Basic and Clinical
NEUROSCIENCE

November & December 2025, Vol 16, No. 6

Research Paper

A New Potassium Channel on the Endoplasmic Reticulum Membrane in a Rat Brain: Electropharmacology and Molecular Evidence

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Citation Nazari, M., Eliassi, A.,Saghiri, R., Nikbakht, F., & Fahanik-babaei, J. (2025). A New Potassium Channel on the Endoplasmic Reticulum Membrane in a Rat Brain: Electropharmacology and Molecular Evidence A New Potassium Channel on the Endoplasmic Reticulum Membrane in a Rat Brain: Electropharmacology and Molecular Evidence. *Basic and Clinical Neuroscience, 16*(6), 1017-1032. http://dx.doi.org/10.32598/bcn.2024.1137.1

http://dx.doi.org/10.32598/bcn.2024.1137.1

Article info:
Received: 15 Jul 2024
First Revision: 13 Aug 2025
Accepted: 10 Sep 2025
Available Online: 01 Nov 2025

Keywords:
Potassium channels, Rough endoplasmic reticulum (RER), ATP-sensitive potassium (KATP) channel, Bilayer lipid membrane, Single channel recording

ABSTRACT

Introduction: Several types of ion channels found in the plasma membrane have also been identified in the membranes of intracellular organelles. These ion channels, including potassium channels, play a crucial role in regulating intracellular ion homeostasis. An ATP-sensitive potassium (KATP) channel with various functional roles has been identified in the endo/sarcoplasmic reticulum membranes of both excitable and non-excitable cells. Our previous studies investigated the electropharmacological and molecular properties of KATP and $BKCa^{+2}$ channels in the rough endoplasmic reticulum (RER) of rat hepatocytes.

Methods: In this study, for the first time, we described the electropharmacological and molecular properties of the RER KATP channel in rat brain cells using an incorporated single-channel in a planar lipid bilayer and Western blotting.

Results: The results of the study revealed the presence of a KATP channel with a conductance of 306 pS, and the open probability was found to be voltage-independent at holding potentials ranging from +40 to -60 in an asymmetric solution (200/50 mM KCl; cis/trans). Additionally, we observed that adding ATP (2.5 mM) to both positive and negative potentials, and 100 µM glibenclamide to the positive voltages inhibited channel activity. The addition of 100 mM 5-HD and 100 nM charybdotoxin to the cis side did not affect the channel behavior. Furthermore, a Western blot analysis provided evidence of the expression of Kir6.2, Kir6.1, sulfonylurea receptor (SUR)1, and/or SUR2B, but not SUR2A, in the RER of rat brain fractions.

Conclusion: In this study, we provide strong evidence for the existence of a KATP channel on the RER membrane of rat brain cells, displaying pharmacological properties distinct from those classically described for the plasma membrane and other intracellular organelles.

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Highlights

- We described the electropharmacological and molecular properties of the RER KATP channel in rat brain cells.
- There was a KATP channel with a conductance of 306 pS, and the open probability was found to be voltage-independent.
- Adding ATP (2.5 mM) to both positive and negative potentials, and 100 µM glibenclamide to the positive voltages inhibited channel activity.
- The addition of 100 mM 5-HD and 100 nM charybdotoxin to the cis side did not affect the channel behavior.
- This study provides strong evidence for the existence of a KATP channel on the RER membrane of rat brain cells.

Plain Language Summary

Modifying the activity of potassium channels can be important therapeutic targets in many incurable diseases such as Alzheimer's disease, cancer and ischemia and also for limb wound repair and regeneration. An ATP sensitive potassium (KATP) channel with various functional roles has been identified in the endo/sarcoplasmic reticulum membranes of both excitable and non-excitable cells. In this study, we aimed to identify the molecular structure of a KATP channel located in the ER membrane of rat brain, as variations in the expression of its constituent subunits can affect its gating behavior. The results revealed the presence of a KATP channel with a conductance of 306 pS, and the open probability was found to be voltage-independent. Additionally, we observed that adding ATP (2.5 mM) to both positive and negative potentials, and 100 µM glibenclamide to the positive voltages inhibited channel activity. The addition of 100 mM 5-HD and 100 nM charybdotoxin to the cis side did not affect the channel behavior. Furthermore, a Western blot analysis provided evidence of the expression of Kir6.2, Kir6.1, sulfonylurea receptor (SUR)1, and/or SUR2B, but not SUR2A, in the RER of rat brain fractions. This study provides strong evidence for the existence of a KATP channel on the RER membrane of rat brain cells, displaying pharmacological properties distinct from those classically described for the plasma membrane and other intracellular organelles.

Introduction

The balance of intracellular potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}) concentrations regulates ion homeostasis, a normal physiological process that preserves the integrity of the plasma membrane and intracellular organelles. Ion channels, including K^+ channels, significantly regulate intracellular ion homeostasis. These channels play a crucial role in cellular processes, such as Ca^{2+} signaling, volume regulation, generation of pH gradients, cell death, oxidative stress production, differentiation, and proliferation, etc. (Averaimo et al., 2010; Edwards & Kahl, 2010; Jehle et al., 2011; Wulff et al., 2009). Regarding their crucial role, modifying the activity of these K^+ channels could be important therapeutic targets in many incurable diseases such as Alzheimer's disease, cancer and ischemia (Hübner & Jentsch, 2002; Jafari et al., 2015; Teisseyre et al., 2019; Waza et al., 2018), and, more recently identified, as targets for limb wound repair and regeneration (Zhang et al., 2020) and the pathophysiology of migraine (Al-Karagholi et al., 2022; Kokoti et al., 2020).

Several types of K^+ channels are identified in the membranes of intracellular organelles, such as mitochondria, nucleus, and endoplasmic reticulum (ER) (Checchetto et al., 2016). Multiple lines of evidence demonstrate that distinct types of K^+ channels, including ATP-sensitive K^+ (KATP) channels, Ca^{2+}-activated K^+ channels (BKCa), and K^+ permeable trimeric intracellular cation channels, have a range of functional roles (Guéguinou et al., 2014; Ng et al., 2010; Salari et al., 2015; Yazawa et al., 2007). Some studies have reported on the endo/sarcoplasmic reticulum membranes in hepatocytes and neuronal HT-22 cells (Khodaee et al., 2014; Richter et al., 2016; Salari et al., 2015). These channels facilitate the flux of K^+ across the membrane of the ER, and their main role appears to be maintaining the balance of charge during Ca^{2+} release and uptake. On the other hand, these channels regulate the volume of the ER lumen and maintain ER calcium homeostasis by keeping the electro-chemical force of

Ca^{2+} ions away from the ER membrane potential (Kuum et al., 2015; Li et al., 2006; Xu et al., 2015). The octameric complex, known as physiological ATP, inhibits KATP. It consists of four pore-forming subunits (Kir6.X) encircled by four regulatory sulfonylurea receptors (SUR), also known as ATP-binding subunit SUR (Clement IV et al., 1997). This channel is found in both the membrane and subcellular membranes, such as the mitochondrial inner membrane (mitoKATP) (Inoue et al., 1991; Paggio et al., 2019), the nuclear membrane (Quesada et al., 2002), and rough endo/sarcoplasmic reticulum (Salari et al., 2015).

