Biochimica et Biophysica Acta 1808 (2011) 454–460

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Electro-pharmacological profile of a mitochondrial inner membrane big-potassium channel from rat brain

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ARTICLE INFO

Article history:
Received 31 July 2010
Received in revised form 25 September 2010
Accepted 12 October 2010
Available online 23 October 2010

Keywords:
Mitochondria
Potassium channels
MitoBK channel
Charybdotoxin
Iberiotoxin

ABSTRACT

Recent studies have indicated a calcium-activated large conductance potassium channel in rat brain mitochondrial inner membrane (mitoBK channel). Accordingly, we have characterized the functional and pharmacological profile of a BK channel from rat brain mitochondria in the present study. Brain mitochondrial inner membrane preparations were subjected to SDS-PAGE analysis and channel protein reconstitution into planar lipid bilayers. Western blotting and antibodies directed against various cellular proteins revealed that mitochondrial inner membrane fractions did not contain specific proteins of the other subcellular compartments except a very small fraction of endoplasmic reticulum. Channel incorporation into planar lipid bilayers revealed a voltage dependent 211 pS potassium channel with a voltage for half activation ($V_{1/2}$) of 11.4 ± 1.1 mV and an effective gating charge z_d of 4.7 ± 0.9. Gating and conducting behaviors of this channel were unaffected by the addition of 2.5 mM ATP, and 500 nM charybdotoxin (ChTx), but the channel appeared sensitive to 100 nM iberiotoxin (IbTx). Adding 10 mM TEA at positive potentials and 10 mM 4-AP at negative or positive voltages inhibited the channel activities. These results demonstrate that the mitoBK channel, present in brain mitochondrial inner membrane, displays different pharmacological properties than those classically described for plasma membrane, especially in regard to its sensitivity to iberiotoxin and charybdotoxin sensitivity.

1. Introduction

Mitochondria are involved in various processes essential for cell survival, including energy production, redox control, calcium homeostasis, and physiological cell death mechanisms. The integrity of mitochondrial membranes is essential to insure the biophysical basis of these complex phenomena [1]. Potassium channels similar to those present in the plasma membrane, including ATP-regulated potassium channels [2], large conductance Ca-regulated potassium channels [3], intermediate conductance Ca-regulated potassium channels [4], voltage-gated potassium channels [5] and twin-pore potassium channels [6] have been found in inner mitochondrial membrane. It has been proposed that mitochondrial potassium channels are involved in the volume regulation in mitochondria [7,8], cytoprotection [9], acidification [7], apoptosis [10], and control of inner mitochondrial membranes' integrity [11].

A putative mitochondrial large conductance Ca^{2+}-activated potassium channel (mitoBK channel) was first described by Siemen et al. [3] in human glioma cells LN229 using patch-clamp technique. The presence of a channel with properties similar to the plasma membrane BK channel was also observed in cardiac mitochondria [10,12] where it could play a prominent role in protecting the heart against ischemic injury [10,13]. Morphological evidence was similarly provided for the presence of a large conductance BK channel in the neuronal inner mitochondrial membrane of rat brain [14]. It was proposed that brain mitoK channels could play an important role in response to hypoxic cell injury. In this regard, Cheng et al. [15] has found that hypoxia increased the mitoBK activity of mitoplasts from rat liver and astrocytes and suggested this response could be interpreted as an anti-apoptotic activity. Notably, activation of mitoBK being localized in the inner membrane of brain mitochondria was reported to inhibit ROS production by respiratory chain complex I [16]. This effect is likely to explain the beneficial effects of BK potassium channel openers on neuronal survival. More recently, it has been observed that Ca-induced mitochondrial membrane potential

Abbreviation: EGTA, ethylene glycol-bis (2 amino-ethylether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) potassium salt; BSA, bovine serum albumin; mitoBK, mitochondrial big Ca^{2+} -activated potassium channel; mitoK$_{ATP}$, mitochondrial ATP-sensitive K^+ channel; Trizma base, Tris [hydroxymethyl] aminomethane; cox, cytochorom oxydase antibody; IbTx, iberiotoxin; ChTx, charybdotoxin; TEA, tetra-ethyl ammonium; 4-AP, 4-aminopyridine; SDS-PAGE, sodium dodecyl sulfate-polyacryl-amide gel electrophoresis; 58KGP, 58K Golgi protein

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doi:10.1016/j.bbamem.2010.10.005

depolarization and respiration can be blocked by iberiotoxin and charybdotoxin [17], while providing single channel evidence for a charybdotoxin-sensitive potassium channel. Depending on the cell type, different BK channels can be observed. This diversity is a consequence of alternative splicing and interaction with regulatory subunits which modulate biophysical and pharmacological properties of BK channels. For example, Meera et al. [18] showed that the human BK channel β-subunit (β_4), highly expressed in brain, renders the BK channel α-subunit resistant to ChTx and IbTx [18]. Notably, the presence of a BK channel β4 subunit has been documented in brain mitochondria [14,19]. Finally, TEA resistant BK channels have been identified in guinea-pig myenteric neurons [20] whereas ATP sensitive BK channels were found in renal proximal tubule cells [21].

