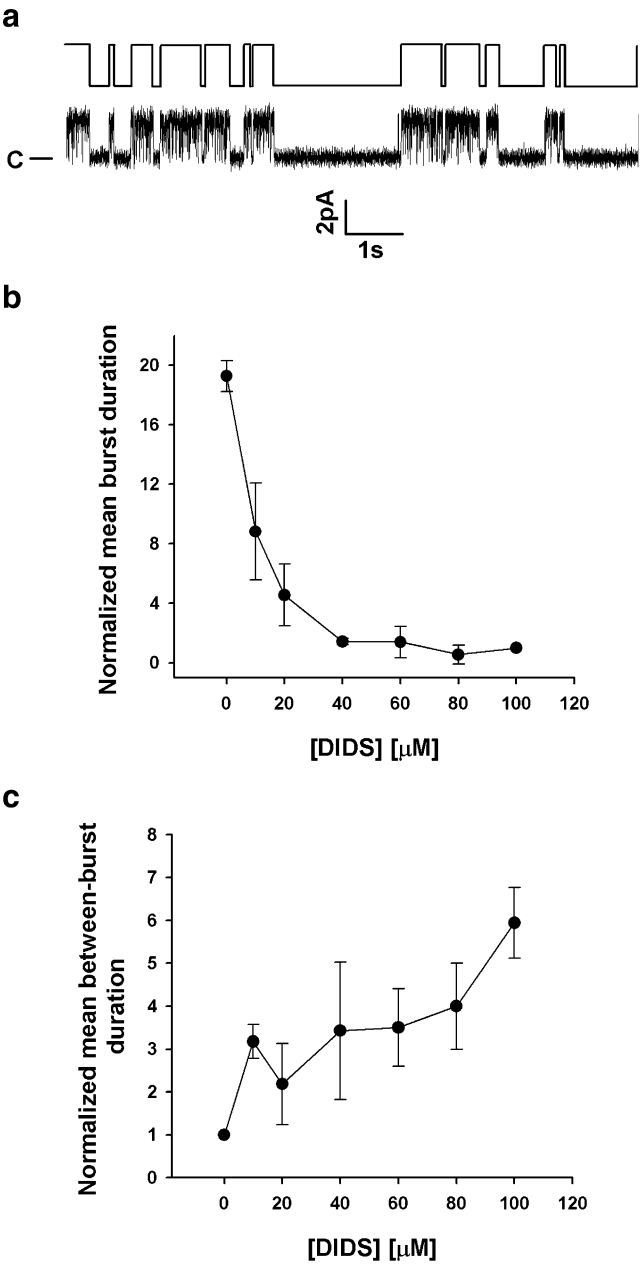

Fig. 6 The concentration-dependent effect of DIDS on burst kinetics of mitochondrial anion channels. **a** Representative current trace of the anion channel modified by 40 μM DIDS. Channel openings are in the upward direction. The *dash* at the *left* of the tracings indicates closed state of the channel (*C*). Upper trace represents the corresponding ideal current trace when channel gating inside the bursts was eliminated. **b** The changes in the mean burst duration as a result of DIDS inhibition of the anion channel (n=5). For this purpose, the open time histogram was constructed from the ideal current traces when closed events shorter than τ_{crit} were not counted during detection of events. **c** The changes in the mean between-burst duration induced by DIDS (n=5)

Discussion

In the present study, we examined in more detail the effect of the anion channel blocker DIDS on the activity of anion channels isolated from rat heart mitochondria. We employed a method of reconstitution of an ion channel into planar lipid membrane. This method allowed us to gain more information about the blocking mechanism of DIDS from the outcomes of detailed analysis of channel gating kinetics. The SMP fraction used in our study contained both inner and outer mitochondrial membranes, in which more types of anion channels were identified. Despite this, in our experiments, all observed anion channels exhibited similar conductive properties. Moreover, we did not reveal a broad heterogeneity in parameters of the channel gating kinetics and all channels were blocked by DIDS in the same manner. This led us to the conclusion that reconstituted anion channels had likely the same origin. Furthermore, the studied channels exhibited only moderate discrimination between Cl^- and cations such as K^+ and choline$^+$ (P_{Cl^-}/P_{K^+}=3.68±0.51; $P_{Cl^-}/P_{choline^+}$=3.00±0.90). The current flow through the channels showed the expected rectification in the presence of a salt gradient. We refer to these as anion channels of their preference for anions.

Anion channels reconstituted in our study exhibited functional properties similar to some extent to those reported so far for mitochondrial anion channels examined by mostly the patch-clamp method. This indicates that our channels studied by the method of planar lipid membrane might be identical to some known type of mitochondrial anion channels. In this regard, we focused on a comparison of permeation properties. The ion conductance of ~100 pS determined in our study under both asymmetric and symmetric KCl conditions clearly argues against the candidacy of mitochondrial anion channels characterized by many orders large of conductance, such as VDAC and MCC (Schein et al. 1976; Pavlov et al. 2005). Likewise, we can exclude the possibility that channel activities recorded in our study represents AAA channels with approximately two times smaller ion conductance determined under similar experimental conditions (Antonenko et al. 1994). Furthermore, it is unlikely that we reconstituted UCP. Albeit, UCP exhibited conductance of 75 pS for monomer and 150 pS for dimmer (Huang and Klingenberg 1996), which are closer to that reported here, we did not observe two populations of anion channels with mono and double ion conductance. In addition, it has been shown that UCP discriminates largely against cations (Bridges et al. 1989)

