Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 31–44
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbabio

Bongkrekic acid and atractyloside inhibits chloride channels from mitochondrial membranes of rat heart

Lubica Malekova [a], Viera Kominkova [a], Miroslav Ferko [b], Peter Stefanik [a], Olga Krizanova [a], Attila Ziegelhöffer [b], Adam Szewczyk [c], Karol Ondrias [a,*]

[a] *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlarska 5, 833 34 Bratislava, Slovak Republic*
[b] *Institute for Heart Research, Slovak Academy of Sciences, 83334 Bratislava, Slovakia*
[c] *Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland*

Received 28 June 2006; received in revised form 22 September 2006; accepted 9 October 2006
Available online 14 October 2006

Abstract

The aim of this work was to characterize the effect of bongkrekic acid (BKA), atractyloside (ATR) and carboxyatractyloside (CAT) on single channel properties of chloride channels from mitochondria. Mitochondrial membranes isolated from a rat heart muscle were incorporated into a bilayer lipid membrane (BLM) and single chloride channel currents were measured in 250/50 mM KCl *cis*/*trans* solutions. BKA (1–100 μM), ATR and CAT (5–100 μM) inhibited the chloride channels in dose-dependent manner. The inhibitory effect of the BKA, ATR and CAT was pronounced from the *trans* side of a BLM and it increased with time and at negative voltages (*trans–cis*). These compounds did not influence the single channel amplitude, but decreased open dwell time of channels. The inhibitory effect of BKA, ATR and CAT on the mitochondrial chloride channel may help to explain some of their cellular and/or subcellular effects.

Keywords: Mitochondrial membrane; Chloride channel; Bongkrekic acid; Atractyloside; Single channel property; Bilayer lipid membrane

1. Introduction

Bongkrekic acid (BKA) inhibits the mitochondrial permeability transition pore (MPTP) via binding to the adenine nucleotide translocator (ANT) in mitochondria and fixing its conformation in the m-state. Conversely, a different ANT inhibitor, atractyloside (ATR), and its derivative carboxyatractyloside (CAT), induce MPTP opening by changing the ANT conformation to its c-state [1–5]. BKA and ATR, used as pharmacological tools to modulate the properties of the MPTP or ANT in mitochondria, have various cellular and subcellular effects.

BKA prevented a number of phenomena linked to apoptosis, e.g. depletion of reduced glutathione, generation of reactive oxygen species, translocation of NFκB, exposure of phosphatidylserine residues on the outer plasma membrane, cytoplasmic vacuolization, chromatin condensation, and oligonucleosomal DNA fragmentation [6]. It also protected multidrug-resistant tumor cells against apoptosis caused by 1,4-anthraquinone [7]. BKA protected MCF-7 cells against the proliferation inhibition effect of the chemotherapy drug Tamoxifen [8] and reduced ischemic-induced neuronal death [9]. BKA inhibited MPTP pore opening in response to pro-oxidants and protonophores, but failed to interfere with MPTP induction by Ca^{2+} and chemical thiol crosslinking [10]. It had a biphasic effect on MPTP; in the first minutes after application it stabilized the mitochondrial membrane, followed by a pronounced opening of PTP [11]. BKA inhibited production of coated platelets [12] and formation of non-specific pores by ANT reconstituted into proteoliposomes [13,14]. It mimicked the effect of diazoxide to suppress calcium-dependent morphological changes in mitochondria [15], prevented $G\alpha_q$-induced cytochrome *c* release and loss of mitochondrial membrane potential, and inhibited cardiomyocyte apoptosis. In contrast to BKA, CsA showed only

Abbreviations: BLM, bilayer lipid membrane; P_{open}, open probability of single channel; E_{rev}, reversal potential; SMP, submitochondrial particles; BKA, bongkrekic acid; ATR, atractyloside; CAT, carboxyatractyloside; CsA, cyclosporine A; MA, myristic acid; TBT, tributyltin; MPTP, mitochondrial permeability transition pore; IMAC, inner membrane anion channel

* Corresponding author. Tel.: +421 2 54774102; fax: +421 2 54773666.
E-mail address: karol.ondrias@savba.sk (K. Ondrias).

0005-2728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2006.10.004

a transient ability to block apoptotic responses in cardiomyocytes [16]. Studies using ATR have implicated MPTP as a potential mediator of myocardial ischemic injury [5,17]. ATR blocked the protective effect of puerarin in isolated rat heart [18] and inhibited the cardioprotection and protection against ischemia–reperfusion injury by nitric oxide donors, isoflurane, CsA, and tumor necrosis factor-alpha [17,19–23]. ATR abolished the beneficial effects of ischemic preconditioning [24], attenuated the neuroprotective effects of diazoxide and CsA [25], and deteriorated the function of donor hearts during preservation [26]. ATR potentiated an arachidonic acid-induced, cyclosporine A (CsA)-insensitive mitochondria matrix swelling [27], and modulated rotenone-induced apoptosis [28]. CAT, a membrane-impermeable analog of ATR, abolished the stabilizing effect of adenine nucleotides on oscillations of the mitochondrial membrane potential [29,30].

In some cases, ATR and BKA have similar cellular and subcellular effects. ATR and BKA prevented oligomycin-induced pyranine fluorescence quenching in submitochondrial particles (SMP) [31] and thyroid hormone-induced efflux of Mg^{2+} from mitochondria [32]. CAT and BKA reduced the uncoupling of mitochondrial oxidative phosphorylation induced by the K(ATP) channel openers diazoxide and pinacidil [33] and abolished palmitate-induced uncoupling in heart mitochondria and in SMP [34].

BKA and ATR influenced membrane channel properties. BKA inhibited K^+ efflux in brain nonsynaptosomal mitochondria [35], reduced inhibition of transient K_{Ca} currents by mitochondrial depolarization in smooth muscle cells of rat cerebral arteries [36] and inhibited glucose-induced electrical activity in the pancreatic beta-cells through the stimulation of ATP-sensitive potassium channel activity [37]. BKA reduced the uncoupling of mitochondrial oxidative phosphorylation induced by K_{ATP} channel openers [33], prevented a loss of mitochondrial inner membrane potential (Delta(Psi)m) induced by H_2O_2 in cerebellar granule neurons [38], inhibited mitochondrial Ca^{2+} efflux, Mg^{2+}, K^+, and adenine nucleotides release, which was promoted by a chelating agent EGTA and stimulated by menadione [39]. ATR inhibited the ryanodine receptor calcium channel in the sarcoplasmic reticulum of skeletal muscle [40] and opened a non-specific ion channel responsible for cytotoxicity [41]. ATR and BKA augmented and reduced IgE-mediated Ca^{2+} store release, respectively [42].

Changes of ion permeability through ion channels of the mitochondrial membranes have a fundamental role in cell function. Ion channels of diverse selectivity have been identified in the mitochondrial membranes [43,44]. These include channels selective for potassium [45,46,47], calcium [48,49], and chloride ions [50,51] that have an important roles in apoptosis [52], necrosis [53], exocytosis [54], synaptic transmission [55], ischemic preconditioning [56], myocardial ischemia–reperfusion injury [57], and cancer [58].

Mitochondrial membranes contain various anion (or chloride)-selective channels that were identified using either patch-clamp or BLM methods on mitoplasts or SMP from brown adipose tissue [59,60], heart [61], liver [13,50,62], and yeast [63]. However, information about the channels is still incomplete. A voltage-dependent anion channel with conductance of about 107 pS [59,60,64] was supposed to be identical to the inner membrane anion channel IMAC, which was first characterized by osmotic swelling of mitochondria [65–68]. Some mitochondrial transporter proteins, such as phosphate carrier protein [69], the uncoupling protein from brown adipose tissue mitochondria [70], and pro-apoptotic BAX were reported to form channels selective for chloride [71]. The first intracellular chloride channel identified, known alternately as mtCLIC or CLIC4, was localized to the mitochondria [51].

Chloride channels are involved in many pathological and physiological cell functions, including cancer and cardioprotection [72–77]. Recently the anion (or chloride)-selective channels in mitochondria have received more attention, since they are involved in apoptosis [78–80] and free radical release [81], and they are considered a potential target for cancer therapy [82]. However, pharmacological modulation of the mitochondrial chloride channels is not well known. Therefore, in our study we evaluated the effects of BKA, ATR and CAT, drugs that are frequently used to modulate mitochondrial functions, on mitochondrial chloride channels. We found that the drugs inhibited mitochondrial chloride channels, which may explain some of their cellular or subcellular effects.

2. Materials and methods

2.1. Chemicals

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Protease inhibitors were from Roche Diagnostics GmbH (Mannheim, Germany), 2,4-dinitrophenol and CAT from Merck (Germany). All other chemicals including BKA, ATR, ATP, ADP, TBT, MA, imidazole (glyoxaline; 1,3-diaza-2,4-cyclopentadiene), thapsigargin, and ouabain were purchased from Sigma-Aldrich (Germany).

2.2. Isolation of mitochondrial inner membrane vesicles

Mitochondria from the hearts of male Wistar rats were isolated essentially as described previously [83]. In brief, rat hearts were removed after thoracotomy and the atria were excised. The ventricles were immersed in ice-cold isolation buffer containing (in mM): sucrose 50, mannitol 200, KH_2PO_4 5, EGTA 1, MOPS 5, and 0.2% BSA (pH 7.3 adjusted with KOH) and rinsed free of blood. The tissue was then cut into approximately 1 mm pieces and homogenized using a Tissue Tearor 985-370 (Biospect products, Bartlesville, USA) at 10,000 rpm in two 10 s cycles. To obtain a finer tissue suspension, three additional homogenization cycles of 20 s were performed manually using a teflon pestle. The homogenate was centrifuged at 750×*g* for 15 min. The supernatant was stored on ice, and the pellet resuspended in isolation buffer and centrifuged at 750×*g* to increase the yield of mitochondria. Both supernatants were further centrifuged at 7000×*g* for 10 min and the resulting pellet of isolated mitochondria was resuspended in the isolation buffer (in further steps without EGTA and BSA) and used immediately for preparation of inner membrane vesicles.

The inner membranes of mitochondria, submitochondrial particles (SMP), were prepared using a modification of the method of Brierley et al. [84] and Paucek et al. [85]. 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 100,000×*g* for 30 min. The final membrane pellet of SMP was resuspended in EGTA- and BSA-free isolation buffer, frozen in liquid N_2 and stored at −70 °C in small aliquots until reconstitution into BLM. All procedures were performed at 4 °C, and buffers contained protease inhibitors: 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 1 μM aprotinin, and 0.2 mM Pefabloc SC.

2.3. Purity of isolated mitochondrial inner membranes

The purity of isolated mitochondrial inner membranes (submitochondrial mitochondrial particles, SMP) was tested by estimation of ATPase activities characteristic for the content of sarcolemma (Na^+/K^+-ATPase) and sarcoplasmic reticulum (Mg^{2+}/Ca^{2+}-ATPase) in the absence and/or presence of their specific inhibitors. Briefly, the basic incubation medium for measurement of the mitochondrial Mg^{2+}-ATPase activity contained 0.1 ml 40 mM $MgCl_2$, 0.2 ml 250 mM imidazole, pH = 7.4, 0.1 ml 0.01 mM 2,4-dinitrophenol (for making all membrane vesicles leaky), and was adjusted with water to a final volume of 0.85 ml. 50 μl of the membrane fraction containing ~1 μg μl^{-1} of protein was preincubated in the basic medium for 10 min at 37 °C. The ATPase reaction was started by the addition of 100 μl 40 mM ATP–Tris pH = 7.4 and it was terminated after 20 min by 1 ml of 12% ice-cold trichloroacetic acid. For estimation of the sarcolemmal Na^+/K^+-ATPase together with the mitochondrial Mg^{2+}-ATPase the basic medium for the latter enzyme was supplemented with 0.1 ml 100 mM NaCl and 0.1 ml 20 mM KCl. Thus, the Na^+/K^+-ATPase activity was evaluated as the difference between the total ATPase (Mg^{2+}-ATPase + Na^+/K^+-ATPase) and the mitochondrial Mg^{2+}-ATPase activity. The validity and reliability of the Na^+/K^+-ATPase activity estimation was also verified via selective inhibition of the enzyme by addition of 0.1 ml 0.1 mM ouabain.

