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

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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 phosphatidylinerse 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 Ca2+ 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 Goq-induced cytochrome c release and loss of mitochondrial membrane potential, and inhibited cardiomyocyte apoptosis. In contrast to BKA, CsA showed only...
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 pyrane fluorescence quenching in submitochondrial particles (SMP) [31] and thyroid hormone-induced efflux of Mg2+ 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 KCa 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 KATP channel openers [33], prevented a loss of mitochondrial inner membrane potential (ΔΨm) induced by H2O2 in cerebellar granule neurons [38], inhibited mitochondrial Ca2+-efflux, Mg2+, 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 Ca2+ 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 (UCP2) [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, KH2PO4 5, EGTA 1, and the atria were excised. The ventricles were immersed in ice-cold isolation buffer containing (in mM): sucrose 50, mannitol 200, KH2PO4 5, EGTA 1, 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).
2.3. Purity of isolated mitochondrial inner membranes

The purity of isolated mitochondrial inner membranes (submitochondrial particles, SMP) was tested by estimation of ATPase activities characteristic for the content of sarcolemma (Na⁺/K⁺-ATPase) and sarcoplasmic reticulum (Mg²⁺/Ca²⁺-ATPase) in the absence and/or presence of their specific inhibitors. Briefly, the basic incubation medium for measurement of the mitochondrial Mg²⁺-ATPase activity contained 0.1 ml 40 mM MgCl₂, 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 Mg²⁺-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 (Mg²⁺-ATPase + Na⁺/ K⁺-ATPase) and the mitochondrial Mg²⁺-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 Mg²⁺/Ca²⁺-ATPase, the basic medium for mitochondrial ATPase was supplemented with 0.1 ml 0.1 mM CaCl₂. 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 0.85 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 Mg²⁺-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 (Mg²⁺-ATPase + Na⁺/ K⁺-ATPase) and the mitochondrial Mg²⁺-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.

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, Abersham 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, Abersham Biosciences, dilution 1:5000) were visualized by the ECL plus Western blotting detection system (ECL plus; Abersham Biosciences). Individual bands were evaluated using the PCBase 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-glycerol-phosphatidylcholine, dioleoyl-glycerol-phosphatidylserine, and dioleoyl-glycerol-phosphoethanolamine at a molar ratio of 3:2:1 in n-decane (20 mg/ml) or dioleoyl-glycerol-phosphatidylcholine and dioleoyl-glycerol-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 MgCl₂, 0.4 CaCl₂, 1 EGTA, 10/5 HEPES/Tris, pH 7.4 (mimicking the intracellular side), and for the cis chamber: 250 KCl, 2 MgCl₂, 0.1 CaCl₂, 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_m 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_m in practice is less than the theoretical. Therefore, in our study we controlled the purity of the Cl⁻ current by applying a voltage of ±50 mV. To study 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 τ₁ and τ₂.

3. Results

3.1. Purity of isolated SMP

Activity of the mitochondrial Mg²⁺-ATPase in the tested preparation was 37.66 ± 1.09 μmol Pi ⋅ mg prot⁻¹ ⋅ h⁻¹. Activity of the Na⁺/K⁺-ATPase was estimated as an additional increase to the mitochondrial Mg²⁺-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 Mg²⁺-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 Mg²⁺/Ca²⁺-ATPase, indicating the presence of sarcoplasmic reticulum membranes in isolated SMP preparation, was 0.72 μmol Pi ⋅ mg prot⁻¹ ⋅ h⁻¹. This value was obtained by the difference between the activity of the mitochondrial Mg²⁺-ATPase estimated in the absence and presence of Ca²⁺ ions. The proportion of Mg²⁺/Ca²⁺-ATPase activity to that of the Mg²⁺-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...
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. $P_{\text{open}}$ 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, $E_{\text{rev}}$, 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. 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.](image1)

![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.](image2)

![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.](image3)
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 ($P_{\text{open}}$) 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 $P_{\text{open}}$ 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 $P_{\text{open}}$ at +40 mV, but BKA decreased $P_{\text{open}}$ 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 ($P_{\text{open}}$) of the single chloride channels in a dose-dependent manner ($n=8/8$) (Fig. 6).

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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 $P_{\text{open}}$ 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 $P_{\text{open}}$ of the single chloride channel at +40 (circles), 0 (triangles) and −40 mV (squares).
Similarly to BKA, the ATR inhibitory effect depended on the site of application. ATR decreased $P_{\text{open}}$ 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 decrease $P_{\text{open}}$, 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 $P_{\text{open}}$ 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 ($n=2/2$). $P_{\text{open}}$ 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 $P_{\text{open}}$; 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 $P_{\text{open}}$ ($>-0.8$; the chloride current of the open chloride channels is upwards). The nonselective channel was mostly closed ($P_{\text{open}}=0.15$; the potassium current of the open nonselective channels is downwards). ATR (40 μM) applied at the cis side slightly decreased the activity of the chloride channels, but significantly activated the nonselective channel ($P_{\text{open}}=0.42$). Further application of ATR at the trans side significantly reduced the activity of the chloride channels and further activated the nonselective channel ($P_{\text{open}}=0.77$) (Fig. 11). The activation effect of ATR on the nonselective channel was evaluated at $-20$ mV, which is approximately the $E_{\text{rev}}$ 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. 5. A—open dwell time distribution and fitted curves of the single chloride channels. Solid line—control: $\tau_1=6.0$ ms, $\tau_2=47$ ms. Dashed line—10 μM BKA (cis): $\tau_1=2.1$ ms, $\tau_2=20$ ms. Dotted line—10 μM BKA (trans): $\tau_1=4.2$ ms, $\tau_2=9.1$ ms. B—close dwell time distribution and fitted curves of the single chloride channels. Solid line—control: $\tau_1=0.7$ ms, $\tau_2=5.4$ ms. Dashed line—10 μM BKA (cis): $\tau_1=1.4$ ms, $\tau_2=11$ ms. Dotted line—10 μM BKA (trans): $\tau_1=2.4$ ms, $\tau_2=26$ ms.](image-url)
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 Mg²⁺/Ca²⁺-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).
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 \( \sim 25 \) or \( \sim 60 \) 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 CLIC4 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\(_{50}=38\) \(\mu\)M) 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
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.

Oberved 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
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, 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 ($P_{\text{open}} \sim 0.9$), 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

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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.

Fig. 10. Concentration and time-dependent inhibition by MA of $P_{\text{open}}$ 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).

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.
used to inhibit ANT. Since they bind to the ANT with very high affinity ($K_d < 1 \mu M$) [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 Goα-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 (Δψm) induced by H2O2 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.

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).
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 acid-induced, CsA-insensitive mitochondria matrix swelling [36], prevented oligomycin-induced pyranine fluorescence quenching in SMP [31], augmented IgE-mediated Ca2+-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.

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