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

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Abstract

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 channel (KATP) with various functional roles has been identified in the endo/sarcoplasmic reticulum membranes of both excitable and non-excitable cells. Our Previous studies have investigated the electro pharmacological and molecular properties of KATP and Bkca+2 channels in the rough endoplasmic reticulum (RER) of rat hepatocytes. In this study, for the first time, we described the electro-pharmacological and molecular properties of the RER ATP-sensitive potassium channel in rat brain cells using an incorporated single channel in the planar lipid bilayer and western blotting analysis. The results of the study revealed the presence of an ATP-sensitive potassium channel with a conductance of 306 pS, and the open probability was found to be voltage-independent at a holding potential 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 the positive and negative potentials and 100 μM glibenclamide to the positive voltages inhibited the channel activity. Interestingly, 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, SUR1, and/or SUR2B, but not SUR2A, in the RER of rat brain fractions. In this study, we provide strong evidence for the existence of an ATP-sensitive potassium channel on the RER membrane of rat brain cells, displaying different pharmacological properties than those classically described for the plasma membrane and other intracellular organelles.

Keywords: Potassium channels, RER K_{ATP} channel, Bilayer lipid membrane, Single channel recording

1. Introduction

The balance of intracellular potassium (K+), sodium (Na+), and calcium (Ca2+) concentrations determines the regulation of ion homeostasis, a normal physiological process that preserves the integrity of the plasma membrane and intracellular organelles. The regulation of intracellular ion homeostasis is significantly facilitated by ion channels, including potassium channels. These channels have an important role in cellular processes such as Ca^{2+} signaling and volume regulation, generation of pH gradient, cell death, oxidative stress production, differentiation, proliferation, etc. (Averaimo, Milton, Duchen, & Mazzanti, 2010; Edwards & Kahl, 2010; Jehle, Schweizer, Katus, & Thomas, 2011; Wulff, Castle, & Pardo, 2009). Regarding their crucial role, modifying the activity of these potassium 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, Palko-Labuz, Sroda-Pomianek, & Michalak, 2019; Waza, Bhat, Hussain, & Ganai, 2018), and, more recently identified, as targets for limb wound repair and regeneration (Zhang, Das, Kelangi, & Bei, 2020) and the pathophysiology of migraine (Al-Karagholi, Hakbilen, & Ashina, 2022; Kokoti, Al-Karagholi, & Ashina, 2020).

Several types of potassium channels are identified in the membranes of the intracellular organelles, such as mitochondria, nucleus, and endoplasmic reticulum (Checchetto, Teardo, Carraretto, Leanza, & Szabo, 2016). Multiple lines of evidence demonstrate that distinct types of potassium channels, including ATP-sensitive potassium channels (K_{ATP}), Ca^{2+}-activated potassium channels (BKCa), and potassium permeable trimeric intracellular cation channels (TRIC channels), have a range of functional roles (Guéguinou et al., 2014; Ng, Schwarzer, Duchen, & Tinker, 2010; Salari et al., 2015; Yazawa et al., 2007), some studies have been reported on the endo/sarcoplasmic reticulum membranes in hepatocytes and neuronal HT-22 cells (Khodaee, Ghasemi, Saghiri, & Eliassi, 2014; Richter et al., 2016; Salari et al., 2015). The channels facilitate the fluxes of potassium through the membrane of the endoplasmic reticulum (ER), and it appears that their main role is to maintain the balance of the charge movement during Ca2+ 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 Ca2+ ions away from the ER membrane potential (Kuum, Veksler, & Kaasik, 2015; Li, Um, & Mcdonald, 2006; Xu, Martinoia, & Szabo, 2015). The octameric complex known as the ATP-sensitive potassium channel (KATP) is inhibited by physiological ATP levels. It consists of four pore-forming subunits (Kir6.X) encircled by four regulatory sulfonylurea receptors, also known as ATP-binding subunits (SUR) (Clement IV et al., 1997). This channel found both in the membrane and subcellular membranes such as mitochondrial inner membrane ($mitoK_{ATP}$) (Inoue, Nagase, Kishi, & Higuti, 1991; Paggio et al., 2019), the nuclear membrane (Quesada et al., 2002) and rough endo/sarcoplasmic reticulum (Salari et al., 2015).

ER K_{ATP} channels and their subunits have been shown to exist in a variety of cells (cell culture), tissues, and organs in recent years, according to a number of electro-pharmacological and molecular studies (Kuum, Veksler, Liiv, Ventura-Clapier, & Kaasik, 2012; Ng et al., 2010; Salari et al., 2015; Zhou et al., 2005). In a study conducted by Zhou et al. (2005), the subcellular distribution of Kir6.2 in neuronal cells was observed 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 and colleagues provided evidence underlying the existence of the $Kir_{6.1}$ subunit on the endoplasmic reticulum membrane of C2C12 and HEK293 cells, which played an important role in modifying the Ca^{2+} release from

intracellular stores (Ng et al., 2010). Kuum et al. (2012) showed that K_{ATP} 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 with 560 pS conductance in the liver that was sensitive to ATP (2.5 mM), Glibenclamide, and Tolbotamide but not sensitive to Iberotoxin and Carybdotoxin. Furthermore, western blot analysis showed the expression of $Kir_{6.2}$, SUR1 and/or SUR2B, and SUR2A in RER fractions (Salari et al., 2015).