For estimation of sarcoplasmic reticulum Mg^{2+}/Ca^{2+}-ATPase, the basic medium for mitochondrial ATPase was supplemented with 0.1 ml 0.1 mM $CaCl_2$. The following steps were similar to those for estimating the sarcolemmal Na^+/K^+-ATPase activity, with the exception that instead of ouabain 0.1 ml 100 nM thapsigargin was applied as a specific inhibitor for verification of reliability of the Mg^{2+}/Ca^{2+}-ATPase estimation. All ATPase activities were estimated spectrophotopmetrically at 700 nm by measuring the amount of P_i liberated by ATP cleavage [86]. Membrane protein concentrations were determined according to the method of Lowry et al. [87]. Specific activities of ATPases were expressed in μmol P_i .mg $prot^{-1}$ h^{-1}.

2.4. Western blot analysis

Protein concentration was measured according to the method of Lowry et al. [87]. Afterwards, proteins were separated by 10% SDS-PAGE and transferred to supported nitrocellulose membranes (Hybond-ECL, Amersham Biosciences) using a semi-dry electrophoretic blotting system. Non-specific binding sites were blocked by immersing the membrane in 5% dry non-fat milk in Tris-buffered saline containing Tween (TBST) overnight at 5 °C. All subsequent washes and primary/secondary antibody incubations were also carried in TBST. Three different primary antibodies were used for hybridization (for 1 h at room temperature): a mouse monoclonal antibody against the Golgi marker protein Gm130 (dilution 1:1000; Abcam), a rabbit polyclonal antibody against the voltage-dependent anion channel VDAC1/Porin as a mitochondrial loading control (dilution 1:1000; Abcam), and a mouse monoclonal antibody against Histone H1 (dilution 1:1000; Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-labeled secondary antibodies (anti-mouse for Gm130 and Histone H1, Calbiochem, dilution 1:10,000; anti-rabbit for VDAC1, Amersham Biosciences, dilution 1:5000) were visualized by the ECL plus Western blotting detection system (ECL plus; Amersham Biosciences). Individual bands were evaluated using the PCBas e2 system.

2.5. Bilayer lipid membrane (BLM) measurements

BLM was formed across an aperture (diameter ~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) or dioleoyl-glycero-phosphatidylcholine and dioleoyl-glycero-phosphoethanolamine at a molar ratio of 3:2 in n-decane (20 mg/ml), similar to the method used in previous study [49]. The composition of the solutions (in mM) was, for the *trans* chamber: 50 KCl, 2 $MgCl_2$, 0.4 $CaCl_2$, 1 EGTA, 10/5 HEPES/Tris, pH 7.4 (mimicking the intracellular side), and for the *cis* chamber: 250 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 10/5 HEPES/Tris, pH 7.4 (mimicking the matrix side). The mitochondrial membranes – SMP – were added to the *cis* chamber. The KCl concentration in the *cis* chamber was increased to 600–800 mM in order to facilitate the fusion of vesicles. SMP fused into BLM spontaneously within a few minutes. After fusion of the vesicles and a chloride single channel was observed, the increased concentration of KCl and excessive proteins were removed by perfusion of the *cis* chamber with 10 volumes of *cis* solution to prevent further incorporation of the vesicles. Ag/AgCl electrodes were placed into each chamber via agar salt bridges and connected to the headstage of the bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT, USA). All the voltages reported here refer to the *trans* side with the *cis* side grounded. With our conditions, the positive current amplitude that increased at the application of positive voltages means a flux of chloride anions from the *cis* to the *trans* side. All procedures were carried out at room temperature (20–22 °C).

Chloride channels were readily distinguished from K^+ channels using *cis*/*trans* solutions of 250/50 mM KCl. The theoretical E_{rev} for K^+, presuming 100% permeability to K^+ and zero permeability for other ions, is +40.7 mV. Since membrane permeability for K^+ is less than 100% and membrane permeability for other ions is higher than zero, E_{rev} in practice is less than the theoretical. Therefore, in our study we controlled the purity of the Cl^- current by applying a voltage of ±30 mV. To study of the effect of drugs we used chloride channels, which have regular single channels with classical stable and constant opening and closing chloride current amplitude. The studied compounds were added to the *cis* or *trans* chamber, followed by continuous stirring for 1 min.

Single channel currents were filtered by a low-pass 8-pole Bessel filter at a corner frequency of 1 kHz and were digitized at a sampling rate of 4 kHz using a DigiData 1200 digitizer (Axon Instruments, Foster City, CA, USA). Data were then stored in an IBM-compatible computer by means of pClamp5 software (Axon Instruments), which was also used for processing of the data. The channel conductance (G) and the reversal potential (E_{rev}) were calculated from the current–voltage relationship within the voltage range of ±30 mV. The single channel open probability (P_{open}) was determined from recordings of =2 min before and after addition of drugs, and calculated from the ratio of the open time/total time intervals. Open dwell times were fitted by second-order exponential functions, giving the open dwell times τ_1 and τ_2.

3. Results

3.1. Purity of isolated SMP

Activity of the mitochondrial Mg^{2+}-ATPase in the tested preparation was 37.66 ± 1.09 μmol P_i .mg $prot^{-1}$ h^{-1}. Activity of the Na^+/K^+-ATPase was estimated as an additional increase to the mitochondrial Mg^{2+}-ATPase activity obtained after the addition of Na^+ and K^+ ions to the basic medium, and it amounted to 0.46 μmol P_i .mg $prot^{-1}$ h^{-1}. The proportion of the Na^+/K^+-ATPase activity to that of the Mg^{2+}-ATPase activity is 0.84%, which represents the median of the range of 0.68–1.47% and indicates the percentage of contamination of the SMP fraction by sarcolemma.

Activity of the Mg^{2+}/Ca^{2+}-ATPase, indicating the presence of sarcoplasmic reticulum membranes in isolated SMP preparation, was 0.72 μmol P_i .mg $prot^{-1}$ h^{-1}. This value was obtained by the difference between the activity of the mitochondrial Mg^{2+}-ATPase estimated in the absence and presence of Ca^{2+} ions. The proportion of Mg^{2+}/Ca^{2+}-ATPase activity to that of the Mg^{2+}-ATPase is 1.59%. This value represents the median of the range of 0.94–2.01% and indicates the percentage of contamination of the SMP fraction by sarcoplasmic reticulum.

In order to further determine contamination of isolated SMP, we used a marker for the Golgi matrix (Gm130), a marker for nuclear membrane (histone H1), and a marker for the outer and inner mitochondrial membranes (VDAC1/porin) (Fig. 1). By Western blot analysis we detected a clear signal for a 97 kDa protein (Gm130) in the whole cell homogenate, but not in

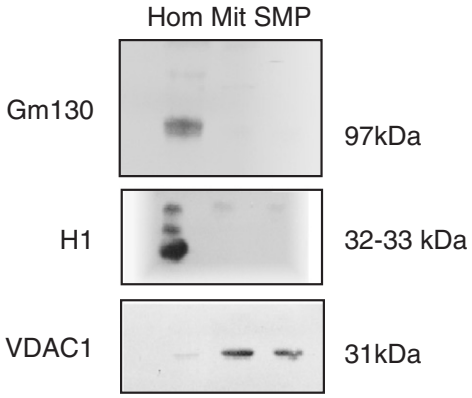

Fig. 1. Western blot analysis of Golgi marker protein 130 (Gm130, top figure), histone (H1, middle figure) and voltage dependent anion channel 1/porin (VDAC1, bottom figure). Hom—whole cell homogenate, Mit—mitochondrial fraction, SMP—mitochondrial inner membrane vesicles.

mitochondrial fraction and the mitochondrial inner membrane vesicles (SMP), indicating no detectable contamination of isolated mitochondrial membranes by Golgi membranes. A clear signal for the 32–33 kDa histone H1 protein was detected only in the whole cell homogenate. The signal was undetectable in the other two fractions tested, total mitochondrial membranes and SMP, indicating no detectable contamination of isolated mitochondrial membranes by nuclear membranes.

Comparison of VDAC signal from total mitochondria homogenate and SMP is shown in Fig. 1. We detected a signal for the 31 kDa VDAC1/porin protein in the mitochondrial fraction and in SMP, but a very low signal in the whole cell homogenate. The calculated ratio of VDAC protein in total mitochondrial homogenate and SMP was 3:2, indicating a high level of contamination of SMP by outer mitochondrial membranes.

3.2. Single channel properties of chloride channels

The single chloride channel properties before application of BKA, ATR, and CAT are shown in Fig. 2 and in controls in Figs. 4 and 6 through 9. P_{open} of the control channels at 0 mV was high before application of the drugs, ranging from 0.80 to 0.95, and it increased or decreased slightly when the *trans–cis* voltage increased or decreased, respectively (Fig. 6). The single channel conductance, obtained from the I–V curve within the range of −30 to +30 mV varied from 104 to 172 pS (Fig. 2). The reversal potential, E_{rev}, was within the range of −14 to −30 mV (Fig. 2A), and the single channel amplitude measured at 0 mV varied from 1.4 pA to 4.9 pA (Fig. 2B).

The activity of the chloride channels was pH dependent. A decrease in pH from 7.4 to 5.8, by addition of HCl at the *trans* side, did not have pronounced effect on the channel activity, but a decrease of pH from 7.4 to 5.8 at the *cis* side blocked the chloride channel (n=8/8). The effect of the HCl at the *cis* side

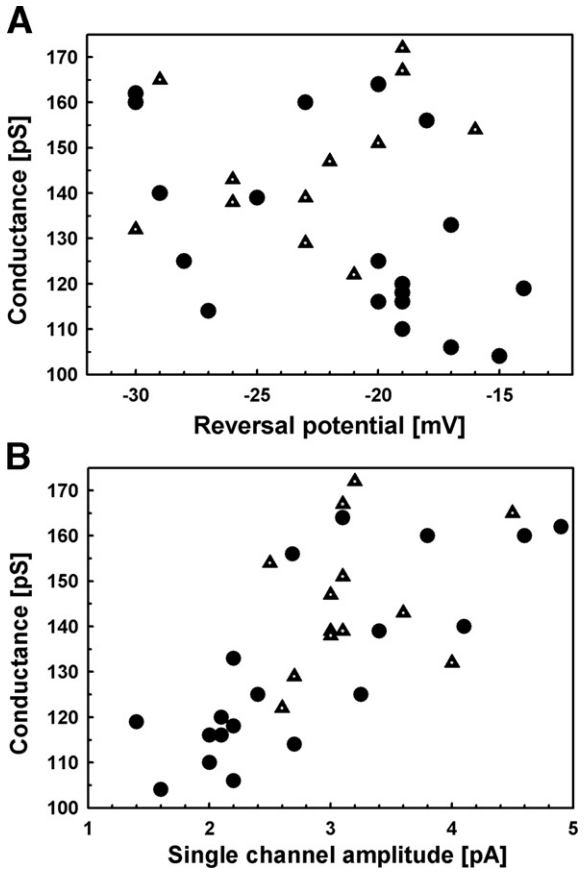

Fig. 2. Correlation between the reversal potential and the conductance of single chloride channels (A) and between the chloride single current measured at 0 mV and the conductance of single chloride channels (B). Circles—BKA experiments. Triangles—ATR and CAT experiments.