ER KATP channels and their subunits have been shown to exist in a variety of cells (cell culture), tissues, and organs in recent years, according to several electropharmacological and molecular studies (Kuum et al., 2012; Ng et al., 2010; Salari et al., 2015; Zhou et al., 2005). Zhou et al. (2005) examined the subcellular distribution of Kir6.2 in neuronal cells using electron microscopy. The research findings revealed the presence of Kir6.2 in various neuronal cell types, such as Purkinje cells, Bergmann glial cells, and glial cells of the corpus callosum and cerebellum (Zhou et al., 2005). Ng et al. provided evidence for the presence of the Kir6.1 subunit on the ER membrane of C2C12 and HEK293 cells, which played a crucial role in modulating Ca^{2+} release from intracellular stores (Ng et al., 2010). Kuum et al. (2012) showed that KATP channels connect with the ER in primary cortical neurons when observed with glibenclamide-BODIPY FL fluorescent dye. In 2015, Salari et al. reported a channel in the liver with a 560 pS conductance that was sensitive to ATP (2.5 mM), Glibenclamide, and tolbutamide but not to iberotoxin and carybdotoxin. Furthermore, Western blot analysis showed the expression of Kir6.2, SUR1, and/or SUR2B, and SUR2A in rough ER (RER) fractions (Salari et al., 2015).

To the best of our knowledge, despite the abundance of research on ER ion channels, particularly K^+ channels, in various cells and tissues, no study has directly examined the electrophysiological characteristics of the brain's ER KATP channel. Therefore, in this study, we examined the biophysical and electropharmacological characteristics of the brain's ER KATP channel using isolated ER membrane particles reconstituted into a planar lipid bilayer. Furthermore, we aimed to identify the molecular structure of a KATP channel located in the ER membrane of rat brain, as variations in the expression of its constituent subunits can affect its gating behavior.

Materials and Methods

Animals and ethical statement

The 200–220 g male Wistar rats were housed in a controlled environment with a 12:12 light/dark cycle, a temperature of 22±2 °C, and a humidity of 50±10%. Animals were acclimatized before being used in experiments and had free access to water and food ad libitum. All procedures involving the animals were conducted in accordance with the guidelines outlined in the guide for care and use of laboratory animals (National Institute of Health Publication No. 80-23, revised 1996), and were approved by the Research Ethics Committee of Laboratory Animal-Tehran University of Medical Sciences.

Materials

According to Singleton et al.'s method, L-a-phosphatidylcholine (L-a-lecithin) was extracted from fresh egg yolk (Singleton et al., 1965). Sucrose, Imidazole, pyrophosphate, K^+ chloride, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, glibenclamide, ATP, 5-HD, and charybdotoxin (ChTx) were purchased from Sigma Aldrich (St Louis, MO, USA). n-decan was purchased from Merck (Darmstadt, Germany). Chamber and cup for bilayer formation (Warner Instrument Corp., Hamden, CT, USA),

RER isolation of whole brain

ER membrane particles derived from the RER of whole rat brain were prepared as previously described (Salari et al., 2015). In brief, five male rats were anesthetized and euthanized by decapitation. The brains were then quickly removed. Brains were washed and placed in a 20 mL ice-cold buffer containing 0.25 M sucrose. The brain tissues were then minced with scissors and homogenized using a motor-driven Teflon-glass Potter homogenizer (step 1). Subsequently, 40 mL of ice-cold 0.25 M sucrose solution was added, and the homogenate was filtered through surgical cotton fabric. The homogenate was centrifuged at 9800×g for 20 minute (Eppendorf model 5415R, Germany). The supernatant was then decanted and centrifuged at 110000×g for 14 minutes (Beckman model J-21B, USA) (step 2). The pellet was dissolved in 15 mL of ice-cold 2 M sucrose. Afterward, the solution was then transferred to a 30 mL glass homogenizer and manually homogenized 20 times using a glass Potter homogenizer to obtain a homogeneous suspension. The suspension was then centrifuged at 300000×g for 67 minutes in a sucrose gradient (step 3). The resulting

pellet was dissolved in 15 mL of a solution containing sucrose, imidazole, and Na pyrophosphate at concentrations of 0.25 mM, 3 mM, and 0.5 mM, respectively. The solution was then centrifuged twice at 140000×g for 47 minutes. RER microsomes was dissolved in 800 µL sucrose 0.25 mM and imidazole 3 mM at a final concentration of 7 mg/mL (Step 4). All procedures were conducted at 4 ˚C. The RER microsomes were then stored in 25 µL aliquots in a solution containing 0.25 mM sucrose and 3 mM imidazole at pH 7.2, and stored at -80 ˚C for one month.

Immunoblot analysis

Protein samples were quantified using the Bradford protocol, with bovine serum albumin as the standard (Bradford, 1976). Using the semi-dry electrophoretic transfer method, 25 µg of protein samples from each purified fraction were separated by SDS-PAGE (12% Bis-Tris Plus gels) and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked for two hours at room temperature using a tris-buffered saline (TBS) solution containing 1% (v/v) Tween 20 and 5% (w/v) bovine serum albumin (BSA). Then, it was immersed in a primary antibody diluted (1:100 to 1:500) for an overnight at 4 ˚C in TBS + 0,1% Tween 20 + 3% BSA. The primary antibodies used included; 58kDa Golgi protein (abcam, ab6284, Cambridge, UK), Cox1 (SC-58347; Santa Cruz Biotechnology Inc., Heidelberg, Germany), Actin (SC-1615; Santa Cruz Biotechnology Inc., Heidelberg, Germany), calnexin (SC-11397; Santa Cruz Biotechnology Inc., Heidelberg, Germany), SUR1, SUR2A and SUR2B (SC-5789, SC-32462 and SC-5793, respectively; Santa Cruz Biotechnology Inc., Heidelberg, Germany), Kir6.1 and Kir6.2 (SC-11228 and SC-11228, respectively; Cruz Biotechnology Inc., Heidelberg, Germany). Following three TBS + 0.1% Tween 20 washes, the membranes were soaked for one hour at room temperature in a secondary antibody that had been diluted (1:1000 to 1:5000) in PBS + 0.1% Tween 20 + 3% BSA. The secondary antibody was horseradish peroxidase (anti-goat and anti-rabbit HRP). The membranes were then treated, in accordance with the manufacturer's instructions, with an excellent chemiluminescent substrate (ECL) kit for chemiluminescence detection, and the immunoreactive bands were visualized using the Amersham ECL Prime Western Blotting detection (GE Healthcare Life Sciences).