To the best of our knowledge, despite the abundance of research on endoplasmic reticulum ion channels - particularly potassium channels- in a variety of cells and tissues, no study has examined directly the electrophysiological characteristics of the brain's ER ATP-sensitive potassium channel. Therefore, using isolated ER membrane particles reconstituted into a planar lipid bilayer, we examined the biophysical and electropharmacological characteristics of the brain's ER ATP-sensitive potassium channel in this study. Furthermore, we aimed to identify the molecular structure of an ATP-sensitive potassium channel located in the endoplasmic reticulum membrane of rat brain, as variations in the expression of constituent subunits of these channels can affect their gating behavior.

2. Materials and Methods

2.1. 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 ºC, 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 (IR.TUMS.AEC.1401.198).

2.2. Materials

According Singleton and colleague's method, L-a-phosphatidylcholine (L-a-lecithin) was extracted from fresh egg yolk (Singleton, Gray, Brown, & White, 1965). Sucrose, Imidazole, Pyrophosphate, Potassium chloride, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trisma base), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), Glibenclamide, ATP, 5-HD and Charybdotoxin (ChTx), were purchased from Sigma Aldrich (St Louis, MO, USA). n-decan was purchase from Merck (Darmstadt, Germany). Chamber and cup for bilayer formation (Warner Instrument Corp., Hamden, CT USA),

2.3. RER isolation of whole brain

ER membrane particles derived from the rough endoplasmic reticulum 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 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 a surgical cotton fabric filter. 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 minute (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 minute in a sucrose gradient (Step 3). The resulting pellet was dissolved in a 15 ml 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 two times at 140000 ×g for 47 minute. 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 a temperature of 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 a pH of 7.2, and stored at -80°C for one month.

2.4. Immunoblot analysis

Protein samples were quantified using the Bradford protocol, with the standard being bovine serum albumin (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 (PVDF) membrane. After that, the membrane was blocked for two hours at room temperature using a TBS solution that contained 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 4º 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, to an ECL kit for chemiluminescence detection, and the immuno-reactive bands were visualized using the Amersham ECL prime western blotting detection (GE Healthcare Life Sciences).

2.5. 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 KCl at a concentration of 200 mM, while the trans chamber contained 4 ml of KCl at a concentration of 50 mM (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 by mechanical means. The formation and thinning of the bilayer were monitored through capacitance measurements and optical observations, with bilayers exhibiting a capacitance of approximately 150 to 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 Instrument, USA). Electrical connections were established using Ag/AgCl electrodes and agar salt bridges (3 M KCl) in order to minimize liquid junction potentials. The cis chamber was voltage-clamped in relation to the grounded trans chamber. Subsequently, the signals underwent filtration at 1 kHz, were digitized at a sampling rate

of 10 kHz using the A/D converter Axone instrument from the 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 the conductance was determined from the current-voltage relationship, which was then averaged from 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 P Clamp 10. Additionally, the mean closed and open times of the channel, as well as P(open), were calculated from segments of continuous recordings lasting 60 s. Any observed differences were assessed for significance using Student's t test, and the data are presented as mean ± S.E.

3. Results

3.1. Purity of endoplasmic reticulum fractions

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

3.2. Single channel properties of ATP-sensitive potassium channel in rat brain RER membrane

In order to offer a more comprehensive account of the electrophysiological properties of the ATP-sensitive potassium channel located on the endoplasmic reticulum (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 (Fig. 1B). Our results revealed a potassium 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, which confirms that the examined channel is selective towards cations. The mean reversal potential calculated from fitting 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). The single-channel I-V relationship obtained from six recordings under the same conditions is illustrated in Figure 2B. The I-V plot showed linearity without any evidence of inward rectification at potentials between +40 and -70 mV.

In the condition of asymmetric concentration (200/50 mM KCl; cis/trans), it was observed that the open probability (Po) of the channel was not influenced by voltage when maintained at a holding potential between +40 to -60 mV. The Po remained constant regardless of positive or negative voltages. The average steady-state Po values as a function of the holding potential for the full open conducting state obtained in six different experiments are presented in Table 1.

3.3. Pharmacological properties of the ion channel

Further experiments were conducted to confirm the nature of the RER potassium channel from the rat brain preparation. So, we examined the effect of several types of potassium channels blockers on the channel activity as described in the fallowing.

