RESEARCH ARTICLE

A new brain mitochondrial sodium-sensitive potassium channel: effect of sodium ions on respiratory chain activity

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ABSTRACT

We have determined the electropharmacological properties of a new potassium channel from brain mitochondrial membrane using a planar lipid bilayer method. Our results show the presence of a channel with a conductance of 150 pS at potentials between 0 and −60 mV in 200 mM cis/50 mM trans KCl solutions. The channel was voltage independent, with an open probability value of approximately 0.6 at different voltages. ATP did not affect current amplitude or open probability at positive and negative voltages. Notably, adding iberiotoxin, charybdotoxin, lidocaine or margatoxin had no effect on the channel behavior. Similarly, no changes were observed by decreasing the pH to 6. Interestingly, the channel was inhibited by adding sodium in a dose-dependent manner. Our results also indicated a significant increase in mitochondrial potassium transport by sodium ions on potassium channel opening could be important for cell protection and ATP synthesis.

KEY WORDS: Mitochondria, Potassium channels, Single channel, Intracellular ion channel, Mitochondrial respiratory chain, Brain

INTRODUCTION

AP-regulated potassium channels (Inoue et al., 1991), large conductance Ca2+-regulated potassium channels (Siemen et al., 1999), intermediate conductance Ca2+-regulated potassium channels (De Marchi et al., 2009), voltage-gated potassium channels (Szbó et al., 2005) and twin-pore potassium channels (Ruszňák et al., 2008) have been found in the inner mitochondrial membrane. Furthermore, our previous work has demonstrated the presence of a 211 pS charybdotoxin (ChTx)-insensitive, ATP-insensitive and iberiotoxin (IbTx)-sensitive mitochondrial big Ca2+-activated potassium (mitoBKCa) channel and a 565 pS ChTx-, ATP- and IbTx-sensitive mitoBKCa channel in rat brain (Fahanik-babaei et al., 2011a,b).

The primary function of mitochondrial potassium channels is to permit K+ transport into the mitochondrial matrix (Garlid and Paucek, 2003). This transport can be observed as a macroscopic ion flux (Bednarczyk et al., 2004; Garlid et al., 1996; Paucek et al., 1992) or at the single channel level with the use of patch-clamp (De Marchi et al., 2009; Inoue et al., 1991; Siemen et al., 1999; Szabó et al., 2005) and planar lipid bilayer techniques (Bednarczyk et al., 2005; Bednarczyk et al., 2004; Choma et al., 2009; Nakae et al., 2003; Skalska et al., 2008). The influx of K+ could be involved in mitochondrial volume homeostasis, cytoprotection (Szewczyk et al., 2009) and integrity of mitochondrial inner membrane (Zoratti et al., 2009), while contributing to changes in mitochondrial membrane potential or pH gradient and respiration (Czyz et al., 1995; Debksa et al., 2002; Debksa et al., 2001; Szewczyk, 1998; Szewczyk et al., 1995).

The pore-forming α-subunits of the potassium channel can be divided into voltage-dependent (Kv) subunits, inward rectifier (Kir) subunits, two pore (K2P) subunits, those activated by intracellular calcium (Ca2+ subunits) and those activated by intracellular sodium (KNa subunits).

Sodium-activated potassium channels (KNa channel) were described for the first time in guinea pig cardiomyocytes (Kameyama et al., 1984). Subsequently, KNa channels have been observed in mammalian neurons (Bader et al., 1985; Bischoff et al., 1998; Dale, 1993; Dryer, 1994; Dryer et al., 1989; Egan et al., 1992b; Haimann et al., 1990, 1992; Hartung, 1985; Safronov and Vogel, 1996; Saito and Wu, 1991; Schwindt et al., 1989). Two genes, Slack (also known as KCNT1 or Slo2.2) and Slick (also known as KCNT2 or Slo2.1), encode KNa channels (Bhattacharjee and Kaczmarek, 2005; Joiner et al., 1998; Yuan et al., 2003). It has been proposed that KNa currents could play an important protective role under ischemic conditions in cardiomyocytes (Kameyama et al., 1984; Luk and Carmeliet, 1990; Mitani and Shattock, 1992). In addition, Wojtovich et al. (2011) suggested that Slou2 protects the cell from hypoxic injury by increasing the potassium ion permeability of the mitochondrial inner membrane (Wojtovich et al., 2011). In contrast to KNa channels, in which Na+ is essential for channel activation, potassium channels such as MaxiK and KcsA are inhibited by Na+ outward flux from the intracellular side (Thompson et al., 2009; Yellen, 1984). This fast blocking action was found to impact the channel conduction properties and impair K+ ion flux.