Recording instrumentation and statistical analysis

A Delrin cup with a 150 µm diameter hole was used to create planar phospholipid bilayers, dividing the setup into cis and trans chambers. The cis chamber contained 4 mL of 200 mM KCI, while the trans chamber contained 4 mL of 50 mM KCI (pH 7.4). Planar phospholipid bilayers were formed by painting a suspension of L-a-lecithin in n-decane (25 mg lipid/mL). Vesicles containing channel proteins were introduced into the cis compartment, and their fusion with the bilayer was initiated mechanically. The formation and thinning of the bilayer were monitored by capacitance measurements and optical observations, with bilayers exhibiting a capacitance of approximately 150-200 pF. Magnetic bars were used to stir the contents of both chambers when necessary. Single-channel currents were measured using a BC-525D amplifier (Warner Instruments, USA). Electrical connections were established using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. The cis chamber was voltage-clamped in relation to the grounded trans chamber. Subsequently, the signals were filtered at 1 kHz, digitized at 10 kHz using the A/D converter (Axone Instruments Inc., USA), and stored on a PC for later analysis utilizing PClamp 10.0 from (Axon Instruments Inc., USA). Single-channel currents were recorded at various voltages, and conductance was determined from the current-voltage relationship, which was then averaged across a minimum of three independent experiments. The total number of experiments is represented by 'n'. The reversal potential was obtained by fitting the experimental data to a second-order polynomial curve. The open-channel probability (Po) was calculated using standard event-detection algorithms in Pclamp Software, version 10. Additionally, the mean closed and open times of the channel, as well as P(open), were calculated from 60-s segments of continuous recordings. Any observed differences were assessed for significance using student's t-test, and the data are presented as Mean±SE.

Results

Purity of ER fractions

The degree of purity of the microsome preparation was assessed using antibodies against ER marker (calnexin, 90 kDa), plasma membrane marker (actin), mitochondrial membrane marker (Cox1), and Golgi matrix marker (58 KGP). As shown in Figure 1A, we prepared separate samples for each isolation step using the specific markers mentioned above. Our results relevant to the final step (step 4) showed that the microsome preparation banded only with the RER marker (calnexin) and did not react with the other markers (Figure 1A). Thus, these data confirm that the microsome preparation obtained from rat ER was pure.

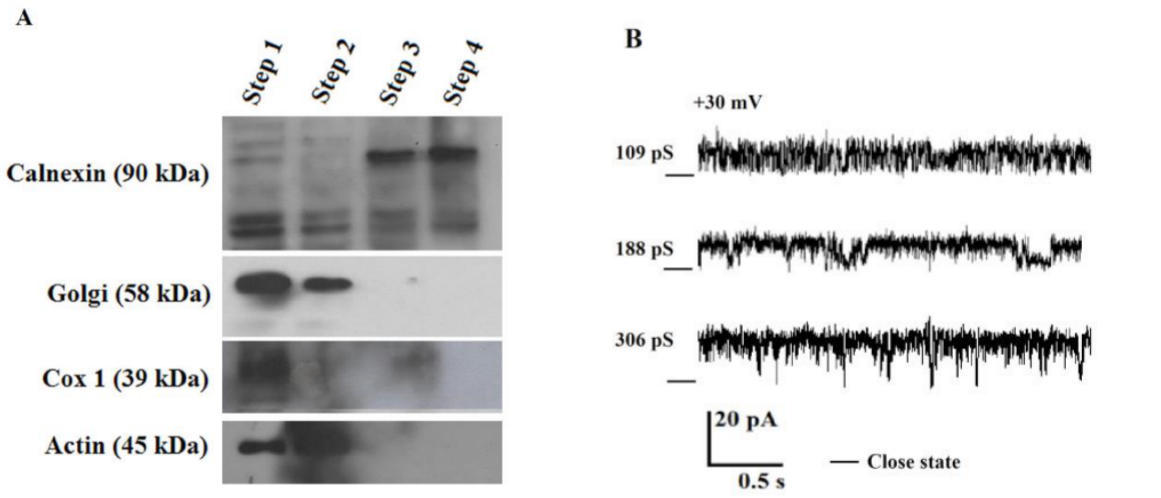

Figure 1. Western blot analysis and single-channel recording

A) Purity of the microsome preparation from whole brain was assessed using plasma membrane markers: Actin (C-11); 45 kDa. Golgi apparatus marker: 58 K Golgi protein; 58 kDa. ER marker: Calnexin, 90 kDa. Mitochondrial membrane marker: Cox1 (1D6), 39 kDa; The method involved the following steps: Step 1: Homogenate; Step 2: High-speed centrifugation; Step 3: Gradient sucrose; Step 4: Final preparation.

B) Single-channel recordings of three different K^+ channels of RER in rat brain in planar lipid bilayer at +30 mV

Single channel properties of KATP channel in rat brain RER membrane

To offer a more comprehensive account of the electrophysiological properties of the KATP channel located on the rough ER (RER) membrane in the rat brain, we conducted observations of single-channel activity under control conditions (200/50 mM KCl; cis/trans) following the incorporation of vesicles obtained from the RER of the brain at various voltage levels (Figure 1B). Our results revealed a K^+ channel with conductance of 306 pS after its incorporation into a bilayer lipid membrane. Additionally, a few chloride channels were also observed, but our focus in this study was on the 306 pS cation channels.