3.3.1. Effect of ATP on channel activity

Initially, we investigated the effects of ATP, a well-known K_{ATP} channel blocker. Figure 3A illustrates the single-channel recordings and open probability 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 face. The presence of ATP had a notable impact on channel amplitude and open probability at both positive and negative voltages, leading to complete blockade of channel activity at these potentials (n=5). These findings are summarized in the bar graph depicted in Fig. 3C.

3.3.2. Effect of glibenclamide on channel activity

An investigation was conducted to analyze the impact of glibenclamide, a well-known sulfonylurea, and K_{ATP} channel blocker, on RER channel activity. As shown in Fig. 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 Fig. 3C.

3.3.3. Effect of 5-HD as a mitochondrial K_{ATP} channel blocker on the channel activity

In the next step, we examined the effect of 5-HD, as a mitoK_{ATP} channel blocker, on channel activity. As shown in Fig. 4A, addition of 5-HD (10 mM) into the cis side had no effect on channel activity (single-channel recordings and open probability) in both positive and negative voltage (n=3). Data are expressed as means ± S.E.

3.3.4. Effect of ChTx as Ca^{2+}-dependent K^+ channels blockers activity

Since both ATP-sensitive BKCa channels (Maxi-KCa) (Fahanik-Babaei, Eliassi, & Saghiri, 2011) and K_{ATP} 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 the use of 1mM ChTx did not inhibit channel activity. Single-channel recordings and open probability at +30 and -40 mV (n=4) are depicted in Figure 4B, with a summary presented in the bar graph on the right panel of Fig. 4C. These findings confirm that the channel derived from rat brain RER preparation corresponds to the KATP channel.

3.4. Investigation of RER K_{ATP} 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 K_{ATP} 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 derived from rat brain cells. Specifically, we employed antibodies targeting the KATP Kir6.2 and Kir6.1 subunits as pore-forming subunits, as well as the SUR1, SUR2A, and SUR2B subunits serving as regulatory elements. The use of anti-$Kir_{6.2}$ and anti-$Kir_{6.1}$ antibodies for labeling revealed a distinct band at approximately 55 kDa, which corresponds to the

anticipated molecular weight of Kir6.2 and Kir6.1 proteins (Fig. 5A) (n = 3). Analysis of the sulphonylurea subunit, as depicted in Fig. 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 $Kir_{6.2}$ and $Kir_{6.1}$ subunits, as well as SUR1 and SUR2A subunits, in both the homogenate and RER membrane preparation.

4. Discussion:

The endoplasmic reticulum (ER) plays a crucial role in the synthesis, folding, and transportation of proteins, and is responsible for various important cellular functions including cell signaling and storage of calcium ions (Ca^{2+}). It is well documented that intracellular organelles like mitochondria, endo/sarcoplasmic reticulum, nucleus, etc., contain potassium channels similar to those found on the plasma membrane (Ballanyi, 2004; Noma, 1983; Rusznak et al., 2008). In this study, we identified the electrophysiological and molecular structure of the ATP-sensitive potassium channel in the endoplasmic reticulum membrane of the rat brain using single channel recording and western blotting techniques. A study conducted on the reconstitution of the endoplasmic reticulum (ER) membrane from rat brain tissue has revealed the presence and activity of a potassium channel with a conductance of 306 pS. This potassium channel was found to be sensitive to ATP and glibenclamide, both of which are well-known blockers of KATP potassium channels. Additionally, the use of specific antibodies targeting Kir6.1, Kir6.2, and SURs 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 the ER KATP channel derived from 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 probability of the channel remained unaffected by different voltages between +40 and -60 mV (Table 1). A growing body of literature provides strong evidence of the K_{ATP} channel properties existing in intracellular organelles such as mitochondria in different tissues, like the liver, heart, lymphocytes, and brain. This evidence is obtained by reconstituting the mitochondria inner membrane into a planar lipid bilayer (Garlid & Paucek, 2001; Leanza et al., 2017; Smith, Nehrke, & Brookes, 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 endoplasmic reticulum ATP-sensitive channel in a rat hepatocyte with 500 ps conductance and voltage dependence on the sublevel (Sepehri, Eliassi, Sauvé, Ashrafpour, & Saghiri, 2007). Then, in 2009, Ashrafpour et al showed the electropharmacological behavior and dose response of ATP in this channel (Ashrafpour, Eliassi, Sauve, Sepehri, & Saghiri, 2008).

In the present research, it was found that the single channel conductance of the endoplasmic reticulum (ER) in the brain measured at 306 pS, which is lower than the previously reported 200 pS for the liver. Additionally, it was observed that the ATP-sensitive potassium 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 potassium channels from brain tissue described here, differ from the ROMK channels expressed in the plasma membrane in renal and brain tissues (Welling & Ho, 2009) and brain mitochondrial inner membrane K_{ATP} channels (Choma et al., 2009). These differences in conductance may be as 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 the potassium channel in brain ER is different from other tissues and organelles of the cells.