Here, we present for the first time results from reconstitution of brain mitochondrial membrane into a black lipid membrane, and describe the presence of a sodium-sensitive K+ channel. This channel is different from the mitoKATP (data not shown) and mitoBKCa channels that we recently reported under the same conditions regarding single channel conductance, open probability (Po) and pharmacological properties (Fahanik-babaei et al., 2011a,b). The channel decreases mitochondrial complex I activity and reactive oxygen species (ROS) production and increases complex IV activity and the mitochondrial...
membrane potential. We propose that inhibition of mitochondrial K\(^+\) transport by the sodium-sensitive K\(^+\) channel might be important for energy production.

**RESULTS**

**Purity of mitochondrial fractions and channel characterization**

We used western blot analysis to determine the purity of preparations (Fig. 1A). Membranes were probed with antibodies to a plasma membrane marker (actin), an endoplasmic reticulum marker (calnexin, 90 kDa), a 58 kDa Golgi matrix marker (58KGP) and a mitochondrial membrane marker (cytochrome c oxidase subunit 1; Cox1). The data indicate that the mitochondrial fraction did not contain other subcellular membranes. Fig. 1B presents an example of single channel recordings measured under control conditions (200 mM/50 mM KCl, \textit{cis}/\textit{trans}) following incorporation of vesicles of brain mitochondrial membrane. After incorporation, we usually observed four different kinds of potassium channels (∼300 records) as follows: channels with conductance of 143 pS (∼34% of recordings) that were blocked by ATP (mitoK\(_{\text{ATP}}\) channel), channels with conductance of 565 pS (22% of recordings) that were blocked by ATP and IbTx (ATP-sensitive mitoBK\(_{\text{Ca}}\) channel) (Fahanik-babaei et al., 2011b), channels with conductance of 211 pS (22% of recordings) that were blocked by IbTx (ATP-insensitive mitoBK\(_{\text{Ca}}\) channel) (Fahanik-babaei et al., 2011a) and a channel with conductance of 150 pS (22% of recordings). In addition, we observed 158 and 301 pS chloride channels (Fahanik-babaei et al., 2017). This paper focuses on the 150 pS cation channel.

**Electrophysiological and biophysical properties of the ion channel**

Typical single potassium channel recordings of brain mitochondrial membrane potassium channels under control conditions at different holding potentials are shown in Fig. 2A. The resulting channel current–voltage (\(I–V\)) curves for different \(\text{trans} K^+\) concentrations are given in Fig. 2B. Under symmetrical 200 mM KCl conditions (open symbols), the \(I–V\) curve showed a slope conductance of 190 pS (\(n=3\)) at potentials between +50 and −40 mV (Fig. 2B).

Furthermore, Fig. 2B shows that a decrease in the \(\text{trans} K^+\) concentration caused a shift of the reversal potential toward more negative values. For instance, in 200 mM KCl \(\text{cis}/50\ \text{mM KCl} \text{trans}\) conditions (closed symbols), the \(I–V\) plot is shown to be inwardly rectifying for the membrane channels, with a slope conductance of 150±5 pS (\(n=6\)) at potentials between 0 and −60 mV and a negative reversal potential close to −30 mV, in accordance with a cationic selective channel. The \(P_o\) was found to be voltage independent at −50 to +50 mV. These results are summarized in Table 1, which presents the average \(P_o\) values as a function of voltage (\(n=6\)).

**Electropharmacological properties of the ion channel**

In the next step, we examined the effects of two well-known BK\(_{\text{Ca}}\) channel blocking agents, ChTx and IbTx, and the K\(_{\text{ATP}}\) channel blockers ATP and glibenclamide. All experiments were carried out under conditions in which the \(\text{cis}\) and \(\text{trans}\) solutions contained 200 and 50 mM KCl, respectively. As shown in Fig. 3A,B, \(\text{cis}\) addition of 100 nM IbTx (\(n=4\)), a specific inhibitor of mitoBK\(_{\text{Ca}}\) channels, or 500 nM ChTx (\(n=4\)) failed to affect the channel \(P_o\) and amplitude at +30 and −50 mV. After adding 1 mM EGTA (\(n=4\)), we did not...
observe any changes in channel behavior (Fig. 3C). Summarized data are shown as bar graphs in Fig. 3. Together, these observations provide evidence that the 150 pS K+ channel in brain mitochondrial membrane is not a Ca2+-activated potassium channel.

In additional experiments, we examined the effect of ATP and glibenclamide, well-known KATP channel blockers, on mitochondrial channel activity. Fig. 4A,B shows that the addition of 2.5 mM ATP (n=4) and 100 nM glibenclamide (n=3) on the cis side did not affect current amplitude or channel P_o at negative and positive voltages.

Kajma and Szewczyk (2012) characterized a new mitochondrial pH-sensitive ion channel in embryonic rat hippocampus (Kajma and Szewczyk, 2012). Therefore, we studied the effect of acidic pH on channel behavior. Fig. 4C presents typical recordings at +40 mV and −50 mV in control solution (pH 7.2) and after changing the cis solution to pH 6. As shown in Fig. 4C, acidic pH had no effect on channel amplitude and activity (n=3). Changing the trans solution to pH 6 also had no effect on channel behavior (n=1) (data not shown).