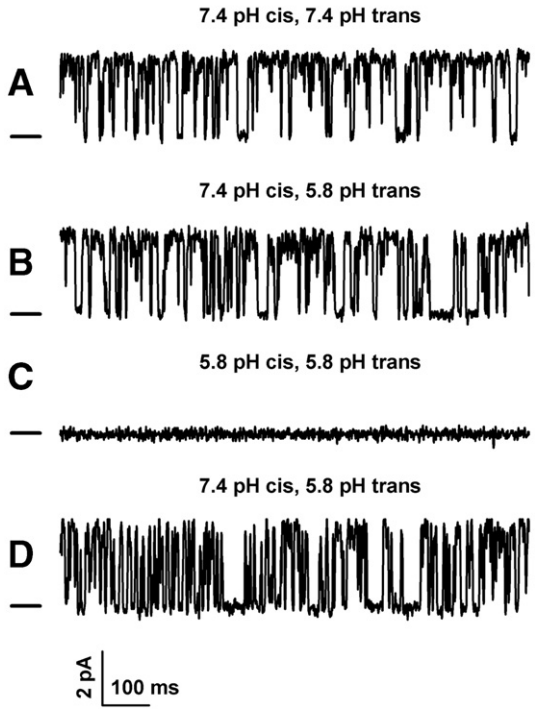

Fig. 3. Effect of pH on the single chloride channel current. A—pH 7.4 *cis*, pH 7.4 *trans*; B—pH 7.4 *cis*, pH 5.8 *trans*; C—pH 5.8 *cis*, pH 5.8 *trans*; D—pH 7.4 *cis*, pH 5.8 *trans*. The voltage is 0 mV. The lines on the left mark the closed state of the channels.

was reversible. A similar pH-dependent effect was observed when the chloride channel was partially inhibited by the studied compounds (Fig. 3). This indicates that the chloride channels were incorporated into BLM in an oriented manner.

To study the effect of the drugs, we used regular single chloride channels having classical stable, constant opening and closing chloride current amplitude for =99% of the observed time (Figs. 3, 4, 6–9).

3.3. Effect of BKA on chloride channels

BKA (1–100 μM) decreased activity (P_{open}) of the single chloride channels in a dose-dependent manner (n=19/19) (Fig. 4). BKA did not have an effect on the single channel amplitude, but it did decrease the open dwell time of the channels after application from the *cis* side, an effect that was more pronounced after application from the *trans* side of the BLM (Figs. 4A, 5A). It also increased the close dwell time of the channels; this effect was most pronounced from the *trans* side of BLM (Figs. 4A, 5B). The BKA inhibitory effect depended on the site of application (n=13/13). BKA decreased P_{open} of the chloride channels slightly from the *cis* side, but the inhibitory effect was pronounced from the *trans* side of the BLM (Fig. 4A, B). There was a slight time-dependent inhibitory effect was observed when BKA was applied at the *cis* side of BLM, but this effect was pronounced after application at the *trans* side (Fig. 4B). The inhibitory effect from the *trans* side gradually increased for several minutes. A similar side-dependent inhibitory effect of BKA was also observed in the experiments where the free calcium concentration at the *trans* side was 100 μM (n=2/2). The inhibitory effect of BKA from the *trans* side was voltage dependent, and it decreased or increased with increasing or decreasing voltage, respectively (Fig. 4C). An example is shown in Fig. 4C. Application of 1 μM and 3 μM BKA at the *trans* side did not have an influence on P_{open} at +40 mV, but BKA decreased P_{open} at 0 mV, and the inhibitory effect was pronounced at −40 mV.

Before application of BKA, the channels were tested for sensitivity to ATP and ADP. In 11/11 experiments, none of the chloride channels were modified by either 1 mM ATP or 1 mM ADP applied to the *cis* side.

3.4. Effect of ATR and CAT on chloride channels

ATR (10–150 μM) decreased activity (P_{open}) of the single chloride channels in a dose-dependent manner (n=8/8) (Fig. 6).

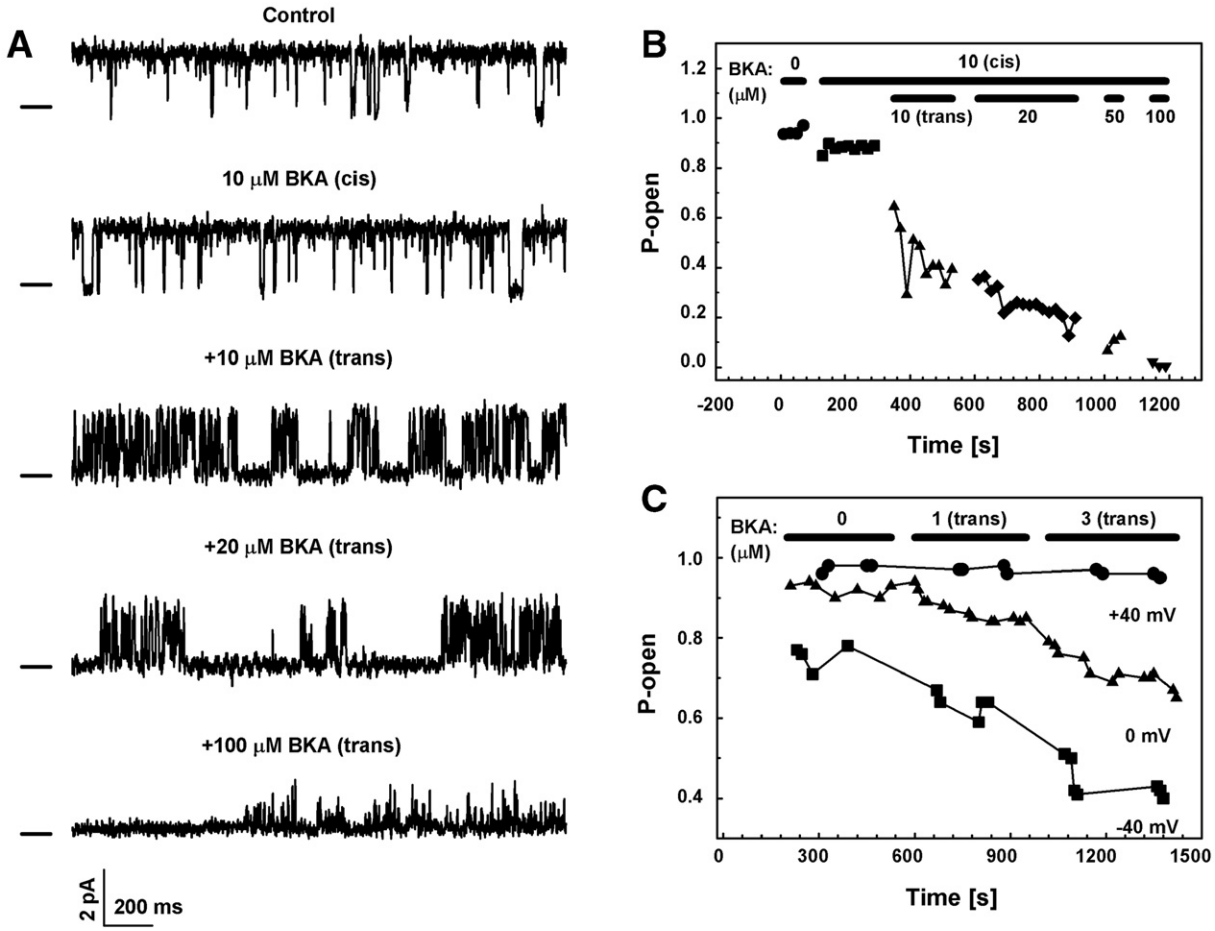

Fig. 4. A—effect of BKA on the single chloride channel current. An application of BKA at the *cis* and the *trans* side of BLM at 10, 20, and 100 μM concentrations. The voltage is 0 mV. The lines on the left mark the closed state of the channels. B—time-dependence of BKA inhibition of P_{open} of the single chloride channel at 0 mV. Comparison of the application of BKA at the *cis* and the *trans* side of BLM at 10 μM *cis* and 10, 20, 50 and 100 μM *trans* BKA concentrations. C—Time-dependent inhibitory effect of 1 and 3 μM BKA from the *trans* side of BLM on P_{open} of the single chloride channel at +40 (circles), 0 (triangles) and −40 mV (squares).

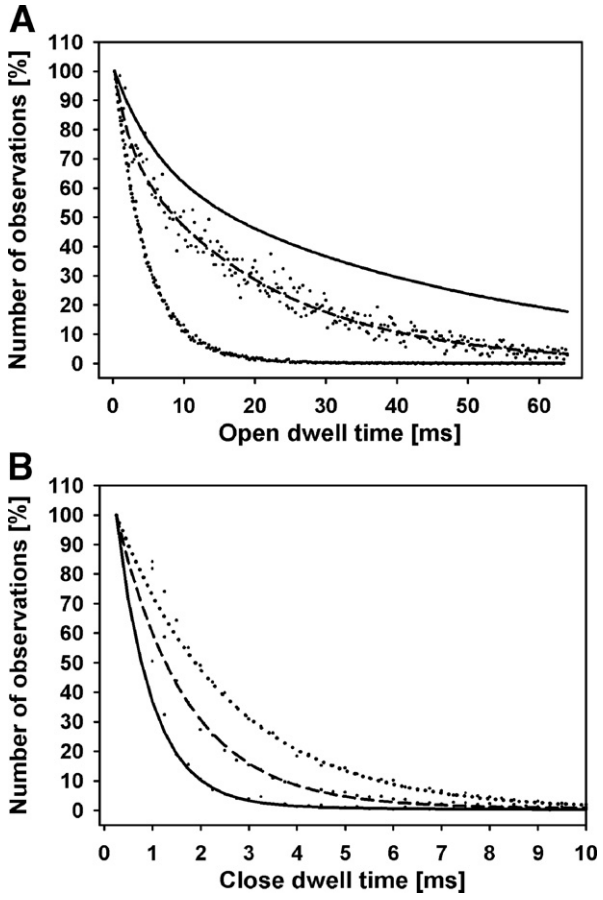

Fig. 5. A—open dwell time distribution and fitted curves of the single chloride channels. Solid line—control: τ_1=6.0 ms, τ_2=47 ms. Dashed line—10 μM BKA (*cis*): τ_1=2.1 ms, τ_2=20 ms. Dotted line — 10 μM BKA (*trans*): τ_1=4.2 ms, τ_2=9.1 ms. B—close dwell time distribution and fitted curves of the single chloride channels. Solid line—control: τ_1=0.7 ms, τ_2=5.4 ms. Dashed line—10 μM BKA (*cis*): τ_1=1.4 ms, τ_2=11 ms. Dotted line—10 μM BKA (*trans*): τ_1=2.4 ms, τ_2=26 ms.

Similarly to BKA, the ATR inhibitory effect depended on the site of application. ATR decreased P_{open} of the chloride channels slightly from the *cis* side, but the inhibitory effect was pronounced from the *trans* side of the BLM (Fig. 6A). The inhibitory effect of ATR was about 5 times smaller than the effect of BKA. The inhibition by ATR from the *trans* side was voltage dependent, and it increased pronouncedly with decreasing *trans–cis* voltage (Fig. 6C). ATR did not have any effect on the single channel amplitude. ATR, similar to BKA, decreased open dwelling time and increased close dwelling time of the chloride channels after application from the *cis* side, but the effect was most pronounced after application from the *trans* side of the BLM (data not shown).

To confirm the side-specific effect of ATR, we used its membrane-impermeable analog CAT. CAT had no effect on the chloride channels from the *cis* side (Fig. 7). Application of 10 and 50 μM CAT to the *cis* side did not decreased P_{open}, but subsequent application of 50 μM CAT to the *trans* side pronouncedly decreased it (Fig. 7B). Similar to BKA and ATR, the *trans* inhibitory effect of CAT decreased at positive voltages and increased at negative ones (Fig. 7C). These results were confirmed through experiments where CAT was applied first at the *trans* side and subsequently at the *cis* side (Fig. 8). CAT (10 and 50 μM) decreased P_{open} from the *trans* side, but its subsequent application to the *cis* side did not have any effect.