Potassium channels of the mitochondrial inner membrane are modulated by inhibitors and activators previously described for plasma membrane potassium channels [22] but little is still known about the pharmacology and the molecular identity of brain mitoBK channel. In this study, we show that rat brain mitochondrial inner membrane contains a voltage-gated 211 pS K channel that is insensitive to ChTx and ATP while it is sensitive to IbTx and 4-AP. Besides, it is sensitive to TEA at positive voltages but not at negative voltages.

2. Materials and methods

2.1. Materials

HEPES, sodium bicarbonate, D-manitol, sucrose, digitonin, potassium chloride, Tris–HCl, BSA, nagarase, potassium chloride, EGTA, IbTx, ChTx, ATP, TEA and 4-AP were purchased from Sigma and n-Decane was obtained from Merck. Salts and all solvents were analytical grade.

2.2. Solutions

Solutions for mitochondrial isolation are as follows: MSE-solution (225 mM manitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml BSA, pH 7.4); MSE-nagarse solution (0.05% nagarse in MSE solution); MSE-digitonin solution (0.02% digitonin in MSE solution).

2.3. Mitochondria isolation

Mitochondria were isolated from the brain of male Wistar rat (weighing 180–200 g) according to the protocol described by Rosenthal et al. [23] with a small modification. All experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). In brief, two rats were anesthetized by ether and brains were rapidly removed and homogenized in 20 ml ice-cold MSE-nagarse solution at 600 units/s using potter homogenizer. After adding 40 ml of ice-cold MSE solution, the homogenate was centrifuged at 2000*g* for 4 min. Thereafter, the supernatant (step 1 for western blotting) was centrifuged at 12000*g* for 9 min at 4 ° C (Beckman model J-21B). After dissolving the pellet in 20 ml of ice-cold MSE (step 2 for western blotting) and digitonin, solution was transferred to a 30 ml glass homogenizer, and it was manually homogenized 8–10 times to obtain a homogenous suspension. Subsequently, the suspension was centrifuged at 12000*g* for 11 min, and the obtained pellet was dissolved in 300 µl of MSE solution (35 mg protein/ml) (step 3 for western blotting).

Mitochondrial inner membranes derived from mitochondria were prepared as previously described method [24]. Briefly, mitochondria were suspended in H_2O at a concentration of 5 mg/ml and were stirred for 20 min on ice. The mixture was homogenized 20 times with a glass homogenizer. Then, the suspension was centrifuged twice at 12000*g* for 5 min. Thereafter, the obtained pellet (mitoplasts) were treated with Na_2CO_3 0.1 M, pH 11.5, at a final concentration of 0.5 mg/ml for 20 min on ice. Finally, the suspension was centrifuged at 100000*g* for 30 min. Mitochondrial inner membrane vesicles were stored in 20 µl aliquots in MSE solution, pH 7.4 at −80 ° C until being used (step 4 for western blotting).

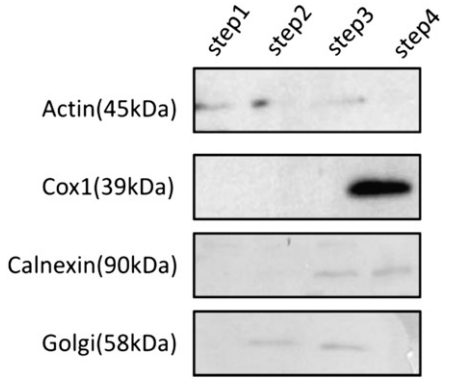

Fig. 1. Purity of cell fractions: western blotting of rat brain. Membranes were probed with organelle specific antibodies. Plasma membrane marker: Actin (C-11); 45 kDa; mitochondrial membrane marker: Cox1 (1D6), 39 kDa; Golgi marker: 58 K Golgi protein; endoplasmic reticulum marker: Calnexin, 90 kDa. Steps are demonstrated in Materials and methods section.

2.4. Immunoblot analysis

2.4.1. Protein samples

Protein concentrations of subcellular fractions were assayed using a DC Protein Assay Kit (Bio-Rad). Protein samples (30 µg) of each