The mitoTASK-3 channel is a lidocaine-sensitive channel. Therefore, we considered the effect of lidocaine on channel activity to determine whether the potassium channel is a mitoTASK-3 channel. Addition of 100 µM lidocaine (n=3) to the cis side did not affect channel amplitude or P_o (Fig. 5A).

We also tested the effect of margatoxin (MgTx), because MgTx-sensitive Kv1.3 channels were characterized in T lymphocyte mitochondria (Stowe et al., 2013). As shown in Fig. 5B, MgTx did not change the channel activities (n=3).

**Effect of sodium ions on channel activity**

Sodium ions have been documented to affect the conduction properties of several K+ channels, including BKCa and KCsA. Fig. 6A shows that the addition of 15 mM Na+ on the cis side had no significant effect on the channel behavior at +30 mV (n=3), whereas Na+ ions at 35 (n=4) and 75 mM (n=4) caused significant decrease in the current amplitude. Summarized results are given in the bar graph of Fig. 6A.

Fig. 6B illustrates the effect of 75 mM trans Na+ ions on channel fluctuations at +50 to −50 mV. Current–voltage curves measured in 200 mM cis/50 mM KCl (closed symbols) and in 200 mM cis/50 mM KCl trans plus 75 mM Na+ trans (open symbols) are presented in Fig. 6C. The curves in Fig. 6C demonstrate that sodium affects the current amplitude at potentials more positive than −10 mV, without apparent effects on the zero current potential value. These observations led to the conclusion that part of the action of sodium is compatible with the effect of a fast blocking agent. The effect of Na+ ions on the channel P_o as a function of voltage is illustrated in Fig. 6D and Table 1. Experiments were performed in which the cis solution remained at 200 mM KCl while trans solutions contained 50 mM KCl plus 75 mM Na+ ions. The results in Fig. 6D show that 75 mM Na+ decreases the current amplitude as a result of a fast flickering block and increases the channel P_o from 0.64 to 0.8 at +40 mV. The results show that the mean value of P_o slightly increased in the presence of Na+ at potentials greater than +20 mV (Table 1), indicating that, in addition to an effect on the channel unitary current amplitude, Na+ appears to modify the channel gating process, with trans Na+ ions inducing enhanced channel activity. This effect cannot be related to a change in the ionic strength of the trans solution because the results in

Table 1. Channel open probability at different membrane voltages

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>Control</th>
<th>NaCl 75 mM</th>
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<tbody>
<tr>
<td>+50</td>
<td>0.63±0.03</td>
<td>0.85±0.03***</td>
</tr>
<tr>
<td>+40</td>
<td>0.62±0.03</td>
<td>0.80±0.01***</td>
</tr>
<tr>
<td>+30</td>
<td>0.64±0.02</td>
<td>0.75±0.03***</td>
</tr>
<tr>
<td>+20</td>
<td>0.63±0.01</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td>+10</td>
<td>0.62±0.01</td>
<td>0.62±0.01</td>
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<tr>
<td>0</td>
<td>0.62±0.02</td>
<td>0.62±0.01</td>
</tr>
<tr>
<td>−10</td>
<td>0.65±0.03</td>
<td>0.63±0.02</td>
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<tr>
<td>−20</td>
<td>0.64±0.01</td>
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<td>−30</td>
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<tr>
<td>−40</td>
<td>0.62±0.05</td>
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<tr>
<td>−50</td>
<td>0.62±0.03</td>
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Channel open probability at different membrane voltages without (control) and with trans addition of 75 mM NaCl. The mean value of P_o slightly increased in the presence of Na+ at potentials greater than +20 mV. Data points are mean±s.e.m. from four experiments. **P<0.01, ***P<0.001.
Fig. 2B (obtained in the presence of K⁺) failed to indicate a positive shift of the $P_o$ versus voltage relationship under similar ionic strength conditions.

Effect of sodium ions on complex I and IV activities in isolated mitochondria

In order to elucidate the effect of Na⁺ ions on the activities of the respiratory chain enzyme complexes, isolated brain mitochondria were incubated with 35 mM NaCl for 15 min. Na⁺ ions decreased mitochondrial complex I activity (Fig. 7A; $P<0.0001$) and increased complex IV activity (Fig. 7B; $P<0.001$).

Effect of sodium ions on ROS production in isolated mitochondria

To investigate whether NaCl affects mitochondrial ROS production, we studied the effects of 35 mM NaCl on ROS synthesis. As shown in Fig. 7C, the rate of generation of ROS in the mitochondria was decreased by NaCl exposure. A t-test indicated that there was a significant difference ($P<0.01$) between ROS production in the experimental group as compared with control.