Tributyltin (TBT) was reported as a potent inhibitor of the inner mitochondrial membrane anion channel (IMAC) [88]. Therefore, we tested its chloride channels inhibition properties. TBT at 4 μM inhibited the chloride channels (n=2/2). P_{open} decreased from 0.8 to close to zero, and the channel behavior was perturbed (Fig. 9).

Since BKA is a lipophilic compound, and BKA, ATR, and CAT have ionizing groups which could affect the surface potential of the bilayer, we examined effect of a "simpler" lipophilic molecule, myristic acid (MA), on chloride channels. MA at 30 and 100 μM did not decrease P_{open}; however, after application of 100 μM MA (9 and 3 min in *cis* and *trans*, respectively), the channel behavior was suddenly perturbed (n=2/2) (Fig. 10). MA decreased the single channel amplitude: the values at 0 mV for control, 30 μM *cis*, +30 μM *trans*, 100 μM *cis* and +100 μM *trans* of MA were 4.01, 3.81, 3.56, 3.20 and 3.00 pA, respectively.

3.5. Effect of ATR and CAT on nonselective channels

ATR and CAT inhibited chloride channels and simultaneously activated nonselective channels in 4/15 experiments. An example of the inhibitory and stimulatory effects of ATR on the chloride and the nonselective channels, respectively, simultaneously incorporated in BLM, are shown in Fig. 11. The single channel current was measured at −10 mV in order to clearly distinguish chloride current passing through chloride channels and potassium current passing through nonselective channels. Three chloride channels at control had high P_{open} (~0.8; the chloride current of the open chloride channels is upwards). The nonselective channel was mostly closed (P_{open}=0.15; the potassium current of the open nonselective channel is downwards). ATR (40 μM) applied at the *cis* side slightly decreased the activity of the chloride channels, but significantly activated the nonselective channel (P_{open}=0.42). Further application of ATR at the *trans* side significantly reduced the activity of the chloride channels and further activated the nonselective channel (P_{open}=0.77) (Fig. 11). The activation effect of ATR on the nonselective channel was evaluated at −20 mV, which is approximately the E_{rev} of the chloride channels. The single channel current traces of the nonselective channel at −20 mV and the effect of 40 μM ATR are shown in Fig. 12A. The single channel current was regular, showing stable, constant open and close levels. ATR activated the nonselective channel, having a conductance of 629 pS within the range of −30 mV to −10 mV, and 466 pS within the range of +10 mV to +30 mV. The conductance of three other ATR- and CAT-activated nonselective channels observed in other experiments was 80, 228, 407 pS. The channel conductance, as well as the dwell open and closed times of the nonselective channel, was several times higher than that of the chloride channel. We did not observe an activation of the nonselective channels by BKA.

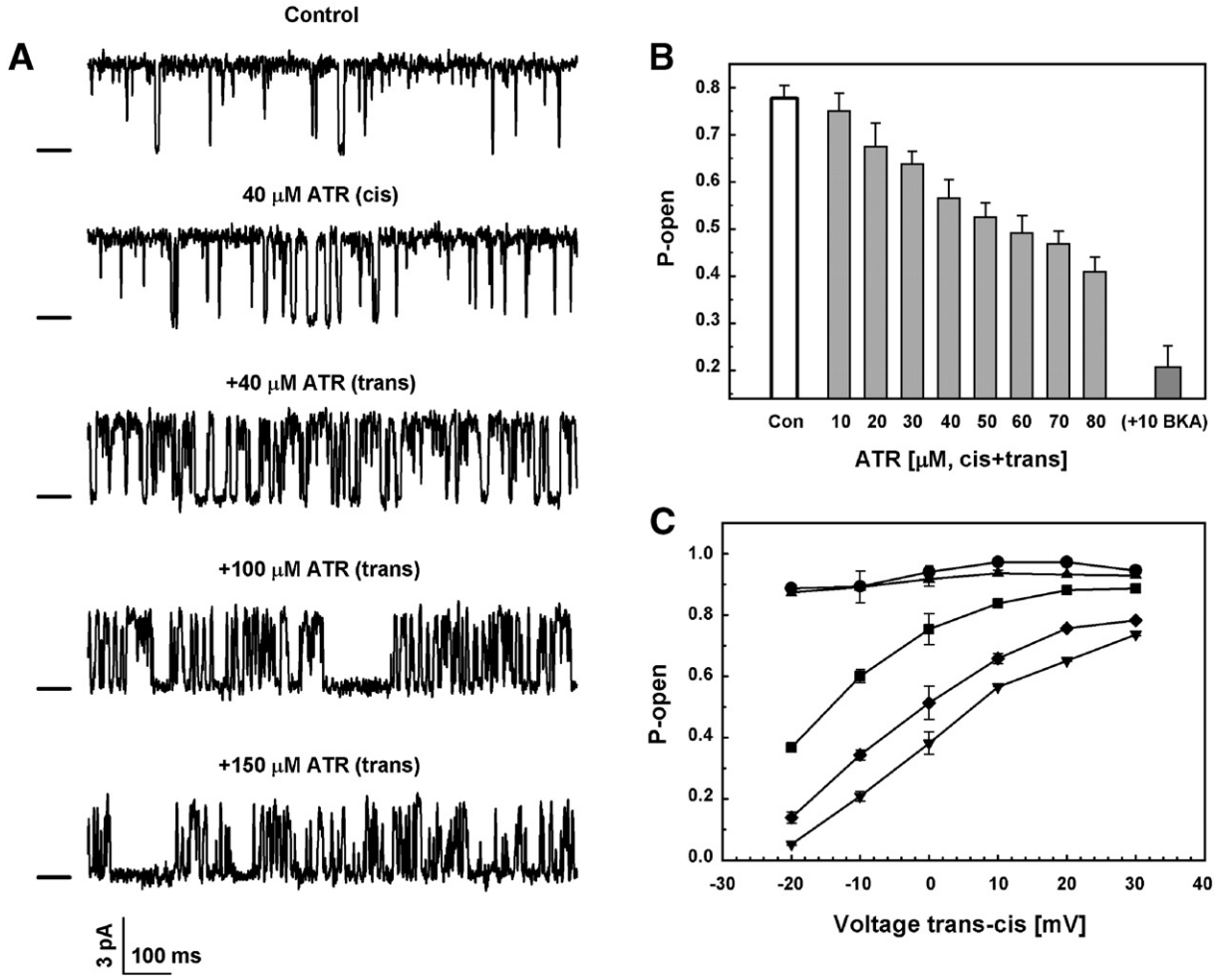

Fig. 6. A—effect of ATR on the single chloride channel current. An application of ATR at the *cis* and the *trans* side of BLM at 40, 100 and 150 μM concentrations. The voltage is 0 mV. The lines on the left mark the closed state of the channels. B — concentration dependence of ATR inhibition of P_{open} of the single chloride channel at 0 mV and with addition of +10 μM BKA after 80 μM ATR. C—voltage-dependent inhibition by ATR. Circles—control; Up-facing triangles—40 μM ATR (*cis*); Squares—40 μM ATR (*trans*); Diamonds — 100 μM ATR (*cis*+*trans*); Down-facing triangles—150 μM ATR (*cis*+*trans*).

The ATR activation effect on the nonselective channel was not voltage dependent (Fig. 12B). The pH decrease at the *trans* side to pH 5.8 did not change the voltage dependence, but a decrease of the pH at the *cis* side decreased the channel amplitude about 50%, and the conductance decreased from 762 pS to 434 pS (Fig. 12C).

4. Discussion

4.1. Purity of SMP isolation

Based on the estimation of Na^+/K^+- and Mg^{2+}/Ca^{2+}-ATPase activities [89], the isolated fraction of SMP contained 0.68–1.47% sarcolemma and 0.94–2.01% sarcoplasmic reticulum, with median values of 0.84% and 1.59%, respectively. This degree of purity is within the range reported in similar studies in the literature [90]. Similarly, based on immunoblot analyses, the isolated SMP were not contaminated by Golgi or nuclear membranes. This indicates that the isolated SMP were not contaminated with sarcolemma, sarcoplasmic reticulum, Golgi or nuclear membranes. We cannot exclude a minor contamination from other intracellular vesicles. However, according to the VDAC1/porin immunoblot results, the isolated SMP contained a significant amount of outer mitochondrial membranes, so the observed chloride channels might be derived from both outer and inner mitochondrial membranes.

4.2. Single channel properties of chloride channels

The single channel properties of the mitochondrial chloride channels were different from the reported voltage dependent anion channel (VDAC) of the mitochondrial outer membrane. The VDAC has a conductance of 4 nS in 1 M KCl and an open channel selectivity of 2:1 for Cl^- over K^+. The VDAC closes to lower conducting states when either positive or negative potentials are applied [91]. On the contrary, we observed regular chloride channels with lower conductance, higher selectivity for Cl^- over K^+, which did not close to lower conducting states when either positive or negative potential was applied. The observed chloride channel had classical stable, constant opening and closing chloride current amplitude for ≥99% of the observed time (Figs. 3, 4, 6–9). We did not

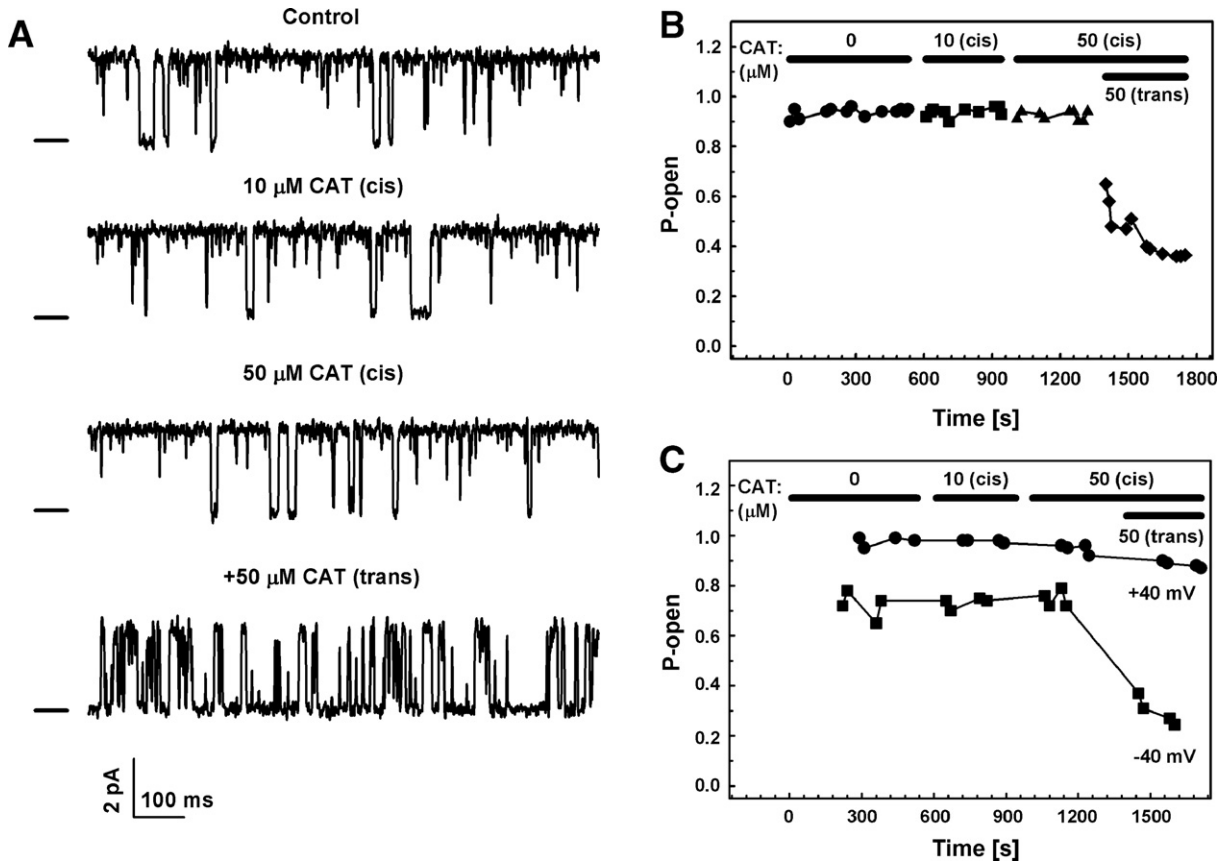

Fig. 7. A—effect of CAT on the single chloride channel current. An application of CAT at the *cis* and the *trans* side of BLM at 10 and 50 μM concentrations, at voltage=0 mV. The lines on the left mark the closed state of the channels. B—time dependence of CAT inhibition of P_{open} of the single chloride channel at 0 mV. Comparison of the application of CAT at the *cis* and the *trans* side of BLM at 10 and 50 μM *cis* and 50 μM *trans* concentrations. C—time dependence of CAT inhibition of P_{open} of the single chloride channel at +40 mV (circles) and −40 mV (squares). Comparison of the application of CAT at the *cis* and the *trans* side of BLM at 10 and 50 μM *cis* and 50 μM *trans* concentrations.

observe subconductance jumps of the chloride current as was reported for VDAC. Therefore, we assume that the observed chloride channels are not VDAC.