According to the Nernst equation, the observed reversal potential in the 200/50 mM KCl (cis/trans) gradient solution was -30 mV, confirming that the examined channel is selective for cations. The mean reversal potential, calculated from the fit to the experimental data, was 30±3 mV (n=6), indicating that the channel was impermeable to chloride ions. Figure 2A displays current traces from brain RER membrane vesicles at various bilayer potentials ranging from +40 to -60 mV, with a determined conductance of 306±12 pS in an asymmetric (200/50 mM KCl; cis/trans) solution (n=6). Figure 2B shows the single-channel I-V relationship obtained from six recordings under the same conditions. The I-V plot showed linearity without any evidence of inward rectification at potentials between +40 and -70 mV.

In the asymmetric concentration condition (200/50 mM KCl; cis/trans), the open Po of the channel was not influenced by voltage when held at potentials between +40 and -60 mV. The Po remained constant regardless of the voltage polarity. Table 1 presents the average steady-state Po values as a function of the holding potential for the fully-open conducting state, obtained from six different experiments.

Pharmacological properties of the ion channel

Further experiments were conducted to confirm the nature of the RER K^+ channel from the rat brain preparation. Therefore, we examined the effect of several types of K^+ channel blockers on the channel activity as described in the following.

Effect of ATP on channel activity

Initially, we investigated the effects of ATP, a well-known KATP channel blocker. Figure 3A illustrates the single-channel recordings and open Po in a solution with 200/50 mM KCl (cis/trans) concentrations at various potentials under control conditions and after the addition of 2.5 mM ATP to the cis side. The presence of ATP had a notable

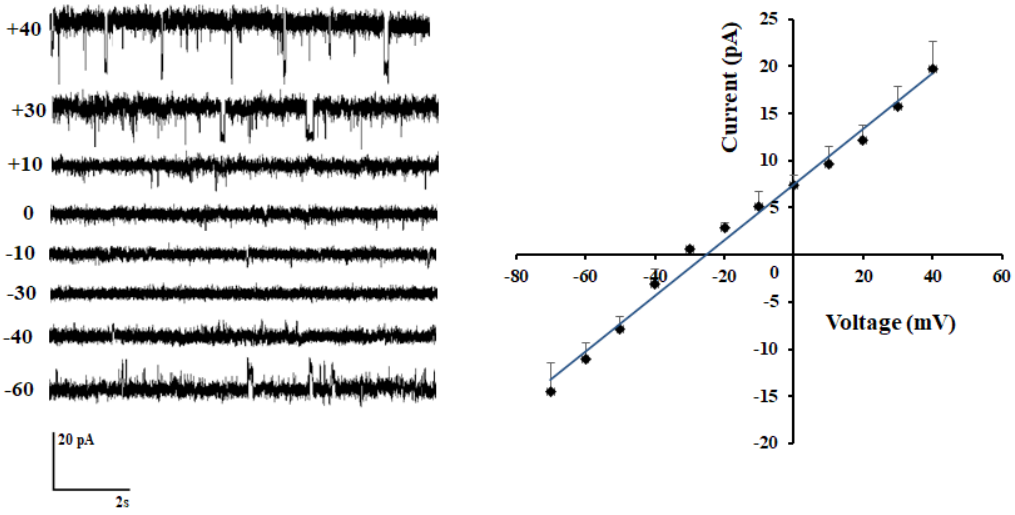

Figure 2. Single channel recordings and current voltage relationship

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A) Single-channel recordings were conducted in asymmetric solutions (200/50 mM KCl, cis/trans) at potentials ranging from −60 to +40 mV: The arrow indicates the closed statem

B) Current voltage relationship for the 306 pS channel under asymmetrical conditions (200 mM KCl cis/50 mM KCl trans)

impact on channel amplitude and open Po at both positive and negative voltages, leading to complete channel blockade activity at these potentials (n=5). These findings are summarized in the bar graph depicted in Figure 3C.

Effect of glibenclamide on channel activity

An investigation was conducted to analyze the impact of glibenclamide, a well-known sulfonylurea, and KATP channel blocker, on RER channel activity. As shown in Figure 3B, the addition of 100 µM glibenclamide into the cis chamber led to a total block of channel activity at positive but not negative voltages (n=4). These results are illustrated in the bar graph presented in Figure 3C.

Effect of 5-HD as a mitochondrial KATP channel blocker on the channel activity

In the next step, we examined the effect of 5-HD, as a mitoKATP channel blocker, on channel activity. As shown in Figure 4A, addition of 5-HD (10 mM) to the cis side had no effect on channel activity (single-channel recordings and open Po) at either positive or negative voltages (n=3). Data are expressed as Mean±SE.

Effect of ChTx as calcium (Ca^{2+})-dependent K^+ channel blockers activity

Since both ATP-sensitive BKCa channels (Maxi-KCa) (Fahanik-Babaei et al., 2011) and KATP channels are sensitive to ATP, we investigated the potential impact of ChTx as a Maxi-KCa channel blocker on channel activity in RER membranes. Our results revealed that 1 mM ChTx did not inhibit channel activity. Single-channel recordings and open Po at +30 and -40 mV (n=4) are depicted in Figure 4B, with a summary presented in the bar graph on the right side of Figure 4C. These findings confirm that the channel derived from rat brain RER preparation corresponds to the KATP channel.

Investigation of RER KATP channel subunits

Since there are two types of subunits in the channel structure, pore subunits and sulphonylurea subunits, in this study, we also investigated the presence of both KATP channel subunits in the RER of brain cells. In our study, we utilized Western blot analysis to investigate the presence of KATP subunits in microsomes from rat brain cells. Specifically, we employed antibodies targeting the KATP pore-forming subunits Kir6.2 and Kir6.1, as well as the regulatory elements SUR1, SUR2A, and SUR2B. The use of anti-Kir6.2 and anti-Kir6.1 antibodies for labeling revealed a distinct band at approximately 55 kDa, corresponding to the anticipated molecular weight of Kir6.2 and Kir6.1 proteins (Figure 5A) (n=3). Analysis of the sulphonylurea subunit, as depicted in Figure 5B, indicated that anti-SUR1, anti-SUR2A, and anti-SUR2B antibodies identified bands at 150 kDa, 143 kDa, and 150 kDa, respectively, in both crude and RER fractions. However, two separate bands were detected in SUR2B at 100 kDa and 150 kDa. These findings confirm the presence of KATP Kir6.2 and Kir6.1 subunits, as well as SUR1 and SUR2A subunits, in both the homogenate and RER membrane preparation.