Further experiments were conducted using well-known ATP-sensitive potassium 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 K_{ATP} channel from the ATP-sensitive BKca channel (Maxi-KCa) (Fahanik-Babaei, Eliassi, Jafari, et al., 2011), Charybdotoxin (100nM) as a Maxi-KCa channel blocker was used which did not have any effect on the channel activity. On the other hand, applying glibenclamide (100 µM) as a specific inhibitor of K_{ATP} channel block the channel activity and make more confirmation that the channel obtained from rat brain preparations is the KATP channel type. Although we showed the purity of the ER preparation by western blotting, but using 5-HD (10 mM) (as mitoK_{ATP} blocker) made more confirmation that the recorded K_{ATP} channel activity was not the mitochondrial type. Note that 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 microsome preparation using the western blotting technique. According to conventional methods of isolating the endoplasmic reticulum using ultracentrifugation in various cells, such as hepatocyte, fibroblast, etc. (Eliassi, Garneau, Roy, & Sauve, 1997; Eriksson, Torndal, & Andersson, 1983; Kan, Jolicoeur, & Paiement, 1992; Williamson, Wong, Bozidis, Zhang, & Colberg-Poley, 2015), and also based on our previous experiences, we isolated the endoplasmic reticulum of a brain rat. The purity of the rat brain endoplasmic reticulum was determined using several antibodies directed against specific marker proteins: actin (plasma membrane), cox1 (mitochondria), calnexin (endoplasmic reticulum), 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 show significant differences or similarities from our previous results on the mitochondrial and endoplasmic reticulum. 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 rough endoplasmic reticulum preparation coming from brain fraction did not contain other subcellular (such as Golgi apparatus and mitochondria) and plasma membranes markers. So, it can be concluded that the recorded channels were related to the rough endoplasmic reticulum of the rat brain.

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

Previous molecular studies have provided more confirmation on this type of channel. ATP-sensitive potassium channels existing on the different intracellular organelles are structurally very similar. For example, in many types of Kir6.x subunits in different parts of the cell with a K_{ATP} 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). In a study

conducted by Wheeler and colleagues in 2008, it was demonstrated that SUR1, SUR2A, and SUR2B can co-assemble in all possible pair-wise combinations to form functional KATP channels, resulting in pharmacological diversity (Wheeler et al., 2008). However, the channel gating and their function may be determined by their respective location and membrane, like what is seen in the cell membrane or mitochondria, etc. These channels play an essential role in each cell membrane, including determining the action potential and/or maintaining the neurotransmitter release (Laniado, Abel, & Lalani, 1997). They can also be co-assembled in a variety of 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 research have suggested that SUR1and SUR2B may also be expressed in a small number of fibers in the brain (Tricarico et al., 2006). Karschin and colleagues indicated the overlapping of $Kir_{6.2}$ with SUR1 in various neurons in the rodent brain using in situ hybridization histochemistry method (Karschin, Ecke, Ashcroft, & Karschin, 1997). Furthermore, there are many reports that K_{ATP} channels are composed of the pore-forming subunit $Kir_{6.2}$ or $Kir_{6.1}$ at the subcellular level and in the different types of regulatory subunits of sulphonylurea. For example, Nitin and colleagues (2013) reported $Kir_{6.2}$ with SUR2 in cardiac mitochondria (Aggarwal, Shi, & Makielski, 2013). In a study by Salari et al (2015), it was also found that Kir6.2 subunits combined with the regulatory subunits SUR1, as well as both SUR2A and SUR2B, in the endoplasmic reticulum of the hepatocyte (Salari et al., 2015). Our analysis showed that both Kir6.2 and Kir6.1 subunits were present, providing further evidence of their existence.

Using the antibodies against $Kir_{6.2}$ and $Kir_{6.1}$, we identified a band with a molecular mass of ~55 kDa for both subunits that was sensitive to 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 $Kir_{6.2}$ and $Kir_{6.1}$. Our analysis using western blotting also indicated two enriched bands of 150 kDa and 100 kDa for the SUR2B subunit. These finding are consistent with previous research findings. 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 fractions of mitochondrial at ~50 kDa after labeling with anti-$Kir_{6.1}$(Brustovetsky, Shalbuyeva, & Brustovetsky, 2005). Bajgar et al reported mitoK_{ATP} channel activity contains 55kDa and 63-kDa SUR subunits in rat brain mitochondria (Bajgar, Seetharaman, Kowaltowski, Garlid, & Paucek, 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, Snipes, Kis, et al., 2003; Lacza, Snipes, Miller, et al., 2003).