The chloride channels observed in our study had a higher conductance than that reported for chloride channels from sheep cardiac mitoplasts measured at pH 8.8 in BLM, which have a multisubstrate conductance of ~25 or ~60 pS in 300/50 choline-Cl^- solutions [61]. Contrary to our observed channels, the reported channels were unaffected by variation in pH (5.5–8.5). However, detailed comparison of the reported channels and our observed channels is limited, since Hayman et al. [61] mostly used 2 mM $CaCl_2$ in BLM solutions.

Our chloride channels had a higher conductance than that reported for the slightly anion selective 45 pS channels from liver mitochondria, measured in 150 mM KCl and at alkaline pH (alkaline-induced anion-selective activity, AAA), which were inhibited by Mg^{2+} [62,92], or the 45 pS channel from yeast mitochondria measured in 150 KCl [63].

The intracellular chloride channel mtCLIC/CLIC4 was localized to mitochondria [51]. Its single channel properties in mitochondria are not known, but over-expressed CLIC4 in the plasma membrane formed anion channel, which had a low conductance of about 1 pS in 140 mM Cl^- [93], and bilayer incorporation of microsomal (mainly ER) membrane vesicles containing recombinant CLIC4 had conductance of about 17 pS in 10/100 mM choline chloride [94]. Since possible oligomerization or coupling of the CLIL4 channels is unknown, we cannot address the possible connection between the observed chloride channels and CLIC4.

It is believed that Cl^- permeates the inner mitochondrial membrane by means of the inner membrane anion channel (IMAC) [65–68,88,95,96]. IMAC is a non-selective anion channel that carries a wide variety of anions ranging from small, singly charged ions, such as Cl^-, to multicharged anions, such as citrate, ferrocyanide, and even ATP (reviewed in [68]). It is regulated by various compounds, and inhibited by matrix Mg^{2+} (IC_{50}=38 μM) and lower matrix pH (pH<7.2) [66,68]. One of the most potent and specific inhibitors of IMAC is TBT [88]. Similarly, the observed chloride channels in our study were reversibly inhibited by low pH (5.8) at the *cis* side of the BLM, and inhibited by a low concentration of TBT (Figs. 3, 9). This may indicate that our chloride channel is IMAC. However, in our study, the chloride channels were observed in the *cis/trans* solutions containing 2 mM Mg^{2+}. More study is needed to clarify this difference.

The 108 pS channel in mitochondria (in 150 mM KCl) [50] has been detected in patch-clamp experiments on mitoplasts from different tissues, including heart [64]. Slightly different

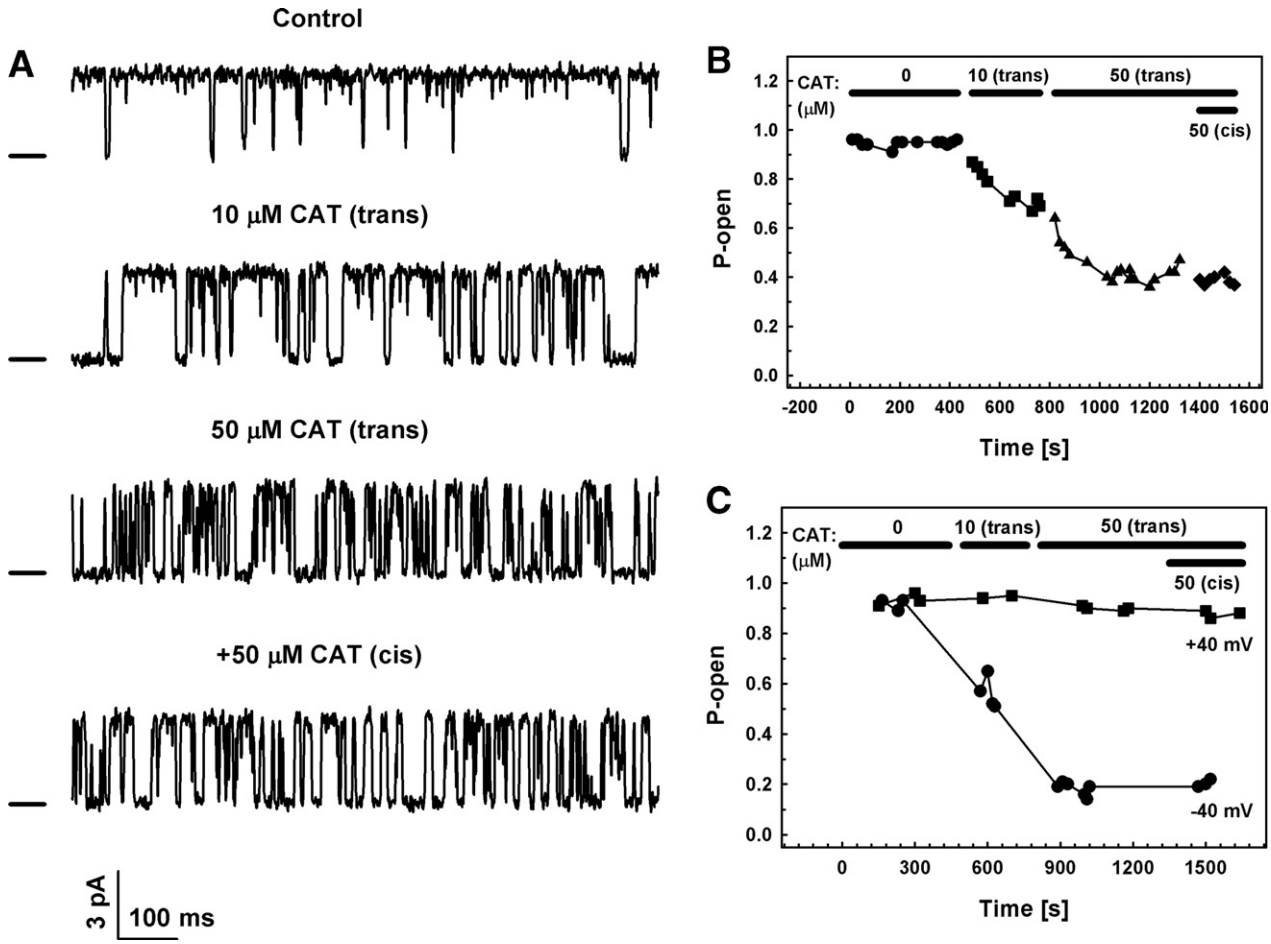

Fig. 8. A—effect of CAT on the single chloride channel current. An application of CAT at the *trans* and the *cis* side of BLM at 10 and 50 μM concentrations, at voltage=0 mV. The lines on the left mark the closed state of the channels. B—time dependence of CAT inhibition of P_{open} of the single chloride channel at 0 mV. Comparison of the application of CAT at the *trans* and the *cis* side of BLM at 10 and 50 μM *trans* and 50 μM *cis* concentrations. C—time dependence of CAT inhibition effect on P_{open} of the single chloride channel at +40 mV (squares) and −40 mV (circles). Comparison of the application of CAT at the *trans* and the *cis* side of BLM at 10 and 50 μM *trans* and 50 μM *cis* concentrations.

properties have been reported for the channel [92,50,64,59,60]. We propose that the observed regular chloride channels could be related to the reported 108 pS channel-IMAC. However, from the variability of the observed single channel properties, chloride channels derived from protein structures such as transporting or uncoupling proteins are not excluded.

The high variation of the single chloride channel conductance observed in our study might result from a cooperative coupling of openings of several chloride channels, as was reported for ryanodine calcium single channels from skeletal and heart muscles [97,98], or from cooperative openings of channel subunits. However, we cannot exclude a minor contamination from non-mitochondrial vesicles.

Observed chloride channels were blocked by 5.8 pH at the *cis* side, but not at the *trans* side. They were markedly inhibited by ATR and BKA from the *trans* side in comparison to the *cis* side. CAT inhibited the chloride channels from the *trans* side only, but not from the *cis* side. From these results, we proposed that the chloride channels were incorporated into the BLM in an oriented manner.

Several studies reported a reversible inhibition of anion-chloride channels derived from inner mitochondrial membranes by lowering the pH below 7.0 at mitochondrial matrix side [62,60,66]. From the observation that the chloride channels in our study were blocked by lowering pH from the *cis* side only, we may assume that the matrix side of the observed chloride channel faced the *cis* side and intracellular side the *trans* side of the BLM.

In our work, we report for the first time that BKA, ATR, and CAT inhibited mitochondrial chloride channels. Reported subcellular effects of BKA, ATR, and CAT were detected at the inner mitochondria membranes [1,2,4,5]. From this, we suggest that the observed chloride channels in our study are probably derived from inner mitochondrial membrane.

The observed inhibitory effect of ATR and CAT on chloride channels was distinct to their known activation effect on MPTP. In our study, ATR and CAT activated a nonselective channel that was distinct from the observed chloride channel. We may suppose that the activated nonselective channel was a part of "classical" MPTP complex. However, we cannot exclude the possibility that the observed chloride channels are also part of the MPTP complex.

The inhibitory effects of the studied compounds were side dependent, and their target on chloride channel was better

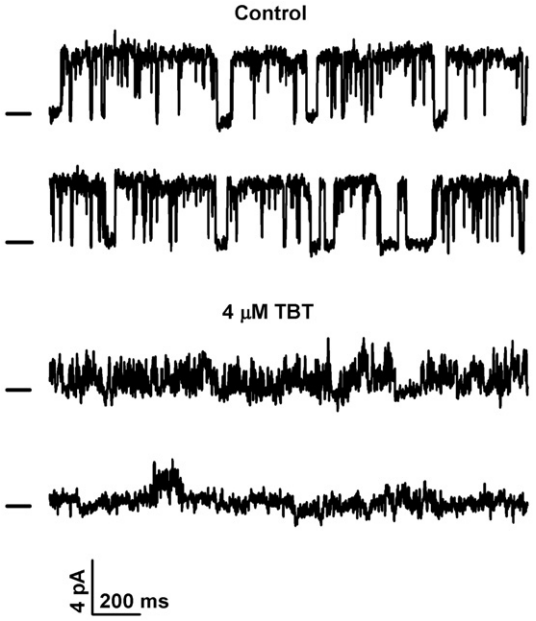

Fig. 9. Effect of TBT on the single chloride channel current. The two upper current traces are controls; the two bottom current traces are application of 4 μM TBT at the *cis* and the *trans* side of BLM. Voltage 0 mV. The lines on the left mark the closed state of the channels.

accessible from the *trans* side then from the *cis* side. We did not observe a sudden block of the chloride channels, but rather a gradual concentration-dependent inhibition. This may indicate that there is not a specific binding side on the channel, the occupation of which would lead to spatial protein conformation change. The inhibitory effect of the compounds was time-dependent from the *trans* side, which indicates that the compounds had to penetrate into a membrane target and accumulate at the target site on the channel. The time-dependent inhibitory effect may also indicate that the compounds influenced the chloride channel indirectly, through another protein or by influencing the channel environment. The studied compounds increased channel kinetics, inhibited channels by decreasing open dwell time and increasing close dwell time,

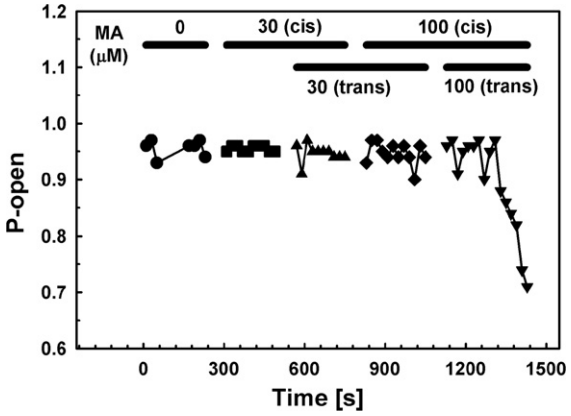

Fig. 10. Concentration and time-dependent inhibition by MA of P_{open} of the single chloride channel at 0 mV. MA concentrations: circles—control; squares—30 μM (*cis*); up-facing triangles—30 μM (*cis*+*trans*); diamonds—100 μM (*cis*) and 30 μM (*trans*); down-facing triangles—100 μM (*cis*) and 100 μM (*trans*).

indicating that they could affect the gating mechanism of the chloride channels.