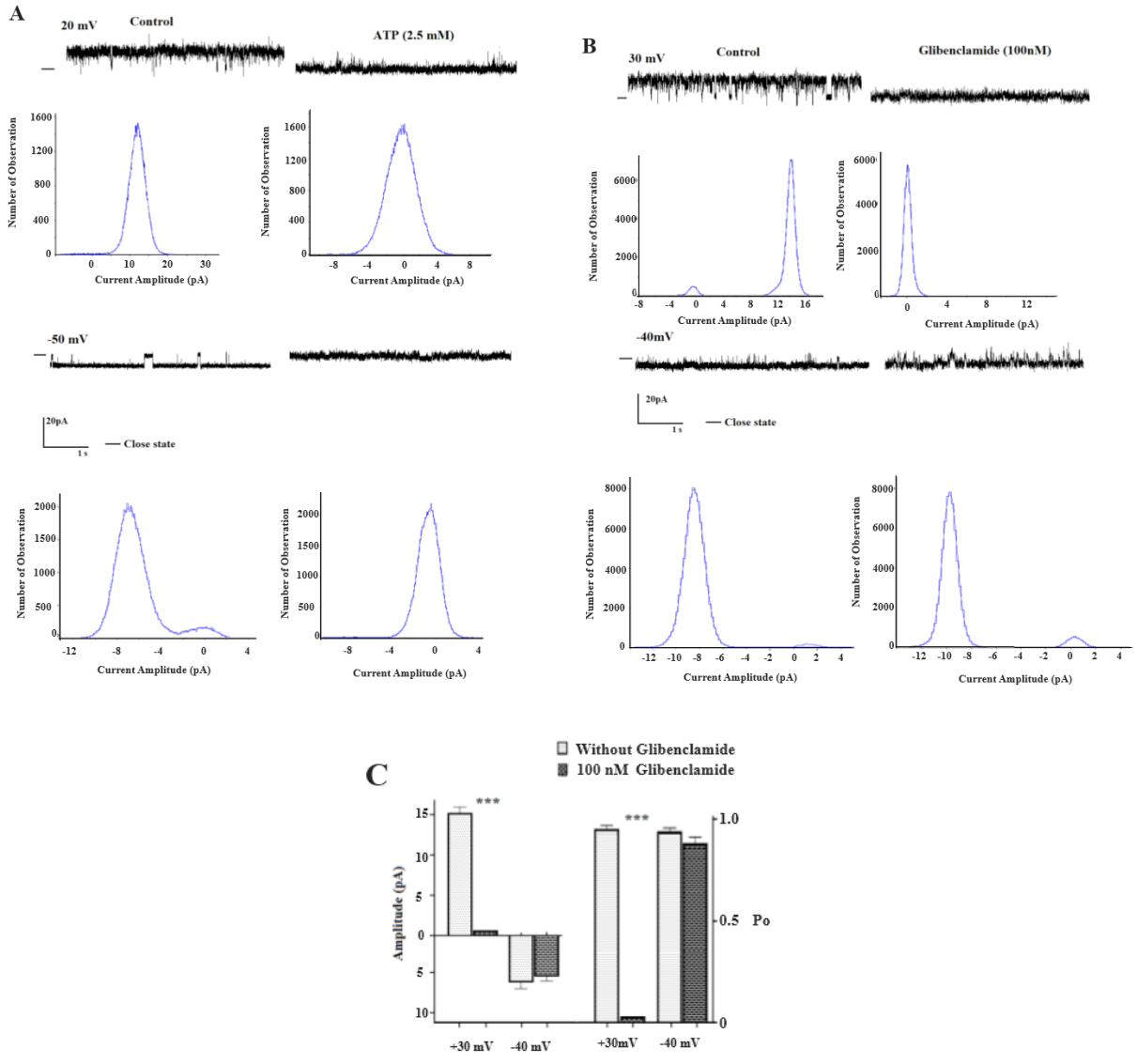

Figure 3. The effect of K^+ channel blocker (ATP, glibnclamide) on channel gating behaviour

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Note: Single-channel recording was conducted to measure the open probability of a 306 pS channel in asymmetric conditions (200/50 mM KCl, cis/trans) with and without cis addition of (A) ATP (2.5 mM) at +20 and −50 mV (n=5), (B) glibenclamide (100 µM) at +30 and -40 mV. (C) The effect of glibenclamide at positive and negative voltages are summarized in the bar graph. ***P<0.001, (n=4). Data are Means±SE. Closed levels are indicated by –.

Discussion

The ER plays a crucial role in the synthesis, folding, and transport of protein and is responsible for various important cellular functions, including cell signaling and the storage of calcium ions (Ca^{2+}). It is well documented that intracellular organelles, such as mitochondria, endo/sarcoplasmic reticulum, and nucleus, contain K^+ channels similar to those found on the plasma membrane (Ballanyi, 2004; Noma, 1983; Rusznak et al., 2008). In this study, we characterized the electrophysiological and molecular properties of the KATP channel in the ER membrane of rat brain using single-channel recording and western blotting. A study on the reconstitution of the ER membrane from rat brain tissue revealed the presence and activity of a K^+ channel with a conductance of 306 pS. This K^+ channel was found to be sensitive to ATP and glibenclamide, both of which are well-known blockers of KATP K^+ channels. Additionally, the use of specific antibodies targeting Kir6.1, Kir6.2, and SUR subunits further confirmed the existence of KATP channels in the ER membrane of rat brain tissue. This groundbreaking research provides the first direct evidence of the electropharmacological properties and structure of