In the RER of the hepatocyte, Salari et al. observed specific bands at ~55 kDa and identified all three subunits of SUR (SUR1, SUR2A, and SUR2B) when labeling rat homogenate and the fraction of ER with anti-$Kir_{6.1}$ (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 results of the study, we suggest that the ER ATP sensitive K^+ channel of the rat brain composed of a $Kir_{6.2}$ 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 needed to obtain more detailed information on how the $Kir_{6.2}$ 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^+ concentrations and [Ca^{2+}] increase in the intracellular space (Perillán et al., 2000; Zawar, Plant, Schirra, Konnerth, & Neumcke, 1999), and in the next 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 take place to balance its membrane potential and maintain efficient Ca^{2+} release/uptake from or into this intracellular calcium store (Fink & Veigel, 1996; Takeshima, Venturi, & Sitsapesan, 2015). Therefore, an Endoplasmic Reticulum membrane potential should result from the relatively quick and large charge translocation that is mediated by both Ca2+ uptake and its release (Fink & Veigel, 1996; Meissner, 1983; MORIMOTO & KASAI, 1986). Ca2+ uptake or release from the ER is inhibited in without the presence of this counter current (Baylor, Chandler, & Marshall, 1984; Oetliker, 1982). Studies suggests that there may be a significant counter-ion flux during the Ca2+ uptake and release phases. This flux could consist of anions like Cl– and HCO3– moving in the same direction as Ca2+, or cations like K+, Mg2+, Na+, and/or H+ moving in the opposite direction. When tetanus begins in frog skeletal fibers, Somlyo and colleagues demonstrate that either Mg2+ or K+ enter the SR during Ca2+ release (Al-Karagholi, Hansen, Severinsen, Jansen-Olesen, & Ashina, 2017; Ashcroft, 2005; Rubaiy, 2016). However, it is evident that potassium 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 K_{ATP} channels to ATP is due to the presence of SUR subunits. Compounds like glibenclamide, which are part of the sulfonylurea group and act as non-specific blockers of K_{ATP} channels, are known to bind to the SUR subunit and inhibit the KATP channel activity (Ashcroft, 2005). Previous studies have shown that SUR1-K_{ATP} channels are expressed in the brain (Al-Karagholi et al., 2017). Additionally, several studies have indicated that glibenclamide has higher affinity for SUR1 than the other subunits that have comparatively less access to the central nervous system (CNS) (Lahmann, Kramer, & Ashcroft, 2019; Rubaiy, 2016). Therefore, to determine if the endoplasmic reticulum potassium 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, at positive potentials, glibenclamide (100 μM) inhibited channel activity, However, at negative potentials, it did not impact the unitary current amplitude and open probability of the channel. The difference between our findings and previous research could be attributed to differences in the method, organ, and/or behavior channel. Some researchers have suggested that the sensitivity of the pathway to sulfonylureas inhibition is affected by the cell's cytoskeleton (Brady, Alekseev, Aleksandrova, Gomez, & Terzic, 1996). (Brady, Alekseev, Aleksandrova, Gomez, & Terzic, 1996). The low affinity in our study for glibenclamide-induced channel inhibition may have been partially increased in the absence of a cytoskeleton since endoplasmic reticulum extraction entails disruption of the cell cytoskeleton. Furthermore, it has been suggested that glibenclamide may function by binding to the inner mouth or the voltage gate of the channel, increasing the hydrophobic interactions between the two and stabilizing the inactivated state of the channel (Mayorga-Wark, Dubinsky, & Schultz, 1996).

Conclusion

In summary, in the present study, we provided the first evidence underlying the presence of a rough endoplasmic reticulum K_{ATP} channel in the rat brain. We demonstrated that potassium channel on the RER membrane is not voltage-dependent, sensitive to ATP and glibenclamide, and insensitive to 5-HD and ChTx. Finally, we proposed that the K_{ATP} channel in the RER membrane may plays a role in regulating the endoplasmic reticulum within neurons, which in turn may enhance neuronal function associated with action potential generation and neurotransmitter release.

Author contributions

Maryam Nazari: Contribution in writing original draft, Resources, Formal analysis. Afsaneh Eliassi: Writing – review and editing, Project administration. Reza Saghiri: Resources; Data curation. Javad Fahanik-babaei: Conceptualization; Data curation; Formal analysis, Methodology; Project administration Writing – review and editing.

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Data availability

Data and material are available upon request to the corresponding author.

Declarations

Ethics approval

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) and approved by the Research Ethics Committee of Laboratory Animal-Tehran University of Medical Sciences (IR.TUMS.AEC.1401.198).

Conflict of Interest

The author declares no conflict of interest.

Consent for publication

Not applicable

Consent to participate

Not applicable

Figure legends

Figure1. Western blots analysis and single channel recording. (A) Purity in the microsome preparation in the whole brain was assessed using plasma membrane markers: Actin (C-11); 45 kDa. Golgi apparatus marker: 58 K Golgi protein; 58 kDa. endoplasmic reticulum marker: Calnexin, 90 kDa. mitochondrial membrane marker: Cox1 (1D6), 39 kDa; The method involved the following steps: Step1: Homogenate, Step2: High speed centrifuge, Step3: After gradient sucrose and Step4: Final preparation. **(B)** Single channel recordings of three different potassium channels of rough endoplasmic reticulum in rat brain in planar lipid bilayer at +30 mV.