The inhibitory effect of BKA, ATR, and CAT cannot be explain by the lipophilicity of BKA or by their effect on the surface potential formed by ionizing groups. In our study, lipophilic MA, which also has ionizing groups, had a different effect on the chloride channels than the studied compounds (Fig. 10). It decreased the single channel amplitude by building a negative charge at the membrane surface. The sudden inhibitory effect after long application of 100 μM MA (Fig. 10) was probably caused by the creation of free volume in the bilayer hydrophobic region, which resulted in a general membrane perturbation [99]. MA was reported to activate the IMAC channel at pH 8.0, which was blocked by TBT [100]. Since the activity of the chloride channel in our study was very high (P_{open} ~0.9), and pH 7.4 was used, we did not observe an activation effect of MA on the channels.

4.3. Comparison with cellular and subcellular effects

In our work, BKA, ATR, and CAT inhibited mitochondrial chloride channels at concentrations that are higher than those

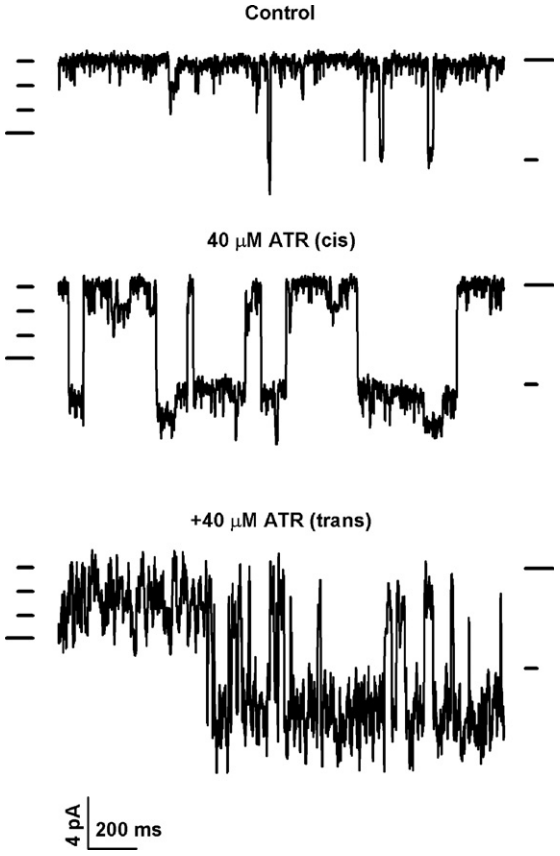

Fig. 11. Effect of 40 μM ATR (*cis* and *trans*) on the chloride current of three single chloride channels and the nonselective potassium channel current simultaneously incorporated into BLM, at a voltage of −10 mV. The longer lines on the left mark the closed state of the three chloride channels, while the shorter lines on the left mark open states of the three chloride channels. The chloride channels are open upwards. The longer lines on the right mark the closed state of the nonselective channel and the shorter lines on the right mark open state of the nonselective channel. The nonselective channel is open downwards.

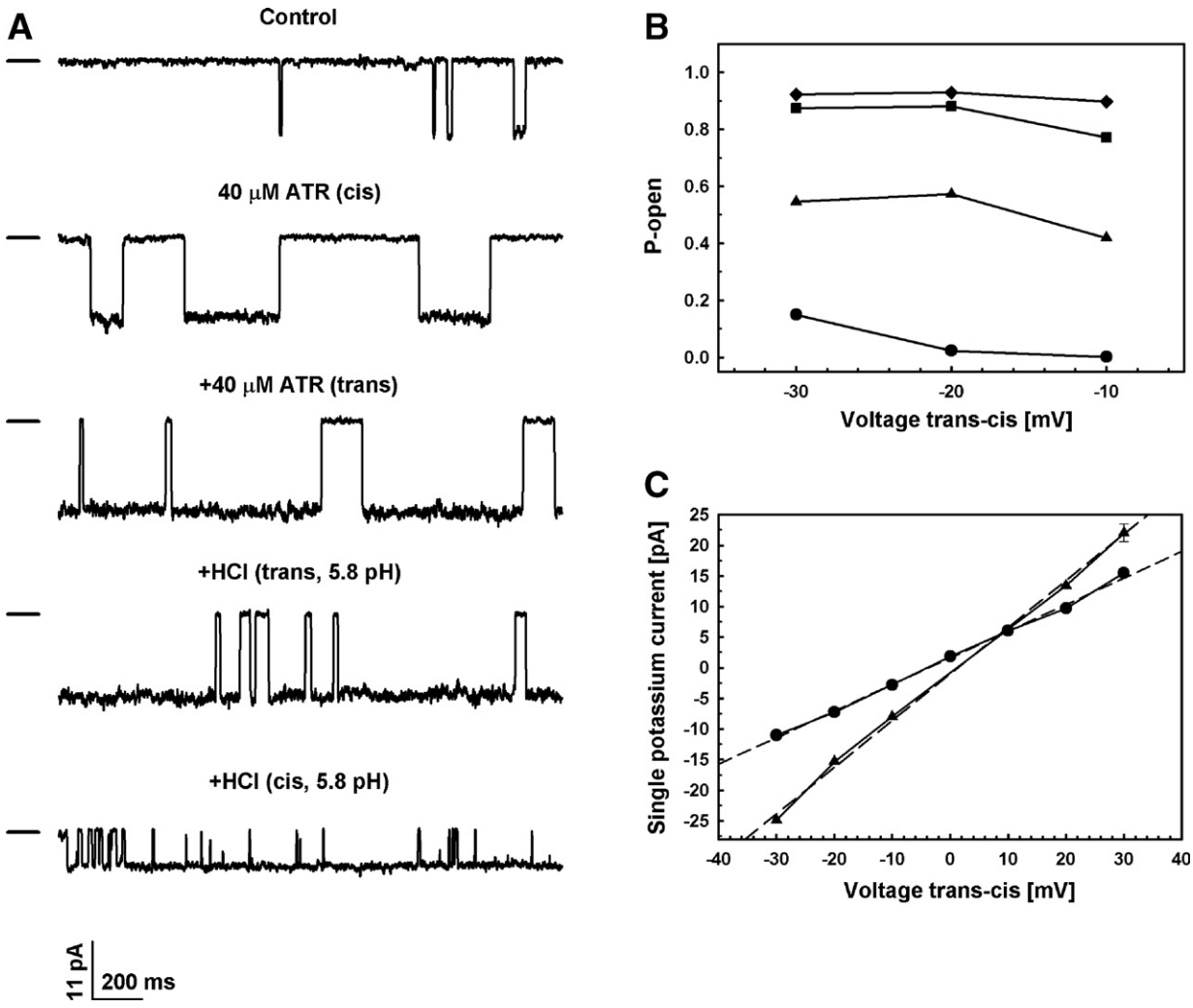

Fig. 12. A—effect of 40 μM ATR (*cis* and *trans*) and pH (*cis* and *trans*) on the nonselective potassium channel current at a voltage of −20 mV. The lines on the left mark the closed state of the channel. B—voltage dependence of the ATR activation effect on P_{open} of the single nonselective channel at −20 mV. Circles—control; triangles — 40 μM ATR (*cis*); squares—40 μM ATR (*trans*); diamonds—pH 5.8 (*trans*). C—Current–voltage relationship of the nonselective channel at pH 5.8 *trans* (triangles) (conductance = 762 pS) and pH 5.8 *cis* (circles) (conductance = 434 pS).

used to inhibit ANT. Since they bind to the ANT with very high affinity (K_d < 1 μM) [101,102], we assume that the inhibitory effect of the compounds on chloride channels is not directly connected with their effect on inhibiting ANT. However, BKA, ATR, and CAT, used as pharmacological tools to modulate properties of the mitochondrial membrane besides their effect on ANT, influenced various cellular and subcellular properties. In many studies, they were used at concentrations that inhibited mitochondrial chloride channels. For example, BKA used at 50 μM, which in our study nearly completely inhibited mitochondrial chloride channels, was reported to prevent a number of phenomena linked to apoptosis, including depletion of reduced glutathione, generation of reactive oxygen species, translocation of NFκB, exposure of phosphatidylserine residues on the outer plasma membrane, cytoplasmic vacuolization, chromatin condensation, and oligonucleosomal DNA fragmentation [6], to protect multidrug-resistant tumor cells against apoptosis caused by 1,4-anthraquinone [7], to inhibit MPTP pore opening in response to prooxidants or protonophores [10], to mimic the effect of diazoxide to suppress calcium-dependent morphological changes in mitochondria [15], to prevent $G\alpha_q$-induced cytochrome *c* release and the loss of mitochondrial membrane potential, and to inhibit cardiomyocyte apoptosis [16], to prevent a loss of mitochondrial inner membrane potential (Delta(Psi)m) induced by H_2O_2 in cerebellar granule neurons [38], to inhibit production of coated-platelets [12], to inhibit glucose-induced electrical activity in the pancreatic beta-cells [37] (at 64 and 128 μM BKA), and to prevent the lost of mitochondrial potential and cell death induced by cysteine starvation (50 and 100 μM BKA) [103].

Lower concentrations of BKA, which in our study significantly inhibited mitochondrial chloride channels, were reported to protect MCF-7 cells against the proliferation inhibitory effects of the chemotherapy drug Tamoxifen [8] (5–40 μM BKA), to prevent oligomycin-induced pyranine fluorescence quenching in SMP [31] (10 μM BKA), to reduce the uncoupling of mitochondrial oxidative phosphorylation induced by the K(ATP) channel openers diazoxide and pinacidil

[33] (20 μM BKA), to inhibit K^+ efflux in brain nonsynaptosomal mitochondria [35], (20 μM BKA), and to reduce inhibition of transient K_{Ca} currents by mitochondrial depolarization in smooth muscle cells of rat cerebral arteries [36] (20 μM BKA).

Similarly, ATR was used at concentrations that were in the range that decreased activity of the studied mitochondrial chloride channels. ATR (100 μM) potentiated an arachidonic acid-induced, CsA-insensitive mitochondria matrix swelling [27], attenuated the neuroprotective effects of diazoxide and CsA [25], prevented oligomycin-induced pyranine fluorescence quenching in SMP [31], augmented IgE-mediated Ca^{2+} store release [42] (10–100 μM ATR), or opened a non-specific ion channel responsible for cytotoxicity [41] (100–600 μM ATR).