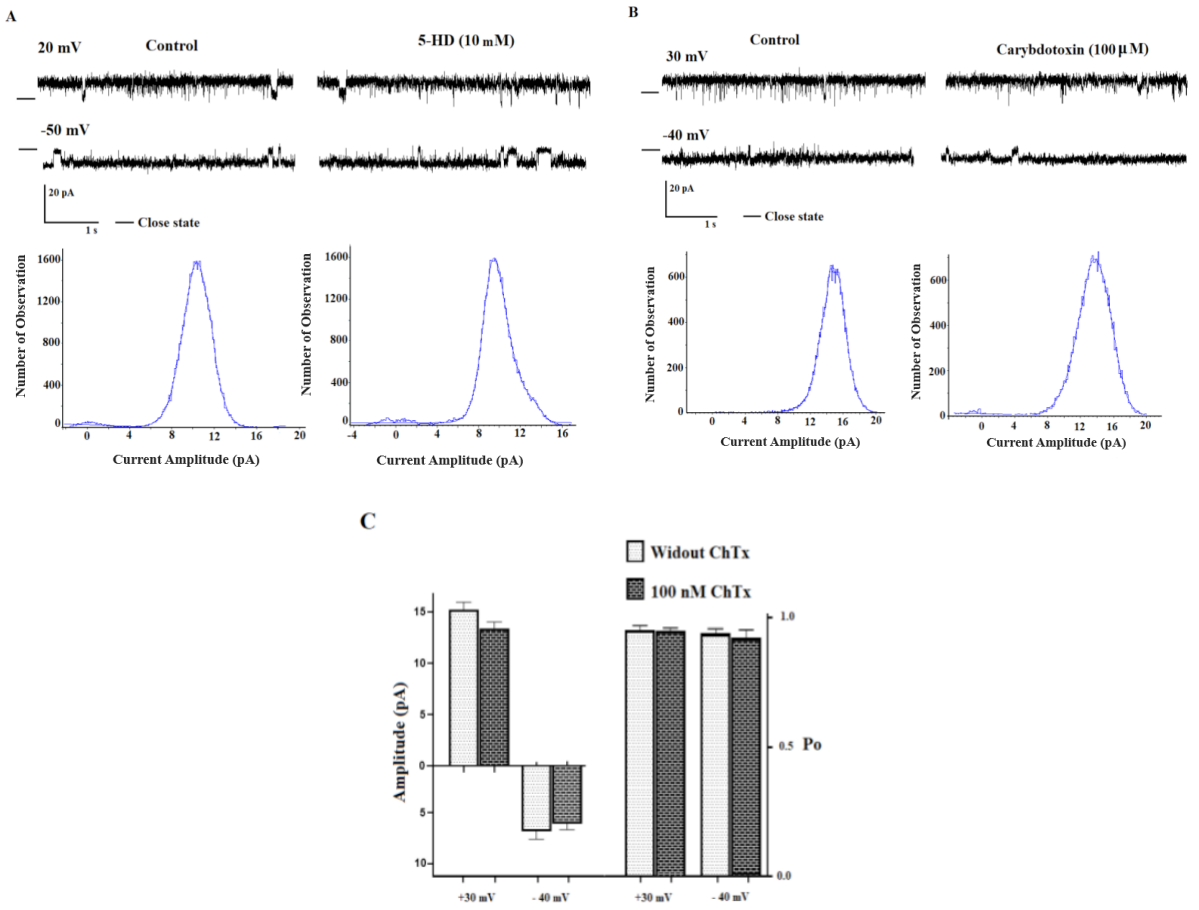

Figure 4. The effect of K^+ channel blocker (ChTx, 5-HD) on channel gating behaviour

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Note: Single-channel recording was conducted to measure the open probability of a 306 pS channel in asymmetric conditions (200/50 mM KCl, cis/trans) s with and without cis addition of (A) 5-HD (10 mM) at +20 and −50 mV, (n=3) and (B) ChTx (100 nM) at +30 and −40 mV, (n=4). There was no significant difference in the current amplitude and Po value in the presence of 5-HD and ChTx. The arrows indicate the closed levels. Data are Mean±SE (n=4).

the ER KATP channel in rat brain tissue. The findings of the current study revealed that the relationship between current and voltage (I-V plot) exhibited linearity, with a conductance of 306 pS under asymmetrical conditions (200/50 mM KCl, cis/trans) within the voltage range of -70 to +40 mV. Furthermore, the open Po of the channel remained unaffected across voltages ranging from +40 to -60 mV (Table 1). A growing body of literature provides strong evidence for the presence of KATP channels in intracellular organelles, such as mitochondria, across different tissues, including the liver, heart, lymphocytes, and brain. This evidence is obtained by reconstituting the mitochondria's inner membrane into a planar lipid bilayer (Garlid & Paucek, 2001; Leanza et al., 2017; Smith et al., 2017; Szabo & Zoratti, 2014) or using the patch-clamp technique (Choma et al., 2009; Costa & Garlid, 2008; Wojtovich et al., 2013). Sepehri et al. (2007) provided the first documentation of the presence of an ER ATP-sensitive channel in a rat hepatocyte with 500 ps conductance and voltage dependence on the sublevel (Sepehri et al., 2007). Then, in 2009, Ashrafpour et al. showed the electropharmacological behavior and dose response of ATP in this channel (Ashrafpour et al., 2008).

In the present study, the single-channel conductance of the ER in the brain was measured at 306 pS, which is lower than the previously reported 200 pS for the liver. Additionally, the KATP channel in the ER was voltage-independent. These differences may be due to the tissue structure and function of the organ. Additionally, biophysical properties, such as a single channel conductance (approximately 306 pS) of ER ATP-regulated K^+ channels from brain tissue described here, differ from the renal outer medullary K^+ (ROMK) channels expressed in the plasma membrane in renal and brain tissues (Welling & Ho, 2009) and brain mitochondrial inner membrane KATP channels (Choma et al., 2009). These differences

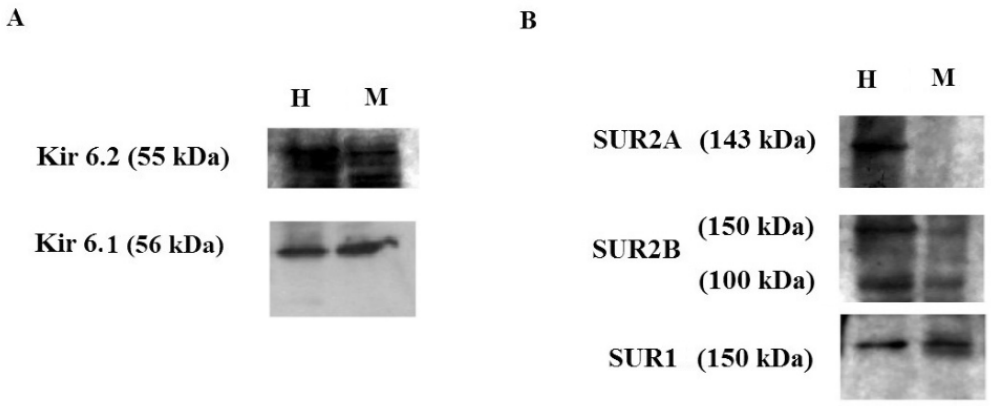

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Figure 5. Representative immunoblot analysis of Kir6.2, Kir6.1 and SUR subunits of RER- KATP in rat microsome fractions revealing the following results

Note: A) Goat anti-Kir6.2 (~55 kDa) and anti-Kir6.1 (~56 kDa) antibodies successfully identified bands in the microsome fractions, corresponding to molecular weights expected for Kir6.2 and Kir6.1 KATP subunits. Labeling indicates a band at ~55 kDa and ~56 kDa, referring to the expressed in ER obtained from brain preparations (n=4). B) Using the goat anti-SUR1 antibody, a ~150 kDa band was detected, whereas the goat anti-human SUR2B antibody labeled two bands at 150 and 100 kDa. 100 kDa may have formed as a result of 150 kDa proteolysis. No bands were identified in the microsome fraction using goat anti-SUR2A (~143 kDa) antibodies (n=4).

in conductance may be a result of various factors, including the composition of organelle membrane lipids, the presence of protein partners, and pre-/ posttranslational modifications of the pore and subunits forming the channel. This evidence confirms that the K^+ channel in the brain ER is different from other tissues and organelles of the cells.