Effect of sodium ions on mitochondria membrane potential in isolated mitochondria

Rhodamine 123 (Rh 123) was used to monitor the mitochondrial membrane potential. As shown in Fig. 7D, 35 mM NaCl induced a significant change ($P<0.01$) in membrane potential compared with the control group (free NaCl).

DISCUSSION

In the current study, we observed four types of mitochondrial potassium channels. The first three were a voltage-dependent 211 pS ATP-insensitive, ChTx-insensitive, IbTx-sensitive potassium channel; a voltage-independent 565 pS ATP-, ChTx-, IbTx-sensitive potassium channel; and a voltage-dependent 143 pS mitoK⁺ channel. The aim of this study was to characterize functionally the fourth type of potassium channel we observed in brain mitochondrial membrane preparations and its possible relationship with the respiratory chain.

A mitoBKCa channel was first described in human glioma cells LN229 (Siemen et al., 1999). Later, the same channel, which was stimulated by the potassium channel opener NS1619 and blocked by charybdotoxin, iberiotoxin and paxilline, was found in cardiac (Ohya et al., 2005; Sato et al., 2005), brain (Kajma and Szewczyk, 2012) and skeletal muscle (Skalska et al., 2008) mitochondrial membranes. Our previous observations concerning the potential presence and regulation of the brain mitoBKCa channel provided evidence for 211 pS and 565 pS mitoBKCa channels, with linear $I$–$V$ curves within the range of −50 to +40 mV in both cases. We previously observed that the 211 pS mitoBKCa channel $P_o$ increases at positive holding potentials to reach a maximum of 0.9±0.01 at +40 mV (Fahanik-babaei et al., 2011a). In contrast to this study and others (Cheng et al., 2008; Szabó et al., 2010), we observed that the 565 pS mitoBKCa channel $P_o$ was not affected by voltages ranging from −40 to +40 mV (Fahanik-babaei et al., 2011a).

In the current study, we characterized a novel potassium channel with respect to voltage dependence, calcium ion sensitivity, conductance and pharmacology. Under symmetrical 200 mM KCl, the $I$–$V$ plot was linear with a conductance of 190 pS (Fig. 2). A decrease in the trans K⁺ concentration (50 mM) caused a shift of reversal potential toward more negative values and the $I$–$V$ curve presented properties characteristic of a slightly inward rectifying channel (with a mean slope conductance for inward
currents of 150±5 pS); these properties were not observed in mitoBKCa channels, where the I–V relationships remained linear under the same ionic conditions (Fahanik-babaei et al., 2011a,b).

Another aspect of the present work concerns the pharmacological profile of the new brain mitoK channel, compared with members of the mitoBKCa channel family. IbTx is considered to be a specific BKCa channel blocker (generally at a concentration of 100 nM), whereas ChTx blocks other potassium channels in addition to BKCa channels (Kaczorowski and Garcia, 1999). A similar channel has been found in guinea pig ventricular mitoplasts (Xu et al., 2002), skeletal muscle (Skalska et al., 2008), brain (Skalska et al., 2009) and human glioma cells LN229 (Siemen et al., 1999). In the current study, we showed that cis addition of IbTx and ChTx did not affect channel activity at different potentials. Under the same conditions, we provided evidence in our previous studies for a 211 pS mitoBKCa channel that can be inhibited by IbTx but not by ChTx and ATP (Fahanik-babaei et al., 2011a), whereas a 565 pS mitoBKCa channel was sensitive to IbTx, ChTx and ATP (Fahanik-babaei et al., 2011b). In contrast, SK Ca protein incorporated into a planar lipid bilayer yielded two conducting state channels with conductances of 230 and 730 pS in the presence of 100 μM [Ca2+] in symmetrical 200 mM KCl conditions. More importantly, the mitochondrial small Ca2+-activated potassium channel (mitoSKCa) protein incorporated into the planar lipid bilayer displayed Ca2+-dependent activity, with a channel Po increasing from 0.5 at 1 μM [Ca2+] to 1.0 at 50 and 100 μM [Ca2+] (Stowe et al., 2013). However, in contrast to that study (Stowe et al., 2013), the present work shows that the 150 pS channel is not affected by Ca2+ at negative or positive voltages. Hence, our results support the presence of a mitoK channel in the brain mitochondrial membrane.