In conclusion, chloride channels are involved in many physiological and pathological processes; many of these processes are also influenced by BKA, ATR, and CAT. Our observation that BKA, ATR, and CAT inhibited mitochondrial chloride channels may imply that some of their cellular or subcellular effects are connected with their inhibitory effect on the chloride channels.

Acknowledgments

The study was supported by grants: APVT 51-027-404, VEGA 2/3001/23, 2/6012/6, 2/6078 and SO VV-CCHS-IPM. This study was also supported partially by NATO collaborative grant LST.CLG.979217.

References

[1] P.J.F. Henderson, H.A. Lardy, Bongkrekic acid an inhibitor of the adenine nucleotide translocase of mitochondria, J. Biol. Chem. 245 (1970) 1319–1326.
[2] B.H. Chua, E. Shrago, Reversible inhibition of adenine nucleotide translocation by long chain acyl-CoA esters in bovine heart mitochondria and inverted submitochondrial particles comparison with atractylate and bongkrekic acid, J. Biol. Chem. 252 (1977) 6711–6714.
[3] M. Klingenberg, Molecular aspects of the adenine nucleotide carrier from mitochondria, Arch. Biochem. Biophys. 257 (1989) 1–14.
[4] A.P. Halestrap, C. Brenner, The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death, Curr. Med. Chem. 10 (2003) 1507–1525.
[5] P. Bernardi, A. Krauskopf, E. Basso, V. Petronilli, E. Blachy-Dyson, F. Di Lisa, M.A. ForteThe mitochondrial permeability transition from in vitro artifact to disease target, FEBS J. 273 (2006) 2077–2099.
[6] P. Marchetti, M. Castedo, S.A. Susin, N. Zamzami, T. Hirsch, A. Macho, A. Haeffner, F. Hirsch, M. Geuskens, G. Kroemer, Mitochondrial permeability transition is a central coordinating event of apoptosis, J. Exp. Med. 184 (1996) 1155–1160.
[7] Y. Wang, E.M. Perchellet, M.M. Ward, K. Lou, D.H. Hua, J.P. Perchellet, Rapid collapse of mitochondrial transmembrane potential in HL-60 cells and isolated mitochondria treated with anti-tumor 1,4-anthracenediones, Anti-cancer Drugs 16 (2005) 953–967.
[8] A. Kallio, A. Zheng, J. Dahllund, K.M. Heiskanen, P. Härkönen, Role of mitochondria in tamoxifen-induced rapid death of MCF-7 breast cancer cells, Apoptosis 10 (2005) 1395–1410.
[9] M. Muranyi, P.A. Li, Bongkrekic acid ameliorates ischemic neuronal death in the cortex by preventing cytochrome c release and inhibiting astrocyte activation, Neurosci. Lett. 26 (2005) 277–281.
[10] N. Zamzami, S.A. Susin, P. Marchetti, T. Hirsch, M. Castedo, G. Kroemer, Mitochondrial control of nuclear apoptosis, J. Exp. Med. 183 (1996) 1533–1544.
[11] Z.Z. Gizatullina, Y. Chen, S. Zierz, F.N. Gellerich, Effects of extramitochondrial ADP on permeability transition of mouse liver mitochondria, Biochem. Biophys. Acta 1706 (2005) 98–104.
[12] G. Remenyi, R. Szasz, P. Friese, G.L. Dale, Role of mitochondrial permeability transition pore in coated-platelet formation, Arterioscler., Thromb., Vasc. Biol. 25 (2005) 467–471.
[13] N. Brustovetsky, M. Klingenberg, Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca2+, Biochemistry 35 (1996) 8483–8488.
[14] A.P. Halestrap, C. Brennerb, The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death, Curr. Med. Chem. 10 (2003) 1507–1525.
[15] M. Akao, B. O'Rourke, H. Kusuoka, Y. Teshima, S.P. Jones, E. Marban, Differential actions of cardioprotective agents on the mitochondrial death pathway, Circ. Res. 92 (2003) 195–202.
[16] J.W. Adams, A.L. Pagel, C.K. Means, D. Oksenberg, R.C. Armstrong, J.H. Brown, Cardiomyocyte apoptosis induced by gaq signaling is mediated by permeability transition pore formation and activation of the mitochondrial death pathway, Circ. Res. 87 (2000) 1180–1187.
[17] D.J. Hausenloy, H.L. Maddock, G.F. Baxter, D.M. Yellon, Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc. Res. 55 (2002) 534–543.
[18] Q. Gao, H.Y. Pan, S. Qiu, Y. Lu, I.C. Bruce, J.H. Luo, Q. Xia, Atractyloside and 5-hydroxydecanoate block the protective effect of puerarin in isolated rat heart, Life Sci. 79 (2006) 217–224.
[19] P.S. Pagel, J.G. Krolikowski, D.A. Neff, D. Weihrauch, M. Bienengraeber, J.R. Kersten, D.C. Warltier, Inhibition of glycogen synthase kinase enhances isoflurane-induced protection against myocardial infarction during early reperfusion in vivo, Anesth. Analg. 102 (2006) 1348–1354.
[20] J.G. Krolikowski, M. Bienengraeber, D. Weihrauch, D.C. Warltier, J.R. Kersten, P.S. Pagel, Inhibition of mitochondrial permeability transition enhances isoflurane-induced cardioprotection during early reperfusion: the role of mitochondrial KATP channels, Anesth. Analg. 101 (2005) 1590–1596.
[21] Q. Gao, S.Z. Zhang, C.M. Cao, I.C. Bruce, Q. Xia, The mitochondrial permeability transition pore and the Ca2+-activated K+ channel contribute to the cardioprotection conferred by tumor necrosis factor-alpha, Cytokine 32 (2005) 199–205.
[22] G. Wang, D.A. Liem, T.M. Vondriska, H.M. Honda, P. Korge, D.M. Pantaleon, X. Qiao, Y. Wang, J.N. Weiss, P. Ping, Nitric oxide donors protect murine myocardium against infarction via modulation of mitochondrial permeability transition, Am. J. Physiol.: Heart Circ. Physiol. 288 (2005) H1290–H1295.
[23] C.P. Baines, C.-X. Song, Y.-T. Zheng, G.-W. Wang, J. Zhang, O.-L. Wang, Y. Guo, R. Bolli, E.M. Cardwell, P. Ping, Protein kinase Cå interacts with and inhibits the permeability transition pore in cardiac mitochondria, Circ. Res. 92 (2003) 873–880.
[24] C.M. Cao, Q. Xia, Q. Gao, M. Chen, T.M. Wong, Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning, J. Pharmacol. Exp. Ther. 312 (2005) 644–650.
[25] L. Wu, F. Shen, L. Lin, X. Zhang, I.C. Bruce, Q. Xia, The neuroprotection conferred by activating the mitochondrial ATP-sensitive K(+) channel is mediated by inhibiting the mitochondrial permeability transition pore, Neurosci. Lett. 402 (2006) 184–189.
[26] K.G. Rajesh, S. Sasaguri, S. Ryoko, H. Maeda, Mitochondrial permeability transition-pore inhibition enhances functional recovery after long-time hypothermic heart preservation, Transplantation 76 (2003) 1314–1320.
[27] M. Di Paola, P. Zaccagnino, C. Oliveros-Celis, M. Lorusso, Arachidonic acid induces specific membrane permeability increase in heart mitochondria, FEBS Lett. 580 (2006) 775–781.
[28] J.S. Isenberg, J.E. Klaunig, Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells, Toxicol. Sci. 53 (2000) 340–351.
[29] O. Vergun, I.J. Reynolds, Fluctuations in mitochondrial membrane