Further experiments were conducted using well-known KATP channel blockers to verify the nature of the new brain ER channel observed in the rat brain preparations. According to pharmacological results, in the first step, addition of ATP (2.5 mM) to the cytoplasmic side (cis chamber) completely blocked the channel activity. To distinguish the KATP channel from the ATP-sensitive BKca channel (Maxi-KCa) (Fahanik-Babaei et al., 2011), ChTx (100nM), a Maxi-KCa channel blocker, was used, which did not affect channel activity. On the other hand, applying glibenclamide (100 µM), a specific inhibitor of KATP channels, blocked channel activity and provided further confirmation that the channel obtained from rat brain preparations was of the KATP channel type. Although we showed the purity of the ER preparation by western blotting, using 5-HD (10 mM) (as mitoKATP blocker) provides further confirmation that the recorded KATP channel activity was not mitochondrial. Ion channels are assembled in the lumen of the ER, moved to the Golgi apparatus, and then carried to their final location on the membrane of an organelle, the cell surface, or other proteins. Raising the question of whether the ER-expressed channel proteins are located in another intracellular or cell membrane, or if they have a functional role on the ER membrane itself. As mentioned, we evaluated the purity of the microsome preparation using western blotting. According to conventional methods of isolating the ER using ultracentrifugation in various cells, such as hepatocyte, fibroblast, etc. (Eliassi et al., 1997; Eriksson et al., 1983; Kan et al., 1992; Williamson et al., 2015), and also based on our previous experiences, we isolated the ER of a brain rat. The purity of

Table 1. Channel open probability (Po) at different membrane voltages

Voltage (mV)	-60	-50	-40	-20	-10	0	+10	+20	+30	+40
Open probability (Po)	0.9	0.91	0.9	0.95	0.94	0.95	0.94	0.96	0.9	0.9

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the rat brain ER was determined using several antibodies directed against specific marker proteins: actin (plasma membrane), cox1 (mitochondria), calnexin (ER), and 58K Golgi protein (Golgi apparatus). Since other markers, as well as pharmacological results, can be used to confirm the purity of an ER sample, a comparison of the electropharmacological properties of the purified channels may reveal significant differences or similarities with our previous results on mitochondrial and ER. In our study, 5HD (mitoKATP inhibitor) did not affect channel activity, and glibenclamide inhibited channel activity at positive but not negative voltages. Therefore, our results revealed that RER preparation from the brain fraction did not contain other subcellular structures (such as Golgi apparatus and mitochondria) or plasma membrane markers. Therefore, it can be concluded that the recorded channels were related to the RER of the rat brain.

KATP channels are octameric structures comprising an inwardly rectifying K^+ channel, and Kir6.x (as pore-forming) is associated with four regulatory SUR subunits (Inagaki et al., 1995). The key to defining pharmacological properties of KATP channels accurately is usually based on the inhibition of channel activity by blockers, such as ATP (Inoue et al., 1991), 5-HD (Jabůrek et al., 1998), and glibenclamide (Paucek et al., 1992) or activation of the channel by K^+ channel openers, such as diazoxide (Garlid et al., 1996).

Previous molecular studies have provided further confirmation of this channel type. KATP channels present in different intracellular organelles are structurally similar. For example, in many types of Kir6.x subunits in different parts of the cell with a KATP channel, the difference between Kir6.1 and Kir6.2 is only 30%, and they share 70% amino acid identity. Also, both subunits can assemble with various SUR subunits until they compose channels with different SUR subunits (Ng et al., 2010; Rodrigo & Standen, 2005). Wheeler et al. (2008) demonstrated that SUR1, SUR2A, and SUR2B can co-assemble in all possible pairwise combinations to form functional KATP channels, thereby conferring in pharmacological diversity. However, channel gating and function may be determined by their respective locations and membranes, as observed in the cell membrane or mitochondria. These channels play an essential role in each cell membrane, including determining the action potential and/or maintaining the neurotransmitter release (Laniado et al., 1997). They can also be co-assembled in various ways. For instance, skeletal muscle and the heart's sarcolemma contain KATP channels composed of the pore-forming subunit Kir6.2 and the regulatory subunit SUR2A (Aguilar-Bryan et al., 1998), while pancreatic tissue contains sKATP, which is made up of Kir6.2/SUR1 and is involved in insulin secretion (Hibino et al., 2010). Other pharmacological studies have suggested that SUR1 and SUR2B may also be expressed in a small number of fibers in the brain (Tricarico et al., 2006). Karschin et al. indicated the overlapping of Kir6.2 with SUR1 in various neurons in the rodent brain using the situ hybridization histochemistry method (Karschin et al., 1997). Furthermore, there are many reports that KATP channels are composed of the pore-forming subunit Kir6.2 or Kir6.1 at the subcellular level, and of different types of regulatory subunits of sulphonylureas. For example, Aggarwal et al. reported Kir6.2 with SUR2 in cardiac mitochondria (Aggarwal et al., 2013). Salari et al. (2015) found that Kir6.2 subunits combined with the regulatory subunits SUR1, SUR2A and SUR2B, in the ER of hepatocytes (Salari et al., 2015). Our analysis showed that both Kir6.2 and Kir6.1 subunits were present, providing further evidence of their existence.