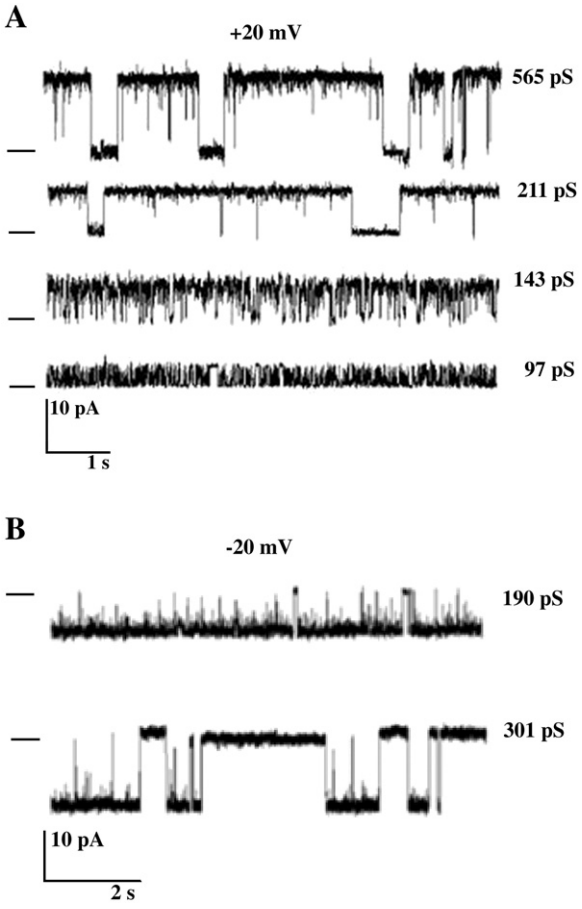

Fig. 2. Single channel recordings of ion channels of brain mitochondrial inner membranes in planar lipid bilayer. (A) Single channel recordings of four different potassium channels at +20 mV and (B) two chloride channels at −20 mV in a 200/50 mM KCl (*cis*/*trans*) gradient solution.

fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ($n=3$) blotted and probed with the following antibodies: cox1 (Santa Cruz, SC-58 347), actin (Santa Cruz, SC-1615), calnexin (Santa Cruz, SC-11 397), 58 K Golgi protein (ABCAM, AB6284). Secondary antibodies linked to horseradish peroxidase were obtained from GE-Biosciences. Blots were finally treated with ECL kit luminogen by chemiluminescence and images were then scanned and were further processed in Adobe Photoshop.

2.5. L-α-Phosphatidylcholine extraction

L-α-Phosphatidylcholine (L-α-lecithin) was extracted from fresh egg yolk according to the protocol described by Singleton and Gray [25].

2.6. Electrophysiological studies

Experiments were performed by using black (bilayer) lipid membrane technique. Bilayer lipid membranes (BLMs) were formed in a 200 μm diameter hole drilled in a Derlin cup (Warner instrument Corp., Hamden, CT USA), which separated two chambers (*cis (cytoplasmic face)/trans (luminal face)*). The chambers contained 200/50 mM KCl (*cis/trans*) solutions. The pH on both sides was adjusted to 7.4 with Tris–HEPES. Planar phospholipid bilayers were painted using a suspension of L-α-lecithin in n-decane at a concentration of 25 mg/ml. Formation and thinning of the bilayers were monitored electrically by capacitance measurements and optical observations. Typical capacitance values ranged from 200 to 300 pF. Single channel currents were measured with a BC-525D amplifier (Warner Instrument). The *cis* chamber was voltage-clamped relative to the *trans* chamber, which was grounded. Electrical connections were made by Ag/AgCl electrodes and agar salt