The mitoKATP channel was first identified using the patch clamp technique in rat liver mitochondria (Inoue et al., 1991). Later, the channel was found in heart tissue (Paucek et al., 1992), human T-lymphocytes (Dahlem et al., 2004), skeletal muscle (Debska et al., 2002), renal mitochondria (Cancherini et al., 2003) and brain (Choma et al., 2009). These channels are inhibited by antidiabetic sulfonylureas (e.g. glibenclamide) and 5-hydroxydecanoic acid (5-HD) (Szewczyk, 1998; Szewczyk and Marban, 1999). To determine whether the potassium channel described in this work corresponds to the KATP channel, we tested the effects of a cis application of ATP on channel activity. Our results had already revealed the presence of an ATP-sensitive potassium channel, a mitoKATP channel. In line with Choma et al. (2009), we observed an ohmic channel with slope conductance of 143 pS (Fig. 1B), which was completely blocked by ATP and antidiabetic sulfonylureas (glibenclamide) but not 5-HD (mitoKATP channel; data not shown). These observations contrast with the results shown in Fig. 4A,B, where we demonstrate that the addition of 2.5 mM ATP or 100 nM glibenclamide to the cytoplasmic side (cis chamber) does not affect K+ channel activity. Very recently, Paggio et al. (2019) identified an ATP-sensitive potassium channel in mitochondria. They observed that ATP did not affect the pore-forming subunit (MITOK) whereas channel ATP sensitivity was conferred by expression of pore forming together with SUR subunits. In contrast with the channel data presented in Fig. 6,
where we demonstrate that Na⁺ blocked the channel activity, it seems that the MITOK does not contain the K⁺ activity of MITOK and an ionic current was observed in the presence of Na⁺ ions (Paggio et al., 2019).

Single channel recordings of the embryonic hippocampus mitochondrial inner membrane revealed the presence of a voltage-dependent potassium channel displaying a conductance of 68 pS at positive voltages and 10 pS at negative voltages in symmetric Fig. 6. Effect of sodium ions on channel activity. (A) Single channel recordings of 150 pS channel in 200/50 mM KCl (cis/trans) conditions with and without trans addition of NaCl at +30 mV. There was no significant difference in $P_o$ value and current amplitude in the presence of 15 mM NaCl ($n=3$), whereas Na⁺ ions at 35 ($n=4$) and 75 mM ($n=4$) caused significant decrease in the amplitude of the current jump and channel $P_o$. (B) Single channel recordings of 150 pS channel in 200/50 mM KCl (cis/trans) condition with trans addition of 75 mM NaCl at potentials ranging from +50 to −50 mV. (C) Single channel current–voltage relationship, showing the effect of 75 mM NaCl on the 150 pS single channel current amplitude. (D) Single channel recordings of 150 pS channel in 200/50 mM KCl (cis/trans) conditions with and without trans addition of 75 mM NaCl at +40 and −40 mV. 75 mM NaCl decreased fast flickering and increased the channel $P_o$ from 0.64 to 0.8 at +40 mV. **$P<0.01$, ***$P<0.001$. 

Fig. 5. Effect of lidocaine and margatoxin on channel gating behavior. (A,B) Single channel recording of 150 pS channel in 200/50 mM KCl (cis/trans) conditions with and without cis addition of 1 mM LDC ($n=3$) at +30 and −40 mV (A) or 10 nM MgTx ($n=3$) at +30 and −50 mV (B). There was no significant difference in $P_o$ value and current amplitude in the presence of LDC or MgTx. Arrows indicate the closed levels.
150 mM KCl. Pharmacological blockers of the mitochondrial potassium channels did not affect the channel gating behavior, but acidic pH (below pH 6.2) inhibited channel activities (Kajma and Szewczyk, 2012). Notably, the two-pore domain channel family is also sensitive to pH and inhibited by lidocaine (Kajma and Szewczyk, 2012). Our results showed that the channel amplitude and $P_o$ were unchanged after the addition of 100 μM lidocaine or acidification (pH 6) of the cis face.

Potassium channels that are activated by an increase in cytoplasmic levels of Na$^+$ ions, commonly termed K$_{Na}$ channels, were first reported in cardiomyocytes (Kameyama et al., 1984) and later in neurons (Bader et al., 1985; Bischoff et al., 1998; Dale, 1993; Dryer, 1991, 1994; Dryer et al., 1989; Egan et al., 1992b; Haimann et al., 1990; Hartung, 1985; Mercer and Hildebrand, 2002; Safronov and Vogel, 1996; Saito and Wu, 1991; Schwindt et al., 1989), cardiac cells from some species (Lawrence and Rodrigo, 1999), diaphragm muscle fibers (Re et al., 1990), developing myoblasts (Zhou et al., 2004) and $Xenopus$ oocytes (Egan et al., 1992a). Two genes that encode K$_{Na}$ channels are Slack and Slick (Bhattarcharjee et al., 2003; Bhattacharjee and Kaczmarek, 2005; Joiner et al., 1998; Yuan et al., 2003). Slack and Slick proteins contain highly conserved PDZ-binding domains that could localize the channels to specific subcellular locations. Slick channel activities are directly reduced by cytoplasmic application of ATP (Bhattacharjee et al., 2003). These features are not in line with the pharmacological profile we obtained for the K$^+$ channel in mitochondrial membranes.