Figure 2. Single channel recordings and current voltage relationship. (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 state. **(B)** The current voltage relationship for the 306 pS channel in asymmetrical condition (200 mM KCl cis/50 mM KCl trans).

Figure 3. The effect of potassium channel blocker (ATP, glibnclamide) on channel gating behaviour. 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 ± S.E. Closed levels are indicated by –.

Figure 4. The effect of potassium channel blocker (charybdotoxin, 5-HD) on channel gating behaviour. 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)** Charybdotoxin (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 Charybdotoxin. The arrows indicate the closed levels. Data are mean ± SE (n = 4).

Figure 5. Representative immunoblot analysis of $Kir_{6.2}$, $Kir_{6.1}$ and SUR subunits of rER-K_{ATP} in rat microsome fractions revealed the following results. (A) The goat anti-$Kir_{6.2}$ (~55 kDa) and anti-$Kir_{6.1}$(~56 kDa) antibodies successfully identified bands in the microsome fractions, corresponding to molecular weights expected for $Kir_{6.2}$ and $Kir_{6.1}$ K_{ATP} subunits. Labeling indicates a band at ~55 kDa and ~56 KDa referring to the expressed in endoplasmic reticulum obtained from brain preparations (n=4). (B) Using the goat anti-SUR1 antibody, ~150 kDa

band was detected, whereas the goat anti-human SUR2B antibody led to the labeling of two bands at 150 and 100 kDa respectively. 100 kDa may have formed as a result of 150 kDa proteolysis. No bands were identified in microsome fraction using the goat anti-SUR2A (~143 kDa) antibodies (n=4).

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Fig 1.

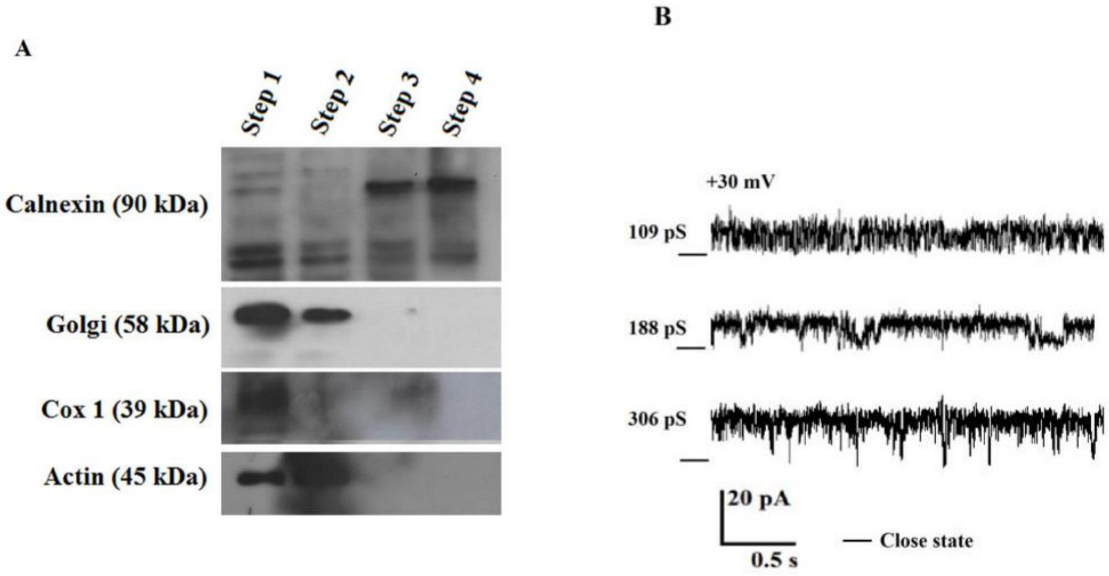


Fig 2.