and we revealed only the moderate anion-cation selectivity. The similar magnitude of the ion conductance determined under symmetric KCl conditions is the first important property that the 108 pS channels (Sorgato et al. 1987; Klitsch and Siemen 1991) and anion channels characterized in our study have in common, suggesting their possible identity. The next line of evidence for the identity of these two types of channels is similar moderate anion–cation selectivity and the linearity of current-voltage dependence obtained under symmetric experimental conditions. The only discrepancy was found in the voltage regulation of compared anion channels. In our study, we showed bell-shaped voltage dependence of the channel activity under both asymmetric and symmetric KCl experimental conditions. This is in contrast to reported voltage-dependent activity of 108 pS channel that was silent at membrane potentials below 0 mV and active at membrane potentials over 0 mV, applied to the matrix side of mitoplast studied by the patch-clamp technique (Sorgato et al. 1989; Borecky et al. 1997). Despite whole-cell current gradually increased when positive potential up to +100 mV where applied, from single channel measurements it is obvious that the channel activity at +70 mV was significantly lower than at +60 mV. Thus, this indicates that the P_o vs voltage relationship of the 108 pS channel examined at single channel level would also be bell-shaped (Sorgato et al. 1989). Analyzing each channel separately enables us to separate two distinct phenomena: the effect of voltage on P_o and the effect of voltage on the current amplitude. Using whole-cell configuration both of these effects are mixed and the presence of maxima in P_o vs voltage dependence is likely masked.

All our experiments were performed in the presence of 2 mM free Mg^{2+} on both sides of the channel and no inhibition of the channel activity was detected when voltage from −100 mV to +100 mV was applied. This is in contrast with the results of Borecky et al. (1997). When they attempted to perform patch clamping of mitoplasts loaded with 1 mM $MgCl_2$, no channels with 108 pS characteristics were found indicating that these channels were efficiently inhibited. This supports the hypothesis that IMAC and the 108 pS channel are identical considering the fact that IMAC has been shown to be inhibited by matrix Mg^{2+} and it was suggested that endogenous matrix Mg^{2+} could be a physiological regulator of IMAC activity (Beavis and Powers 1989). On the other hand, when Sorgato et al. (1989) reconstituted the 108 pS channel into a planar lipid membrane, they also included 2 mM Mg^{2+} into solutions and they were still able to record active channels. Taken together, it seems that the inhibition of mitochondrial anion uniport by matrix Mg^{2+} requires some other matrix factor which may be absent in our SMP fraction in respect to intact mitochondria and mitoplasts. However, the planar lipid bilayer technique provides a unique way to study regulation of a channel in detail by precise control of solution content on both sides of a channel.

The main goal of the present study was to examine the blockage of anion channels derived from mitochondrial membranes by DIDS. We showed that these anion channels are indeed inhibited by DIDS with IC_{50}=11.7±3.1 μM (n= 5). The blockade was complete and irreversible when the concentration of DIDS was increased up to 200 μM. Furthermore, the inhibition was observed only when DIDS was added to the *cis* side of a planar lipid membrane. This result suggests that channels were inserted asymmetrically in the membrane. Consequently, this finding could explain why the 108 pS channel was not modified by DIDS using the planar lipid membrane technique. It was not specified to which side of a membrane the inhibitor was applied (Sorgato et al. 1989).

In principal, a drug might inhibit the channel by one or both of two mechanisms: open-channel or allosteric block (Lansdell et al. 2000; Cai et al. 2004). It has been suggested that disulfonic stilbenes belong to permeant open-channel blockers inhibiting anion channels by binding within the pore and preventing anion flow by occluding the permeation pathway (Bridges et al. 1989; Laver and Bradley 2006). This scenario is also supported by the fact that the sulfonate groups of DIDS are present as fully ionized divalent anions at physiological pH, thus this compound does not penetrate the lipid membrane. One of the features indicating open-channel blockage is concentration-dependence of open time distributions, while the distribution of blocker induced closures remains unchanged. To gain more information about the mechanism of DIDS inhibition, we performed an analysis of the channel gating kinetics. Increasing the concentration of DIDS led to remarkable dose-dependent changes in both, intraburst and burst kinetics, indicating a more complex nature of DIDS inhibitory action. The decrease in P_o was associated with shortening of all populations of open events and prolongation of all populations of closed events. However, the most dramatic change was revealed for the mean burst duration that was reduced by about twenty times. Interestingly, the blockade of channel activity was not accompanied by a generation of new populations of closings as it has been proposed for the action of an open-channel blocker. On the basis of these findings we can conclude that the effect of DIDS on studied anion channels from mitochondrial membranes is more likely attributed to allosteric blockage. This is in contrast to the open channel block induced by DIDS and other stilbenes on anion channels from sarcoplasmic reticulum of rabbit skeletal muscle (Laver and Bradley 2006). Till now, DIDS has been considered to be a nonspecific inhibitor of anion channels. However, our results provide the evidence that anion channels from membranes of mitochondria described in our study are inhibited by DIDS

in a specific way and this could be used as a tool for reliable identification of this type of anion channels on the functional level.

Acknowledgments We thank Lubica Malekova for her excellent technical assistance, Viera Kominkova for helping in experiments and in isolation of SMP, Zdena Sulova and Mario Seres for helping with a dot blot assay and Karol Ondrias for stimulating discussions. This work was supported by grant VEGA 2/3001/23, VEGA 2/6011/26, VEGA 2/5111/26 and APVV-51-027404.

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