We decided to test the effect of Na$^+$ ions on channel activity and determine the Na$^+$ permission through the channel. We found that trans Na$^+$ ions inhibited K$^+$ currents, a process typical of a fast blocking action (Fig. 6A). The effects of Na$^+$ ions appear not to be limited to a decrease in the channel current jump amplitude. Fig. 6D and Table 1 reveal that trans application of Na$^+$ ions also resulted in an increase in channel $P_o$ as a function of voltage. This effect cannot be related to a change in the trans solution ionic strength, because the results in Fig. 2B obtained in the presence of K$^+$ (200 mM KCl cis/200 mM KCl trans) failed to indicate a positive shift of the $P_o$ versus voltage relationship under near similar ionic strength conditions (200 mM KCl cis/50 mM KCl plus 75 mM NaCl trans). These two effects, namely the fast blockade by sodium observed at positive voltages and the sodium-dependent variations of $P_o$ measured at higher than +20 mV can be interpreted in terms of a model in which the channel contains two binding sites for Na$^+$: one binding site located in the ion conducting pathway involved in a blocking process by Na$^+$ and a second binding site responsible for the effect of Na$^+$ on the channel gating process. Because the experimental conditions used to estimate the shift in reversal potential in the presence of trans Na$^+$ (Fig. 6C) were limited to low...
Na⁺ concentrations because of the important channel block at sodium concentrations greater than 150 mM we cannot rule out the possibility that Na⁺ could permeate the channel. The Na⁺ block reported here for the K⁺ channels of brain mitochondria is very similar to that observed in the KcsA, a prototypical bacterial K⁺ channel (Doyle et al., 1998; Zhou et al., 2001) and in the MaxiKCa channel (Yellen, 1984). The interaction of Na⁺ ions in the presence of K⁺ ions has been explored by using KcsA, as a K⁺ channel model (Thompson et al., 2009). It has been indicated that KcsA is blocked by Na⁺ from the intracellular side during outward current (Nimigean and Miller, 2002; Thompson et al., 2009). In this case, the blocking effect of Na⁺ is voltage dependent, becoming significant at large positive potentials (>120 mV). In addition, it was proposed that low concentrations of intracellular Na⁺ ions (5–10 mM) could not pass through a K⁺-occupied selectivity filter, but could reach the nonselective aqueous cavity and block the K⁺ flux with low affinity and fast kinetics (Nimigean and Miller, 2002). Our results shown in Fig. 6C do not support a significant voltage dependence for the fast blocking action by Na⁺; the ratio of control currents to currents with Na⁺ remained approximately equal to 1.5 within the −10 mV to 50 mV voltage range. We cannot, however, rule out the possibility of a voltage-dependent block by Na⁺ ions at very negative potentials. Furthermore, according to electrostatic potential calculations based on the MthK channel structure, the central cavity of large conductance channels, such as the one described in this work, should constitute a continuous isopotential region with the cytoplasmic medium (Jiang et al., 2002).

Assuming that the mitochondrial channel in the present case is incorporated such that the vestibule is facing the trans side, one would not expect the Na⁺ concentration in the channel cavity to be affected by a potential drop between the cavity and the trans medium. Under these conditions, it is unlikely to modulate the Na⁺ fast block effect directly by voltage, as seen in Fig. 6C. Furthermore, if the Na⁺ binding site is not directly in contact with the selectivity filter on the trans side, the K⁺ ion occupancy profile in the filter should not contribute to a voltage regulation of the Na⁺ block. These observations suggest the presence of a low affinity Na⁺ binding site, accessible from the trans compartment, capable of affecting the energy balance between the channel open and closed configurations. This mechanism would be distinct from the Slack and Slick activation process, in which Na⁺ is essential for channel activation.