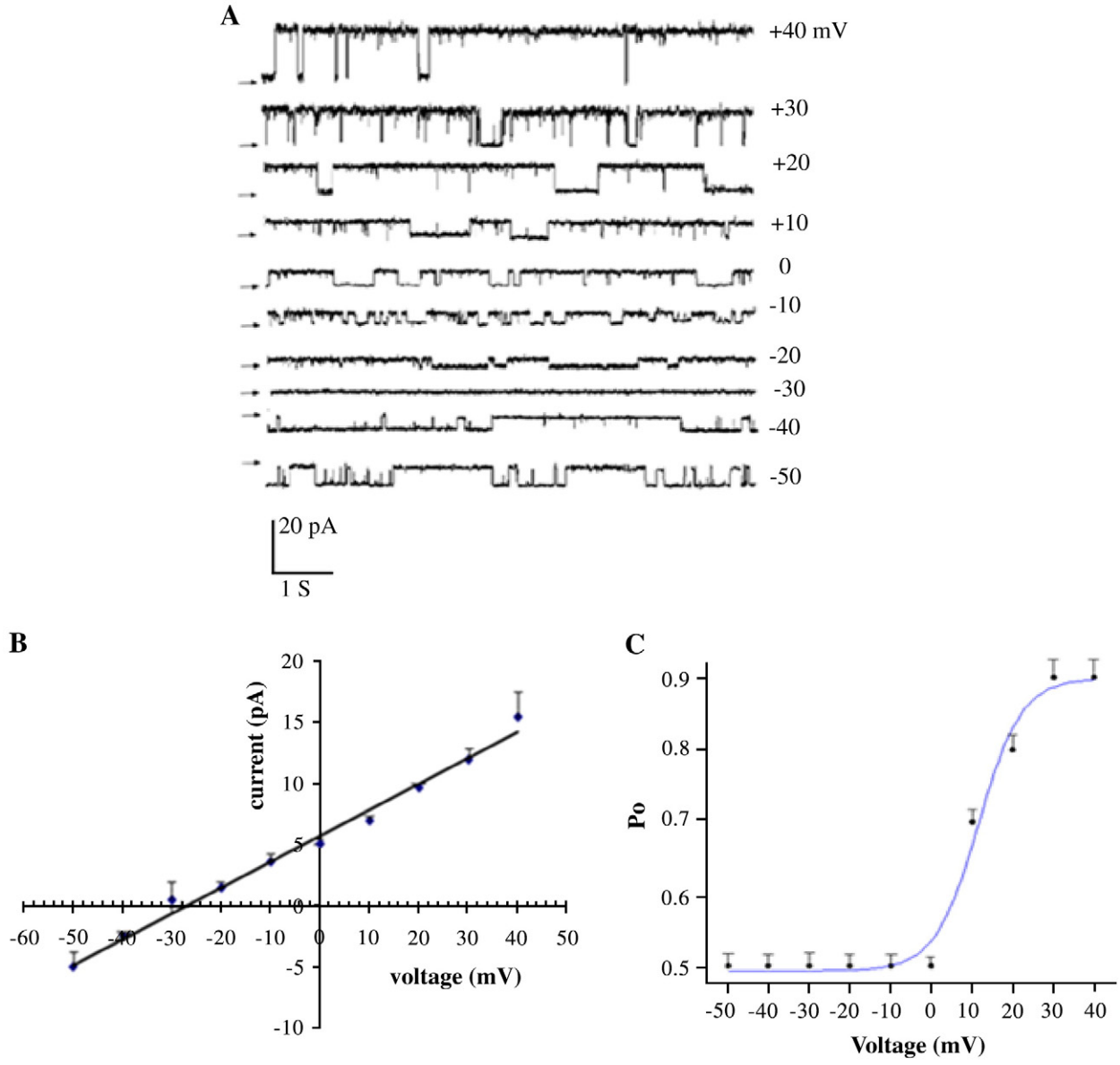

Fig. 3. Single channel recordings, current voltage relationship and open probability as a function of voltages. (A) Single channel recordings in 200/50 mM KCl (*cis/trans*) gradient after reconstitution of brain mitochondrial inner membrane vesicles in planar lipid bilayer at potentials ranging from −50 to + 40 mV. The arrow indicates the closed state. (B) Single channel current voltage relationship. Data points are mean ± SE, obtained from 5 experiments. (C) Open probability (P_o) as a function of voltage. Data fitted to a Boltzmann distribution: $P_o = V_{min} + \frac{V_{max} - V_{min}}{1 + e^{ZdF(V - V_{mid})/RT}}$; with V_{mid}: 11.4 ± 1.1 mV; $z_d = 4.7 \pm 0.9$. Each point represents the average open probability as a function of voltages in five different experiments.

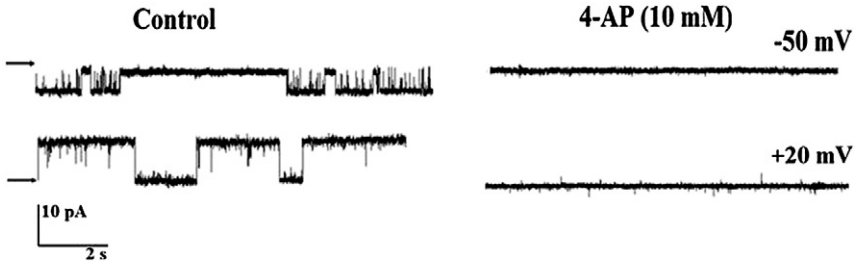

Fig. 4. The effect of 4-AP on channel gating behavior at +20 and −50 mV. Single channel recordings of under control conditions (200/50 mM KCl; *cis*/*trans*), and immediately after cis addition of 4-AP 10 mM ($n=5$). Arrows indicate the closed levels.

bridges (3 M KCl). All recordings were filtered at 1 kHz using a 4-pole Bessel filter, digitized at a sampling rate of 10 kHz and stored on a personal computer for off line analysis by Pclamp10 (Axon Instruments Inc). Unitary channel conductance was calculated from the current–voltage relationship. Open channel probability (P_o) was calculated using the standard event detection algorithms in Pclamp10. P_o was calculated from segments of continuous recordings lasting 50 s. The significance of differences was determined by Student's *t* test. Data are expressed as mean ± S.E. (standard error). The permeability ratios for K^+ and Cl^- were calculated according to the Goldman–Hodgkin–Katz voltage equation.

3. Results

3.1. Purity of mitochondrial fractions

We used western blotting and antibodies directed against various cellular proteins considered unique to particular subcellular regions (Fig. 1). Membranes were probed with antibodies to a plasma membrane marker (actin), an endoplasmic reticulum marker (calnexin, 90 kDa), a Golgi matrix marker (58KGP), and a mitochondrial membrane marker (cox). These data indicate that the mitochondrial fraction did not include specific proteins of the other subcellular compartments except a small fraction of endoplasmic reticulum.