Using antibodies against Kir6.2 and Kir6.1, we identified a ~55 kDa band for both subunits that was sensitive to the blocking peptide. Additionally, when using the antibody against sulphonylurea subunits, we observed two subunits, SUR1 and SUR2B, with a molecular mass of ~150 kDa for both Kir6.2 and Kir6.1. Western blotting analysis also indicated two bands at 150 kDa and 100 kDa for the SUR2B subunit. These findings are consistent with previous studies. Suzuki et al. proposed the presence of a Kir6.1-like 51 kDa protein in mitochondria (Suzuki et al., 1997). In the rat brain, Brustovetsky et al. noted two enriched bands that were sensitive to blocking peptide in mitochondrial fractions at ~50 kDa after labeling with anti-Kir6.1 (Brustovetsky, et al., 2005). Bajgar et al. reported that the mitoKATP channel activity in rat brain mitochondria contains 55kDa and 63-kDa SUR subunits (Bajgar et al., 2001). In two studies, Lacza et al. detected 51 kDa proteins as well as two enrichment bands ~130- and ~30-kDa in the heart and brain mitochondria using antibodies against Kir6.1 (Lacza et al., 2003a; Lacza et al., 2003b).

In the RER of the hepatocyte, Salari et al. observed specific bands at ~55 kDa. They identified all three SUR subunits (SUR1, SUR2A, and SUR2B) when labeling rat homogenate and the fraction of ER with anti-Kir6.1 (Salari et al., 2015). However, they also found two bands, ~150- and ~100-kDa, for SUR1 and SUR2B. In contrast to their results, we observed only bands of ~150- and ~100-kDa in the SUR2B subunit, but not in the SUR1 subunit, in the ER of the rat brain. Based on the results of the study, we suggest that the ER ATP-sensitive K^+ channel of the rat brain composed of a Kir6.2 subunit and SUR1 or SUR2B

subunits, as well as the presence of the Kir6.1 subunit, may be due to protein synthesis in the ER and do not play a functional role in these organelles. However, more studies are required to obtain more detailed information on how the Kir6.2 and SUR subunits combine.

One of the roles of the ER in the intracellular space is the regulation of [Ca^{2+}] during the neurotransmitter release and uptake. Studies have shown that during neuronal activity, K^+ and [Ca^{2+}] concentrations increase in the intracellular space (Perillán et al., 2000; Zawar et al., 1999), and in the subsequent phase, Ca^{2+} is recaptured in the ER lumen. Studies have indicated that Ca^{2+} is released from the ER membrane during nerve cell activity, leading to a negative charge inside the ER. Then, during Ca^{2+} uptake, a positive potential is generated within the ER lumen, which inhibits the Ca^{2+}-pumping function. Potent counter-ion movements via K^+ channels on the ER membrane occur to balance its membrane potential and maintain efficient Ca^{2+} release/uptake from or into this intracellular calcium store (Fink & Veigel, 1996; Takeshima et al., 2015). Therefore, an ER membrane potential should result from the relatively quick and significant charge translocation that is mediated by both Ca^{2+} uptake and its release (Fink & Veigel, 1996; Meissner, 1983; Morimoto & Kasai, 1986). Ca^{2+} uptake or release from the ER is inhibited in absence of this countercurrent (Baylor et al., 1984; Oetliker, 1982). Studies suggest that there may be a significant counter-ion flux during the Ca^{2+} uptake and release phases. This flux could consist of anions, such as Cl^- and HCO_3– moving in the same direction as Ca^{2+}, or cations, such as K^+, magnesium($^{2+}$) (Mg^{2+}), sodium (Na^+), and/or hydrogen ion (H^+), moving in the opposite direction. When tetanus begins in frog skeletal fibers, Somlyo et al. demonstrated that either Mg^{2+} or K^+ enters the sarcoplasmic reticulum (SR) during Ca^{2+} release (Al-Karagholi et al., 2017; Ashcroft, 2005; Rubaiy, 2016). However, it is evident that K^+ channels, such as ER ATP-sensitive K^+ channels, play a crucial role in the homeostasis of neurons' intracellular calcium, and defects in these channels can lead to neuronal death (Kuum et al., 2015).

The sensitivity of the KATP channels to ATP is due to the presence of SUR subunits. Compounds, such as glibenclamide, which are part of the sulfonylurea group and act as non-specific blockers of KATP channels, bind to the SUR subunit and inhibit KATP channel activity (Ashcroft, 2005). Previous studies have shown that SUR1-KATP channels are expressed in the brain (Al-Karagholi et al., 2017). Additionally, several studies have indicated that glibenclamide has a higher affinity for SUR1 than for the other subunits, which have comparatively less access to the central nervous system (Lahmann et al., 2019; Rubaiy, 2016). Therefore, to determine if the ER K^+ channel in a rat's brain corresponds to the ATP-sensitive K^+ channel, we investigated the effects of applying glibenclamide on channel activity. According to our results, glibenclamide (100 µM) inhibited channel activity at positive potentials. However, at negative potentials, it did not impact the unitary current amplitude and open Po of the channel. The difference between our findings and previous research could be attributed to differences in the method, organ, and/or behavior channels. Some researchers have suggested that the pathway's sensitivity to sulfonylurea inhibition is affected by the cell's cytoskeleton (Brady et al., 1996). The low affinity for glibenclamide-induced channel inhibition in our study may have been partially increased in the absence of a cytoskeleton, since ER extraction disrupts the cell cytoskeleton. Furthermore, it has been suggested that glibenclamide may function by binding to the inner mouth or the voltage-gated of the channel, increasing the hydrophobic interactions between the two and stabilizing the channel in its inactivated state (Mayorga-Wark et al., 1996).

Conclusion

This study provides the first evidence for the presence of an RER KATP channel in the rat brain. We demonstrated that the K^+ channel on the RER membrane is not voltage-dependent, sensitive to ATP and glibenclamide, and insensitive to 5-HD and ChTx. Finally, we propose that the KATP channel in the RER membrane may play a role in regulating the ER within neurons, which in turn may enhance neuronal function associated with action potential generation and neurotransmitter release.

Ethical Considerations

Compliance with ethical guidelines

All experiments were executed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996). this study was approved by the Research Ethics Committee of Laboratory Animal of Tehran University of Medical Sciences, Tehran, Iran (Code: IR.TUMS.AEC.1401.198).

Funding

This study was supported by grants from the Electrophysiology Research Center, Tehran University of Medical Sciencesm, Tehran, Iran, and the Neuroscience Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Authors' contributions

Conceptualization, methodology, and formal analysis, Javad Fahanik-Babaei; Resources: Maryam Nazari and Reza Saghiri; Data curation Reza Saghiri and Javad Fahanik-Babaei; Formal analysis, and writing the original draft: Maryam Nazari; Project administration, review and editing: Afsaneh Eliassi and Javad Fahanik-Babaei.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors thank Neuroscience Research Center of Shahid Beheshti University of Medical Sciences ND, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran.

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