Our study showed that Na⁺ ions increase complex IV activity and Δψm, and decrease complex I activity and ROS production. It has been observed that decreased Δψm reduces complex I-mediated ROS formation (Malinska et al., 2010; Szewczyk et al., 2010). In contrast, at higher Δψm, the mitochondrial respiratory chain becomes a significant producer of ATP, ROS and reverse electron transfer (Korshunov et al., 1997; Starkov and Fiskum, 2003). Fig. 7D shows that Rh 123 fluorescent intensity increased in the presence of Na⁺ ions. There is an inverse relationship between the magnitude of fluorescent intensity of Rh 123 and Δψm (Baracca et al., 2003). Thus, our results suggest that Na⁺ ions increase Δψm together with sodium-induced complex IV activity, which results in more ATP synthesis. Furthermore, Fig. 7A,C shows that Na⁺ ions decrease complex I activity and ROS production. Why did we observe reduced ROS production instead of more production at higher Δψm? Mitochondria produce ROS including hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻) and the hydroxyl radical (‘OH) (Boveris and Cadenas, 1975; Turrens, 2003). ROS production occurs as electrons pass onto oxygen instead of the next electron carrier in the electron transport chain during cellular respiration (Cadenas and Davies, 2000; Murphy, 2009). A higher rate of ROS production occurs at respiratory chain complex I either through forward electron transfer (NADH to Q) or reverse electron transfer (QH₂ to NAD⁺) (Dröse et al., 2016). It has been suggested that in forward electron transfer, if rotenone blocks complex I, electrons cannot be transferred to coenzyme Q (CoQ). Under these conditions, NADH accumulation and over-reduction of the complex I IF site results in electron leakage and increased ROS production (Murphy, 2009). In contrast, in reverse electron transfer, the blocking effect of rotenone on complex I activity (blocking IQ site) prevents CoQ from electron back transfer to complex I and decreases ROS generation (for a review, see Scialò et al., 2017). Our results show that Na⁺ ions decreased complex I activity and ROS production. Therefore, we hypothesize that Na⁺ ions may act similarly to rotenone during reverse electron transfer. It should be mentioned that recent studies suggest that complex I ROS production by reverse electron transfer could have a physiological role (Arias-Mayenco et al., 2018; Scialò et al., 2017). These effects might protect mitochondria against harmful ROS levels during ATP synthesis.

Patch-clamp experiments on the mitochondria of human astrocytoma have shown local coupling of complex IV of the respiratory chain and the mitoBKCa channel (Bednarczyk et al., 2013). In line with this study, our data show that Na⁺ ions decrease the brain mitochondrial sodium-sensitive potassium channel as well as complex I activity. Further studies are needed to clarify the relationship between the channel and complex I of the respiratory chain. In brief, during neuron excitation and increased intracellular sodium concentration, mitochondrial sodium-sensitive channels may be inhibited via Na⁺ ions and increased mitochondrial Δψm, which in turn increases ATP synthesis. On the other hand, it is likely that sodium-decreased complex I activity reduces ROS production via reverse electron transfer. These are preliminary data and further studies are needed to clarify these relationships.

In summary, our data suggest the presence of a novel potassium channel that can be inhibited by Na⁺ ions in the mitochondrial membrane of rat brain. The existence of this channel suggests novel signaling between the mitochondrial membrane potential and the respiratory chain. As a result, we propose that inhibition of mitochondrial K⁺ transport by Na⁺ ions on the potassium channel opening might be important for cell protection and ATP synthesis.

MATERIALS AND METHODS
Electrophysiological studies
Materials
HEPES, BSA, iberiotoxin (IbTx), charybdotoxin (ChTx), ATP, margatoxin (MgTx), EGTA and lidocaine were obtained from Sigma.

Mitochondrial inner membrane isolation
Rat brain mitochondria were isolated according to the protocol described previously (Rosenthal et al., 1987). Briefly, after anesthetizing rats (weighing 180–200 g) using ether, the brains were removed and homogenized at 6000 rpm in MSE solution (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml BSA, pH 7.4) containing 0.05% nagarase. The homogenate was centrifuged at 2000×g for 4 min. Then, the supernatant (step 1 for western blotting) was centrifuged at 12,000×g for 9 min and the resulting pellet homogenized in MSE (step 2 for western blotting) containing digitonin to disrupt synaptosomal membranes and release the mitochondria.
The suspension was subsequently centrifuged at 12,000g for 11 min and the resulting pellet dissolved in 300 µl of MSE solution (35 mg protein/ml) (step 3 for western blotting). Brain mitochondrial inner membranes were prepared as previously described (Da Cruz et al., 2003). Briefly, after suspending and stirring the mitochondria in H2O for 20 min, the mixture was homogenized 20 times with a glass homogenizer. The suspension was subsequently centrifuged twice at 12,000g for 5 min. Thereafter, the obtained pellet (mitoplasts) was treated with 0.1 M Na2CO3 (pH 11.5) at a final concentration of 0.5 mg/ml for 20 min on ice. Finally, the suspension was centrifuged at 100,000g for 30 min. Mitochondrial membrane vesicles were stored in 20 µl aliquots in MSE solution pH 7.4 at −80°C until use (step 4 for western blotting). The final concentration of preparation ranged from 13 to 15 mg protein/ml.

All experiments were carried out based on guidelines from the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.NRC.REC.1393.23).

Immunoblot analysis
Cytochrome c oxidase subunit 1 (Santa Cruz, SC-58347), actin (Santa Cruz, SC-1615), calnexin (Santa Cruz, SC-11397) and 58K Golgi protein (ABCAM, AB6284) antibodies were used to test the purity of preparation. Secondary antibodies linked to horseradish peroxidase were obtained from GE-Biosciences. Blots were treated with enhanced chemiluminescence kit luminogen and the images scanned for further processing in Adobe Photoshop.