3.2. Electrophysiological and biophysical properties of the ion channel

Fig. 2 presents an example of single channel recordings measured under control conditions (200 mM/50 mM KCl; *cis*/*trans*) following incorporation of rat brain mitochondrial inner membrane vesicles into planar bilayers. After incorporation, we usually observe six types of ion channels that had different gating, amplitude and behavior. Four different kinds of potassium channels that were observed are as follows: channels with conductance of 97 pS ($n=42$) that were only sensitive to 4-AP, channels with conductance of 143 pS ($n=64$) that were blocked by ATP, channels with conductance of 565 pS ($n=30$) that were blocked by ATP and IbTx, and channels with conductance of 211 pS ($n=32$) (Fig. 2A). In addition, we observed two types of chloride channels with conductance of 190 pS and 301 pS (Fig. 2B). The BK channel with a conductance of 211 pS has been focused in this article.

Fig. 3A shows typical recordings of brain mitochondrial inner membrane potassium channels in asymmetrical 200 mM KCl *cis*/50 mM KCl *trans* solutions at holding potentials of - 50 to + 40 mV as indicated. The voltage dependence of channel gating, with long open events at positive potentials and marked bursting behavior and long lasting silent period at negative potentials, was typical. Current–voltage (I–V) plot was linear with no evidence of inward rectification at potentials between +40 and −50 mV ($n=5$) (Fig. 3B). This channel displayed a conductance value of 211 pS and a negative reversal potential close to −30 mV, which attest its cationic selectivity under these conditions.

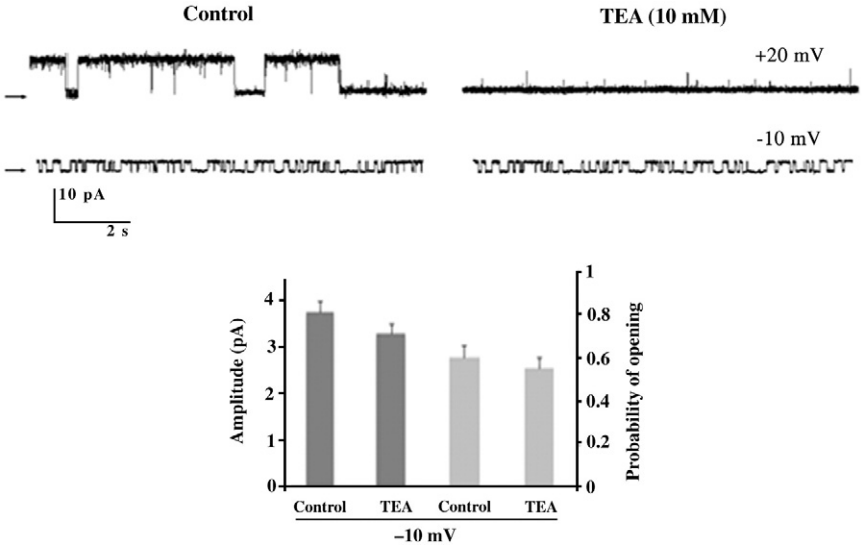

Fig. 5. The effect of TEA on channel gating behavior at −10 and +20 mV. Single channel recordings of under control conditions (200/50 mM KC; *cis*/*trans*) and immediately after *cis* addition of TEA 10 mM. The significant differences in the open probability value and amplitude are not observed at −10 mV, whereas channel activity is completely blocked at +20 mV. Data are means ± SE ($n=4$). Arrows indicate the closed levels.