potential in single isolated brain mitochondria: modulation by adenine nucleotides and Ca2+, Biophys. J. 87 (2004) 3585–3593.
[30] O. Vergun, I.J. Reynolds, Distinct characteristics of Ca(2+)-induced depolarization of isolated brain and liver mitochondria, Biochim. Biophys. Acta 1709 (2005) 127–137.
[31] M. Ziegler, H.S. Penefsky, The adenine nucleotide translocase modulates oligomycin-induced quenching of pyranine fluorescence in submitochondrial particles, J. Biol. Chem. 268 (1993) 25320–25328.
[32] A. Romani, C. Marfella, M. Lakshmanan, Mobilization of Mg^{2+} from rat heart and liver mitochondria following the interaction of thyroid hormone with the adenine nucleotide translocase, Thyroidology 6 (1996) 513–519.
[33] D.M. Kopustinskiene, A. Toleikis, N.E. Saris, Adenine nucleotide translocase mediates the K(ATP)-channel-openers-induced proton and potassium flux to the mitochondrial matrix, J. Bioenerg. Biomembr. 35 (2003) 141–148.
[34] V.I. Dedukhova, E.N. Mokhova, V.P. Skulachev, A.A. Starkov, E. Arrigoni-Martelli, V.A. Bobyleva, Uncoupling effect of fatty acids on heart muscle mitochondria and submitochondrial particles, FEBS Lett. 295 (1991) 51–54.
[35] T. Brustovetsky, N. Shalbuyeva, N. Brustovetsky, Lack of manifestations of diazoxide/5-hydroxydecanoate-sensitive KATP channel in rat brain nonsynaptosomal mitochondria, J. Physiol. 568 (2005) 47–59.
[36] S.Y. Cheranov, J.H. Jaggar, Mitochondrial modulation of Ca2+ sparks and transient KCa currents in smooth muscle cells of rat cerebral arteries, J. Physiol. 556 (Pt. 3) (2004) 755–771.
[37] B. Kiranadi, J.A. Baugham, P.A. Smith, Inhibition of electrical activity in mouse pancreatic beta-cells by the ATP/ADP translocase inhibitor, bongkrekic acid, FEBS Lett. 283 (1991) 93–96.
[38] Y. Teshima, M. Akao, R.A. Li, T.H. Chong, W.A. Baumgartner, M.V. Johnston, E. Marban, Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress, Stroke 34 (2003) 1796–1802.
[39] A. Toninello, M. Salvi, M. Schweizer, C. Richter, Menadione induces a low conductance state of the mitochondrial inner membrane sensitive to bongkrekic acid, Free Radical Biol. Med. 37 (2004) 1073–1080.
[40] N. Yamaguchi, T. Kagari, M. Kasai, Inhibition of the ryanodine receptor calcium channel in the sarcoplasmic reticulum of skeletal muscle by an ADP/ATP translocase inhibitor, atractyloside, Biochem. Biophys. Res. Commun. 258 (1999) 247–251.
[41] D. Haouzi, I. Cohen, H.L. Vieira, D. Poncet, P. Boya, M. Castedo, N. Vadrot, A.S. Belzacq, D. Fau, C. Brenner, G. Feldmann, G. Kroemer, Mitochondrial permeability transition as a novel principle of hepatorenal toxicity in vivo, Apoptosis 7 (2002) 395–405.
[42] Y. Suzuki, T. Yoshimaru, T. Inoue, C. Ra, Mitochondrial Ca^{2+} flux is a critical determinant of the Ca^{2+} dependence of mast cell degranulation, J. Leukocyte Biol. 79 (2006) 508–518.
[43] A. Szewczyk, The intracellular potassium and chloride channels: properties, pharmacology and function (review), Mol. Membr. Biol. 15 (1998) 49–58.
[44] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (1999) 1127–1155.
[45] I. Inoue, H. Nagase, K. Kishi, T. Higuti, ATP-sensitive K+ channel in the mitochondrial inner membrane, Nature 352 (1991) 244–247.
[46] P. Paucek, G. Mironova, F. Mahdi, A.D. Beavis, G. Woldegiorgis, K.D. Garlid, Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K+ channel from rat liver and beef heart mitochondria, J. Biol. Chem. 267 (1992) 26062–26069.
[47] P. Bednarczyk, A. Kicinska, V. Kominkova, K. Ondrias, K. Dolowy, A. Szewczyk, Quinine inhibits mitochondrial ATP-regulated potassium channel from bovine heart, J. Membr. Biol. 199 (2004) 63–72.
[48] Y. Kirichok, G. Krapivinsky, D.E. Clapham, The mitochondrial calcium uniporter is a highly selective ion channel, Nature 427 (2004) 360–364.
[49] V. Kominkova, M. Novotova, K. Ondrias, T. Ravingerova, A. Szewczyk, Mitochondrial channels permeable by calcium ions, Toxicol. Mech. Methods 14 (2004) 35–39.
[50] M.C. Sorgato, B.U. Keller, W. Stuhmer, Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel, Nature 330 (1987) 498–500.
[51] E. Fernandez-Salas, M. Sagar, C. Cheng, S.H. Yuspa, W.C. Weinberg, p53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein, J. Biol. Chem. 274 (1999) 36488–36497.
[52] M.R. Duchen, Mitochondria and calcium: from cell signalling to cell death, J. Physiol. 529 (2000) 57–68.
[53] A.L. Nieminen, Apoptosis and necrosis in health and disease: role of mitochondria, Int. Rev. Cytol. 224 (2003) 29–55.
[54] D.R. Giovannucci, M.D. Hlubek, E.L. Stuenkel, Mitochondria regulate the Ca(2+)-exocytosis relationship of bovine adrenal chromaffin cells, J. Neurosci. 19 (1999) 9261–9270.
[55] E.A. Jonas, J. Buchanan, L.K. Kaczmarek, Prolonged activation of mitochondrial conductances during synaptic transmission, Science 286 (1999) 1347–1350.
[56] S.A. Javadov, S. Clarke, M. Das, E.J. Griffiths, K.H. Lim, A.P. Halestrap, Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart, J. Physiol. 549 (2003) 513–524.
[57] E.J. Griffiths, A.P. Halestrap, Protection by cyclosporin a of ischemia/reperfusion-induced damage in isolated rat hearts, J. Mol. Cell. Cardiol. 25 (1993) 1461–1469.
[58] K.S. Suh, S.H. Yuspa, Intracellular chloride channels: critical mediators of cell viability and potential targets for cancer therapy, Curr. Pharm. Des. 11 (2005) 2753–2764.
[59] T. Klitsch, D. Siemen, 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.
[60] J. Borecky, P. Jezek, D. Siemen, 108-pS channel in brown fat mitochondria might be identical to the inner membrane anion channel, J. Biol. Chem. 272 (1997) 19282–19289.
[61] K.A. Hayman, T.D. Spurway, R.H. Ashley, Single anion channels reconstituted from cardiac mitoplasts, J. Membr. Biol. 136 (1993) 181–190.
[62] Y.N. Antonenko, D. Smith, K.W. Kinally, H. Tedeschi, Single-channel activity induced in mitoplasts by alkaline pH, Biochim. Biophys. Acta 1194 (1994) 247–254.
[63] C. Ballarin, M.C. Sorgato, An electrophysiological study of yeast mitochondria. Evidence for two inner membrane anion channels sensitive to ATP, J. Biol. Chem. 270 (1995) 19262–19268.
[64] M.C. Sorgato, O. Moran, V. DePinto, B.U. Keller, W. Stühmer, Further investigation on the high-conductance ion channel of the inner membrane of mitochondria, J. Bioenerg. Biomembr. 21 (1989) 485–496.
[65] K.D. Garlid, A.D. Beavis, Evidence for the existence of an inner membrane anion channel in mitochondria, Biochim. Biophys. Acta 853 (1986) 187–204.
[66] A.D. Beavis, K.D. Garlid, The mitochondrial inner membrane anion channel: regulation by divalent cations and protons, J. Biol. Chem. 262 (1987) 15085–15093.
[67] S.C. Halle-Smith, A.G. Murray, M.J. Selwyn, Palmitoyl-CoA inhibits the mitochondrial inner membrane anion-conducting channel, FEBS Lett. 236 (1988) 155–158.
[68] A.D. Beavis, Properties of the inner membrane anion channel in intact mitochondria, J. Bioenerg. Biomembr. 24 (1992) 77–90.
[69] K. Herick, R. Kramer, H. Luhring, Patch clamp investigation into the phosphate carrier from *Saccharomyces cerevisiae* mitochondria, Biochim. Biophys. Acta 1321 (1997) 207–220.
[70] S.G. Huang, M. Klingenberger, Chloride channel properties of the uncoupling protein from brown adipose tissue mitochondria: a patch-clamp study, Biochemistry 35 (1996) 16806–16814.
[71] P.H. Schlesinger, A. Gross, X.-M. Yin, K. Yamamoto, M. Saito, G. Waksman, S.J. Korsmeyer, Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11357–11362.
[72] K.S. Suh, M. Mutoh, M. Gerdes, S.H. Yuspa, CLIC4, an intracellular chloride channel protein, is a novel molecular target for cancer therapy, J. Investig. Dermatol. Symp. Proc. 2 (2005) 105–109.

[73] K. Kunzelmann, Ion channels and cancer, J. Membr. Biol. 205 (2005) 159–173.
[74] X. Wang, N. Takahashi, H. Uramoto, Y. Okada, Chloride channel inhibition prevents ROS-dependent apoptosis induced by ischemia–reperfusion in mouse cardiomyocytes, Cell. Physiol. Biochem. 16 (2005) 147–154.
[75] M. Batthish, R.J. Diaz, H.P. Zeng, P.H. Backx, G.J. Wilson, Pharmacological preconditioning in rabbit myocardium is blocked by chloride channel inhibition, Cardiovasc. Res. 55 (2002) 660–671.
[76] H. Chen, L.L. Liu, L.L. Ye, C. McGuckin, S. Tamowski, P. Scowen, H. Tian, K. Murray, W.J. Hatton, D. Duan, Targeted inaction of cystic fibrosis transmembrane conductance regulator chloride channel gene prevents ischemic preconditioning in isolated mouse heart, Circulation 110 (2004) 700–704.
[77] T.J. Jentsch, M. Poët, J.C. Fuhrmann, A.A. Zdebik, Physiological functions of CLC Cl-channels gleaned from human genetic disease and mouse models, Annu. Rev. Physiol. 67 (2005) 779–807.
[78] H. Azoulay-Zohar, A. Israelson, S. Abu-Hamad, V. Shoshan-Barmatz, In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death, Biochem. J. 377 (2004) 347–355.
[79] E. Fernández-Salas, K.S. Suh, V.V. Speransky, W.L. Bowers, J.M. Levy, T. Adams, K.R. Pathak, L.E. Edwards, D.D. Hayes, C. Cheng, A.C. Steven, W.C. Weinberg, S.H. Yuspa, mtCLIC/CLIC4, an organellular chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53, Mol. Cell. Biol. 22 (2002) 3610–3620.
[80] K.S. Suh, M. Mutoh, K. Nagashima, E. Fernandez-Salas, L.E. Edwards, D.D. Hayes, J.M. Crutchley, K.G. Marin, R.A. Dumont, J.M. Levy, C. Cheng, S. Garfield, S.H. Yuspa, The organellular chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis, J. Biol. Chem. 279 (2004) 4632–4641.
[81] D. Han, F. Antunes, R. Canali, D. Rettori, E. Cadenas, Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol, J. Biol. Chem. 278 (2003) 5557–5563.
[82] K.S. Suh, M. Mutoh, M. Gerdes, S.H. Yuspa, CLIC4, an intracellular chloride channel protein, is a novel molecular target for cancer therapy, J. Investig. Dermatol. Symp. Proc. 2 (2005) 105–109.
[83] E.L. Holmuhamedov, L. Wang, A. Terzic, ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria, J. Physiol. 519 (1999) 347–360.
[84] G.P. Brierley, M.H. Davis, D.W. Jung, Intravesicular pH changes in submitochondrial particles induced by monovalent cations: relationship to the Na^+/H^+ and K^+/H^+ antiporters, Arch. Biochem. Biophys. 264 (1988) 417–427.
[85] P. Paucek, V. Yarov-Yarovoy, X. Sun, K.D. Garlid, Inhibition of the mitochondrial KATP channel by long-chain acyl-CoA esters and activation by guanine nucleotides, J. Biol. Chem. 271 (1996) 32084–32088.
[86] H.H. Taussky, E. Shorr, A microcolorimetric method for the determination of inorganic phosphorus, J. Biol. Chem. 202 (1953) 675–685.
[87] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[88] M.F. Powers, A.D. Beavis, Triorganotins inhibit the mitochondrial inner membrane anion channel, J. Biol. Chem. 266 (1991) 1725–17256.
[89] M. Fedelesova, A. Ziegelhöffer, O. Luknarova, A. Dzurba, S. Kostolansky, Influence of K^+,Mg^{++}-(D,L)-aspartate on various ATPase activities of the dog heart, Arzneim. Forschg. Drug Res. 23 (1973) 1048–1053.
[90] H. Lecoeur, A. Langonne, L. Baux, D. Rebouillat, P. Rustin, M.-Ch. Prevost, C. Brenner, L. Edelman, E. Jacotot, Real-time flow cytometry analysis of permeability transition in isolated mitochondria, Exp. Cell Res. 294 (2004) 106–117.
[91] M. Colombini, Voltage gating in the mitochondrial channel, VDAC, J. Membr. Biol. 111 (1989) 103–111.
[92] Y.N. Antonenko, K.W. Kinnally, S. Perini, H. Tedeschi, Selective effect of inhibitors on inner mitochondrial membrane channels, FEBS Lett. 285 (1991) 89–93.
[93] I. Proutski, N. Karoulias, R.H. Ashley, Overexpressed chloride intracellular channel protein CLIC4 (p64H1) is an essential component of novel plasma membrane anion channels, Biochem. Biophys. Res. Commun. 297 (2002) 317–322.
[94] R.R. Duncan, P.K. Westwood, A. Boyd, R.H. Ashley, Rat Brain p64H1, expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum, J. Biol. Chem. 272 (1997) 23880–23886.
[95] A.D. Beavis, M. Powers, Temperature dependence of the mitochondrial inner membrane anion channel. The relationship between temperature and inhibition by magnesium, J. Biol. Chem. 279 (2004) 4045–4050.
[96] F.G. Akar, M.A. Aon, G.F. Tomaselli, B. O'Rourke, The mitochondrial origin of postischemic arrhythmias, J. Clin. Invest. 115 (2005) 3527–3535.
[97] S.O. Marx, K. Ondrias, A.R. Marks, Coupled gating between individual skeletal muscle Ca^{2+} release channels, Science 281 (1998) 818–821.
[98] S.O. Marx, J. Gaburjakova, M. Gaburjakova, C. Henrikson, K. Ondrias, A.R. Marks, Coupled gating between cardiac calcium release channels (ryanodine receptors), Circ. Res. 88 (2001) 1151–1158.
[99] P. Balgavy, F. Devinsky, Cut-off effects in biological activities of surfactants, Adv. Colloid Interface Sci. 66 (1996) 23–63.
[100] P. Schönfeld, I. Sayeed, R. Bohnensack, D. Siemen, Fatty acids induce chloride permeation in rat liver mitochondria by activation of the inner membrane, anion channel (IMAC), J. Bioen. Biomem. 36 (2004) 241–248.
[101] M. Klingenberg, K. Grebe, B. Scherer, The binding of atractylate and carboxy-atractylate to mitochondria, Eur. J. Biochem. 52 (1975) 351–363.
[102] M. Klingenberg, M. Appel, W. Babel, H. Aquila, The binding of bongkrekate to mitochondria, Eur. J. Biochem. 131 (1983) 647–654.
[103] J.S. Armstrong, M. Whiteman, H. Yang, D.P. Jones, P. Sternberg, Cysteine starvation activates the redox-dependent mitochondrial permeability transition in retinal pigment epithelial cells, Invest. Ophthalmol. Visual Sci. 45 (2004) 4183–4189.