L-α-phosphatidylcholine extraction
We extracted L-α-phosphatidylcholine (L-α-lecithin) from egg yolk as previously described (Singleton et al., 1965). Lecithin purity (∼98%) was compared with a standard lecithin (Merck) using thin layer chromatography.

Electrophysiological analysis
A black (bilayer) lipid membrane technique was used to characterize the single channel gating behavior. The cis (cytoplasmic face) and trans (luminal face) chambers contained 4 ml of 200 and 50 mM KCl, respectively. The pH was adjusted to 7.2 with Tris-HEPES. To fuse the channel into bilayer lipid membrane, the vesicle was made electrical connections and minimize liquid junction modification. The sample of isolated mitochondria was added to a published protocol (Spinazzi et al., 2011) with a small modification. The sample of isolated mitochondria was added to a well-mixed solution containing distilled water, fatty acid-free BSA (50 mg/ml), 0.5 M potassium phosphate buffer pH 7.5, 10 mM KCN and 10 µl of 10 mM NADH in a 1 ml cuvette. The reaction was started by adding 10 mM ubiquinone. The decrease in absorbance was measured at 340 nm for 3 min at 15 s interval. The extinction coefficient for NADH was 6.2 mM cm−1. The total protein concentration of mitochondria was 400 µg/ml.

Biochemical studies
Materials
Sucrose, mannitol, EDTA, Tris-HCl, BSA, KCN, NADH, sodium dithionite, potassium phosphate monobasic (KH2PO4), potassium phosphate dibasic (K2HPO4), reduced cytochrome C, rhodamine 123, and 2',7'-dichlorofluorescein diacetate (DCFH2-DA) were purchased from Sigma.

Mitochondrial isolation
Mitochondria were extracted as previously described (Navarro et al., 2005). Briefly, adult male rat (weighing 180–200 g) brains were rapidly removed and minced finely in isolation buffer (230 mM mannitol, 70 M sucrose, 1.0 mM EDTA and 10 mM Tris–HCl, pH 7.40). After homogenizing in isolation buffer, the suspension was centrifuged at 7000g for 10 min at 4°C. The supernatant fraction was collected and centrifuged again at 8000g for 10 min. Then, extracted mitochondria were stored in isolation buffer. To consider the effect of Na+ ions on brain mitochondrial activity, 230 mM mannitol was replaced with 165 mM mannitol and 35 mM NaCl. Mitochondrial protein content was assayed as previously described (Bradford, 1976).

Assay of brain mitochondrial ROS
Detection of brain mitochondrial ROS was done using a Multi-Mode Micro plate reader, (synergy HTX) using 2',7'-dichlorofluorescein diacetate (Pipatpiboon et al., 2012; Thummasom et al., 2011). Isolated mitochondrial samples (0.7 mg/ml protein) were added to microplate wells and incubated with 2 μM 2',7'-dichlorofluorescein diacetate. After 20 min, the fluorescence was measured at 490 nm excitation and 530 nm emission wavelength.

Mitochondrial membrane potential detection
Mitochondrial membrane potential (ΔΨm) was measured by quantitation of rhodamine 123 (Rh 123) quenching. The mitochondrial fractions were incubated with Rh 123 for 5 min. Then, Rh 123, a cationic fluorescent dye, was excited at 490 nm wavelength and detected at the emission wavelength of 530 nm (Luo and Shi, 2005). The change in ΔΨm was measured as fluorescence intensity using a fluorescent microplate reader.

Spectrophotometric analysis of electron transport chain complexes
Complex I
The enzymatic activity of complex I (NADH-CoQ oxidoreductase) of the brain mitochondrial respiratory chain was assayed according to a published protocol (Spinazzi et al., 2011) with a small modification. The sample of isolated mitochondria was added to a well-mixed solution containing distilled water, fatty acid-free BSA (50 mg/ml), 0.5 M potassium phosphate buffer pH 7.5, 10 mM KCN and 10 µl of 10 mM NADH in a 1 ml cuvette. The reaction was started by adding 10 mM ubiquinone. The decrease in absorbance was measured at 340 nm for 3 min at 15 s interval. The extinction coefficient for NADH was 6.2 mM cm−1. The total protein concentration of mitochondria was 400 µg/ml.

Complex IV
Complex IV (Cox1) activity was assayed according to the method described previously (Spinazzi et al., 2011). The reaction buffer contained distilled water, reduced cytochrome c (1 mM) and potassium phosphate buffer (100 mM). The reaction was started by adding homogenized brain mitochondria. The decrease in absorbance was monitored at 550 nm wavelength for 20 min at 30 s intervals.
Data analysis
For all experiments, mitochondria were prepared from six animals. All data are represented as the mean ± s.e.m. Student’s unpaired t-test was used to evaluate differences between two groups. In statistical analysis, P<0.05 was considered significant.

Competing interests
The authors declare no competing or financial interests.

Author contributions

References