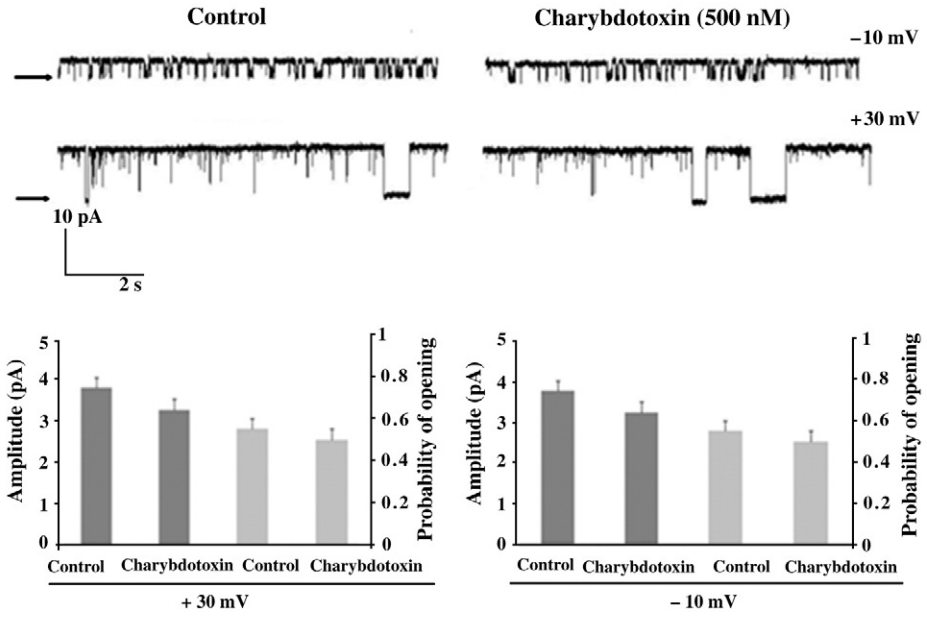

Fig. 6. The effect of charybdotoxin on channel activity at −10 and +30 mV. Representative recordings of channel currents in under control conditions and after addition of 500 nM charybdotoxin to *cis* face. Summarized data show current amplitudes and P_o of reconstituted channels in absence or presence of charybdotoxin. Significant differences in the open probability value and amplitude are not observed. Data are mean ± SE ($n = 4$). The arrows indicate the closed levels.

The open probability (P_o) was also found to be voltage-dependent. Fig. 3C indicates the average steady-state open probability values as a function of the holding potential for full open conducting state obtained from five different experiments. As seen, channel open probability increased at positive holding potentials to reach a maximum of 0.9 ± 0.01 at + 40 mV. Curve fitting the experimental data to a Boltzmann equation led to a voltage for half maximum activation of 11.4 ± 1.1 mV with an equivalent gating charge z_d of 4.7 ± 0.9.

3.3. Electro-pharmacological properties of the ion channel

3.3.1. Effect of K channel blockers

In the next step, we examined the blockade by a nonspecific K channel blocker, 4-AP, and well known BK channel blockers, TEA, ChTx, and IbTx. All the following experiments were carried out under a condition in which the *cis*/*trans* solutions contained 200 and 50 mM KCl, respectively.

Fig. 4 illustrates an example of single channel recordings at −50 and + 20 mV before and after addition of 4-AP. As seen, addition of 10 mM 4-AP to *cis* chamber completely blocked the channel activity ($n = 5$).

The effects of TEA on channel activity are summarized in Fig. 5. TEA (10 mM in *cis* face) had no significant effect on channel amplitude and open probability at −10 mV, but it completely blocked the channel activity at +20 mV ($n = 4$).

We also tested the influence of ChTx on channel activity. As seen in Fig. 6, addition of 500 nM ChTx to *cis* compartment failed to modify the channel conducting or gating behavior ($n = 4$).

Additionally, the activity of the channel was studied after application of IbTx, a specific inhibitor of the K_{Ca}-channel of BK type. Fig. 7 shows that the K current was completely inhibited by addition of IbTx (100 nM) to *cis* compartment at +20 mV and −40 mV ($n = 5$).

3.3.2. Effect of ATP on channel activity

To further characterize this channel, we tested the effect of ATP on the channel behavior. Fig. 8 presents single channel recordings in a 200/50 mM KCl (*cis*/*trans*) solution at −50 mV and +20 mV under control conditions, and after addition of 2.5 mM ATP to the *cis* face ($n = 4$). Channel activity was not affected by the application of the ATP. The amplitude of the currents and probability of opening under control conditions and after addition of ATP is shown in Fig. 8. Current amplitude and open probability were not significantly affected.

4. Discussion

We have succeeded to characterize the electro-pharmacological profile of a BK channel present in mitochondrial inner membrane of neuronal cells.

Electrophysiological techniques showed functional ion channels in mitochondrial inner membrane [2]. A putative mitochondrial large

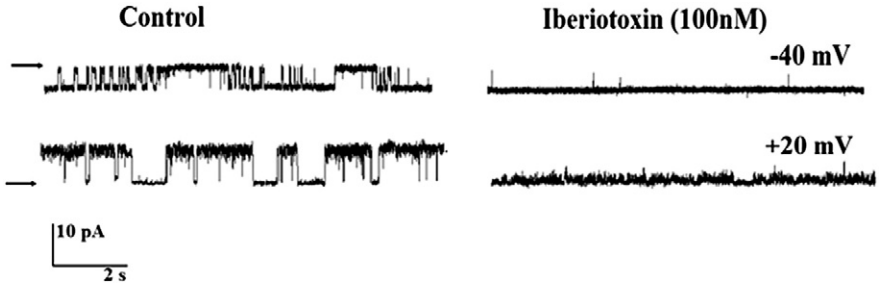

Fig. 7. The effect of iberiotoxin on the activity of the channel at +20 and −40 mV. Representative single channel recording of under control conditions (200/50 mM KCl; *cis*/*trans*) and after addition of 100 nM iberiotoxin to *cis* compartment. The arrows indicate the closed levels.