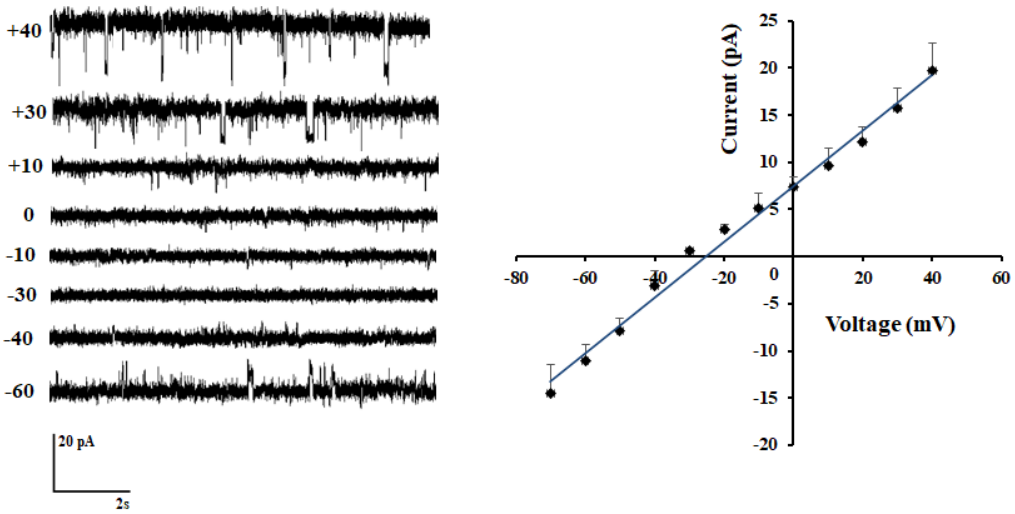
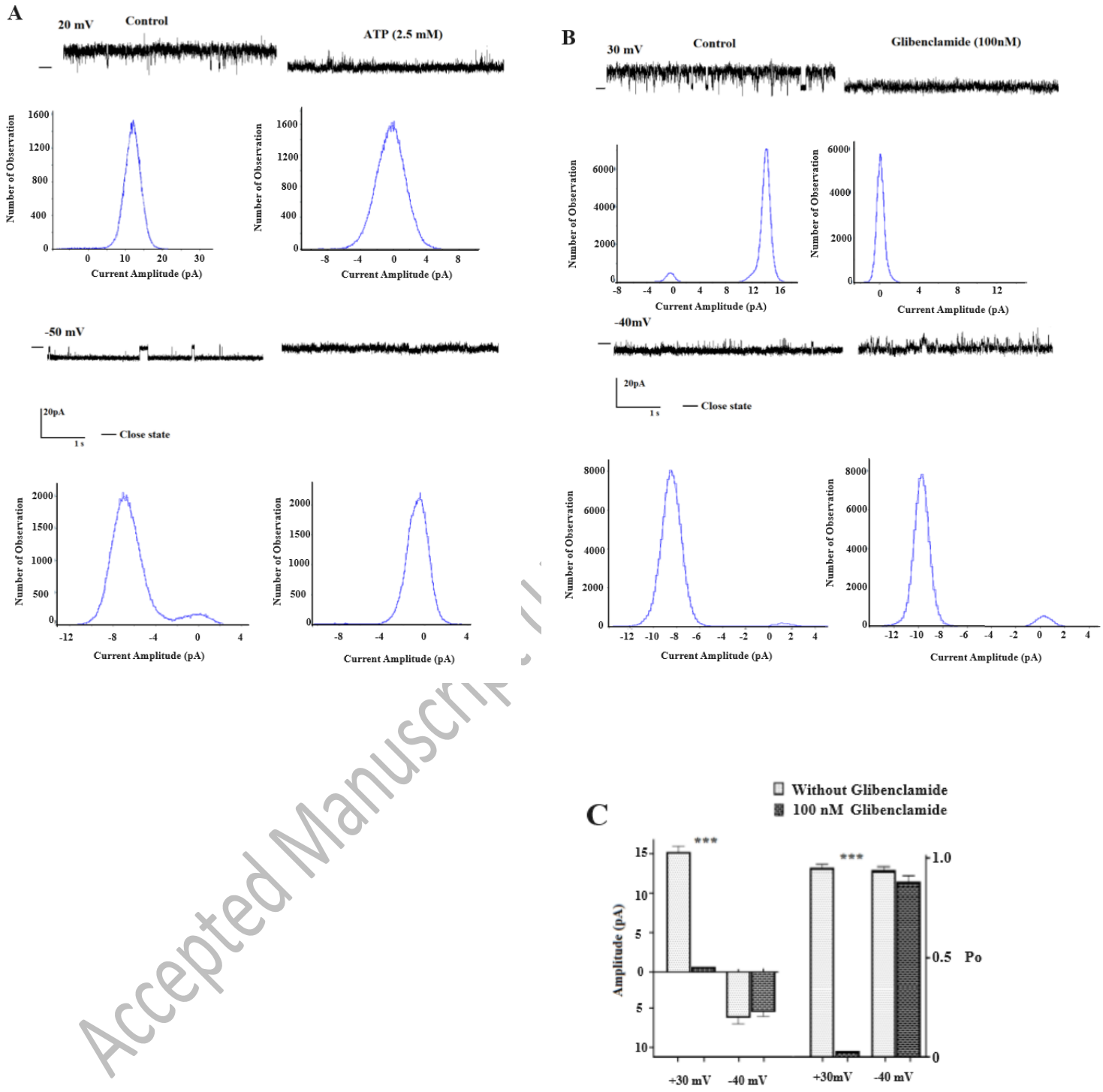


Fig3.