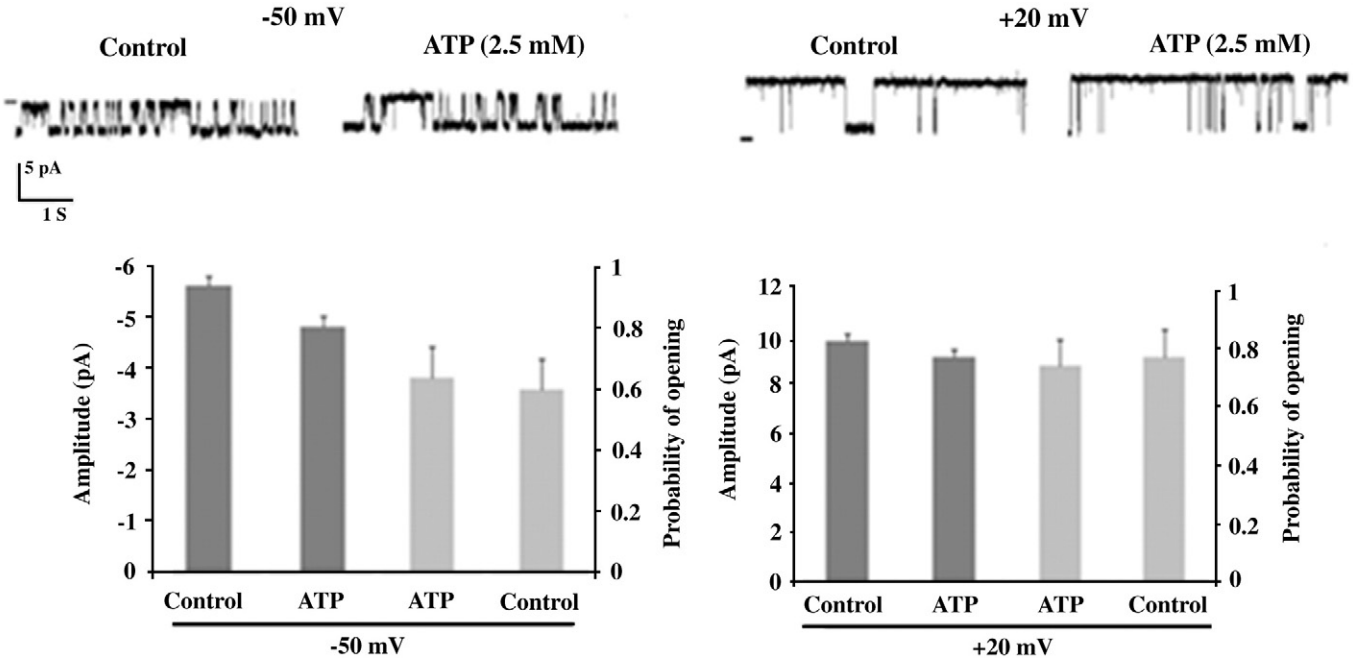

Fig. 8. The effect of ATP on channel activity at −50 and +20 mV. Representative recordings of channel currents in under control conditions and after addition of 2.5 mM ATP to *cis* face. Summarized data show current amplitudes and P_o of reconstituted channels in absence or presence of ATP. No significant difference was observed in the open probability value and amplitude. Data are mean ± SE ($n = 4$). Closed levels are indicated by "—".

conductance Ca-activated potassium channel (mitoBK channel) has been described in human glioma cells LN229 using patch-clamp technique [3]. This channel with a conductance of 295 pS was stimulated by Ca^{2+} and was blocked by charybdotoxin. Later, the presence of a channel with properties similar to the plasma membrane BK channel (stimulated by the potassium channel opener NS1619 and blocked by charybdotoxin, iberiotoxin, and paxilline) was observed in cardiac mitochondria [10,12]. It has been suggested that mitoK channel activation confers cardioprotection in manner similar to but independent of mitoK$_{ATP}$ channel activation [10]. Our results showed that the channel I/V curve was linear within the range of −50 to +40 mV with a slope conductance of 211 pS. BK channels with similar features were reported for channels identified in brain mitochondrial inner membrane by patch clamp [15] and by channel incorporation techniques in lipid bilayer membrane [17,26,27]. Supportive evidence for the potential presence of a BK channel in the inner membrane of brain mitochondria has been also obtained from the use of immunocytochemistry and immune-gold electron microscopy [14,19]. We observed the channel open probability increased at positive holding potentials to reach a maximum of 0.9 ± 0.01 at +40 mV with an equivalent gating charge z_d of 4.7 ± 0.9. These results suggest that channel activity can be modulated by putative changes in mitochondrial membrane potential occurring during Ca^{2+} fluxes. Kulawiak et al. [26] observed that brain mitochondrial membrane potassium channel activity decreased at negative voltages, similar to what was reported on the mitoBK channel in glioma [3]. In our experiments, channel did not totally close at negative voltages. Such behavior is at variance with the result obtained by Skalska et al. [17]. Several factors including the possibility of regulatory subunits being lost when working on isolated channel incorporated into a bilayer, and the fact that our experiments were performed in the absence of EGTA and at contaminant Ca^{+2}, can be responsible for this discrepancy. To resolve this issue, further studies on single channel behavior and structure–function relationship will be needed.

Another aspect of the present work concerns the pharmacological profile of the brain inner mitoBK channels, compared with other members of the plasma membrane big potassium channel family. Fig. 4 demonstrates that addition of 10 mM of 4-AP to the cytoplasmic side (*cis* chamber) completely blocked channel activity while lower concentration of 4-AP (5 mM) had no detectable effect. The blocking effect of 4-AP was not voltage-dependent. The channel isolated from liver mitochondrial inner membrane could be blocked by 4-AP (5 mM) [2]. Several pharmacological blockers of BK channels are known. These include TEA [28] and both scorpion-derived peptide charybdotoxin and iberiotoxin [29]. We showed that TEA inhibited the channel gating in a voltage-dependent manner. IbTx is considered to be a specific BK channel blocker, whereas ChTx blocks other potassium channels as well as BK channels [30]. Because of its specificity, IbTx is used as a pharmacological agent to dissect the role of BK channels in physiological processes, and generally it is used at a concentration of 100 nM. We showed that addition of 100 nM of IbTx to the *cis* side of the reconstituted K channel derived from brain mitochondrial membrane inhibited the channel activity at positive or negative potentials. In contrast, unitary current amplitude, and gating behavior of the channel were not affected by the addition to the *cis* chamber of ChTx (500 nM). Hence, our results would support the presence of a BK channel in the brain mitochondrial inner membrane with a conductance similar to the channel reported from mitochondria of human glioma cell line [3], brain [17] astrocytes [15], and ventricular tissues [10,12]. However, our findings of the effect of ChTx which are shown in Fig 6 differ from the observations reported for the mitoBK channel of the brain [17] and glioma cell line [3]. Tissue-specific modulatory β subunits may account for much behavior [31]. β subunits increase the apparent Ca^{+2}/voltage sensitivity of BK α subunit [32], modify channel kinetics [33] and alter its pharmacological properties [32]. For example, in contrast to smooth and skeletal muscles, where BK channels are normally blocked by 100 nM IbTx or ChTx, both sensitive- and insensitive-ChTx [30] as well as IbTx sensitive [34] BK channels have been observed in neurons. The β4-subunit is expressed mainly in brain and decreases the ChTx binding strength [35]. Meera et al. [18] reported that co-expression of a neuronal BK channel β subunit (β4) leads to a BK channel phenotype that displays a low apparent IbTx and ChTx sensitivity due to dramatically decreased toxin association rates. There is also evidence showing that the β4 glycosylation affects the β4 ability to modulate BK channels by protecting them against instantaneous toxin block [36]. The study of Piwonska et al. [14] showed presence of β4 subunit in brain mitochondrial inner membrane,

suggesting that the β4 subunit is a regulatory component of neurons' mitochondrial BK channels. Our results support the presence of a ChTx-insensitive–IbTx-sensitive brain mitoBK channel in rat brain, enabling a qualitative comparison of the effect of β4 subunit on defining the ChTx and IbTx sensitivity. However, this result contradicts the observation of inhibition of brain mitoBK channel activity by ChTx [17]. This discrepancy may be explained by the possible presence of various BK channels. In this regard, we announce the presence of an ATP- and IbTx-sensitive 565 pS BK channel in brain mitochondrial membrane (Fig 2 A) (article is being prepared for submission).

It was reported that the activity of several BK channels was altered by intracellular ATP [37]. Regarding the ATP effect, two mechanisms have been demonstrated. One mechanism is that channel activity changes, induced by ATP, are elicited by phosphorylation processes [38]. The other mechanism is that ATP directly affects channel activity without protein phosphorylation [37,39]. Furthermore, activity of BK channel of renal proximal tubule is inhibited by cytoplasmic ATP [21]. Our experiments showed that ATP does not affect brain mitoBK channel activity. The function of mitochondrial K_{Ca} channel is unknown. There is growing evidence that mitochondrial channels play an important role in buffering intracellular Ca^{2+} and cytoprotection of brain, liver, skeletal muscle, and heart tissue [2,40].

Another observation provided by this study is that western blotting of discrete subcellular fractions demonstrated that cytochrome c oxidase or complex IV-subunit 1 was only expressed in the mitochondrial inner fraction whereas actin, calnexin, and 58 kDa Golgi protein, were not, demonstrating the purity of the mitochondrial fraction. Cytochrome oxidase is a marker of mitochondria but cox1 was detected only in the mitochondrial fraction in step 4 (mitoplasts formation). This raises the possibility that cox1 binding site(s) may be located in matrix face or cox1 binds to a special cytochrome oxidase conformation.

We conclude that brain mitoBK channels are voltage dependent, sensitive to 4-AP, TEA, IbTx, and insensitive to ChTx and ATP. In the context of recent studies providing evidence for the presence of brain mitoBK channel β4 subunit, we propose that β4 subunit acts as a regulatory component that alters the pharmacological behavior of brain miroBK channels.

Acknowledgments

This work was supported by the Neuroscience Research Center of Shahid Beheshti University of Medical Sciences. We thank Dr B. Vaziri for western blotting technical support.

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