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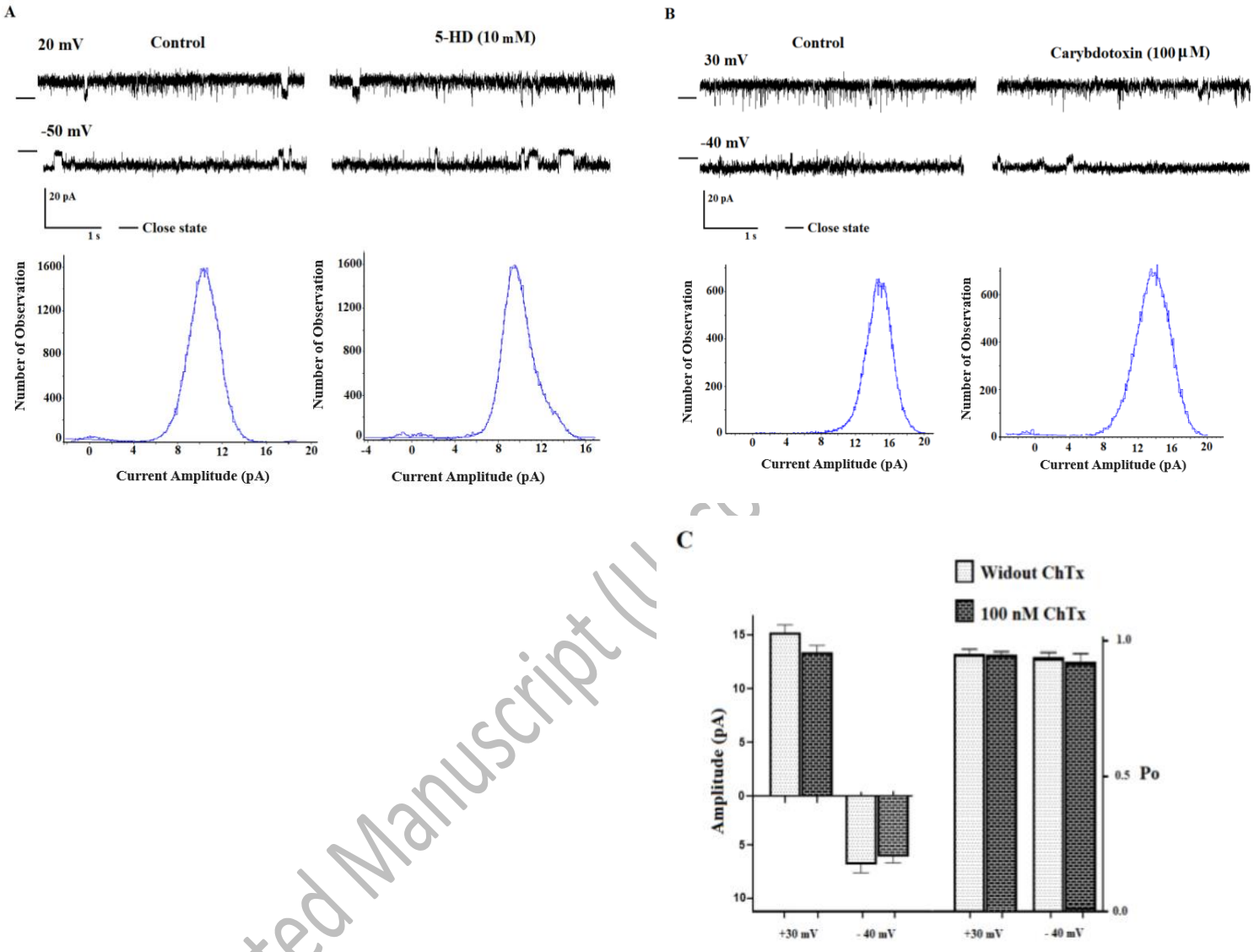

Fig 5.

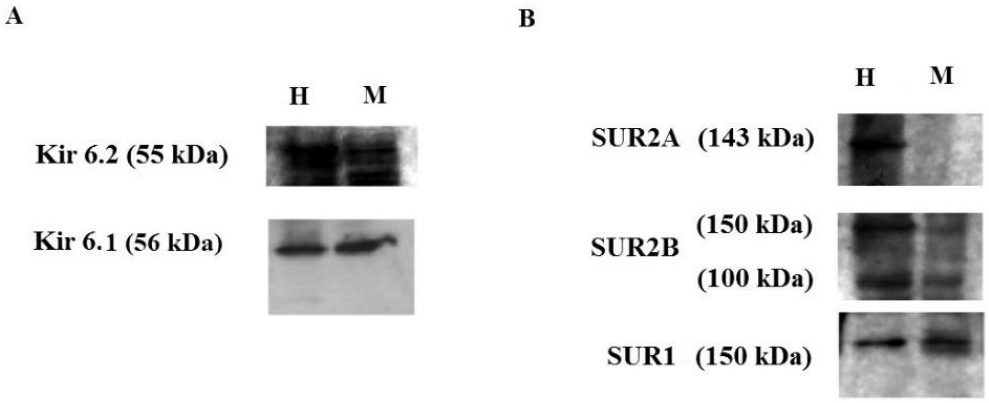

Table 1. Channel open probability (Po) at different membrane voltages

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**