Chapter 4

The Role of Calcium-activated Potassium Channel in Mitochondria-Associated ER Membrane and Its Functional Link to Cell Survival and Death

Ackuvinas Swakin Annammal Vargheese,
Krishnan Sundar and Thandavarayan Kathiresan

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Abstract

The process of apoptosis is not only regulated by molecular gens but it is also regulated by cellular ionic homeostasis especially K^+ homeostasis in the cell. In the past decade, molecular mechanisms of ionic regulation of apoptosis have been extensively investigated. The ionic mechanism of apoptosis are involves Ca^{2+} influx and accumulation of intracellular Ca^{2+} is convincing evidence to excessive K^+ efflux resulting in early steps in apoptosis. The BK channels play a critical role in mediating the K^+ efflux linked with apoptotic cell shrinkage. Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondria. The BKα subunits are localized in the inner mitochondrial and ER membrane and directly interact with other BK channel associated proteins (BKAPs) like, IP3R-1, calreticulin at the ER face of the MAMs, and the molecular chaperone grp78, which bridges the IP3R-1 with voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (OMM). The present chapter clearly depicts that how BK channels are associated with BKAPs and how they are involved in apoptosis through regulation of K^+ efflux.

Keywords: BK channel, Ca^{2+} signaling, ER-mitochondria juxtaposition, elution of protein complexes, protein-protein interactions

1. Introduction

In cochlea, the BK channels are localized in both presynaptic and extrasynaptic zone near the apical portion of inner hair cells (IHC) and outer hair cells (OHC). The BK channels are

known to be involved in noise-induced hearing loss (NIHL) [1] through activation of Ca^{2+} induced Ca^{2+} release and ROS pathway by association of BKAPs like SOD, peroxidase, catalase and GSTμ, [2]. In addition, BK channel is known to be associated with deafness proteins like γ-actin and methylthioadenosine phosphorylase (MTAP) [3]. The molecular mechanisms that regulate the BK channel and their role in NIHL and deafness remain unclear. Therefore, understanding mechanisms of BK channel regulation and its associated proteins (BKAPs) will provide insights in understanding the problems in deafness and NIHL.

Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondria. We found that BKα subunits [2] are localized in the inner mitochondrial membrane and interacted directly with other BKAPs like, IP3R1, calreticulin at the ER face of the MAMs, and the molecular chaperone grp78, which bridges the IP3R-1 with voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (OMM) [4]. The BK channel is associated with all other proteins having a contribution in mitochondria-associated ER membranes. Therefore, the functional regulation of BK channel and its role in MAMs remains unclear.

The novel concept of mechanism of apoptosis is in addition to molecular genes, ionic homeostasis also induces apoptosis especially K^+ in cell [5]. The ionic mechanism of apoptosis associates the accumulation of intracellular Ca^{2+} leading to uncontrolled K^+ efflux resulting in the early steps in apoptosis [6]. The BK channels play a critical role in mediating the K^+ efflux linked with apoptotic cell shrinkage. Inhibition of BK channel with iberiotoxin dramatically reduced K^+ efflux and prevents apoptosis. Therefore, enhanced K^+ efflux is an essential mediator not only for early apoptotic cell shrinkage but also for downstream of caspase-3 activation and DNA fragmentation.

2. Structure and functions of BK channel

BKα channels are involved in regulating a diversity of physiological processes such as metabolism, signaling, phosphorylation, neurotransmitter release, and modulation of smooth muscle contractions [7]. The BKα channels are activated by the cooperative effects of two distinct stimuli, membrane depolarization, and elevation of free cytoplasmic Ca^{2+} concentration. BK channels are assembled in membrane as tetramers of pore-forming α-subunits enclosing two regions, transmembrane spanning region containing two domains. They are voltage-sensing domain (VSD), which senses membrane potential and pore-gated domain (PGD) which opens and closes to control the permeability of K^+ ions. The other region, the cytoplasmic C-terminus region comprises many protein phosphorylation sites [8] such as RCK1, RCK2, leucine zipper, heme and caveolin-binding motif and Ca^{2+} bowl that regulates PGD and permeability of K^+ ions (**Figure 1**).

The pore-forming and the C-terminus domain of the BKα subunits contain several protein kinases (cAMP-dependent PKA, PKC, cGMP-dependent PKG, c-Src) and phosphatase (**Figure 1**) binding motifs which are mainly associated with a number of interacting partners to regulate the channel gating and signaling pathways. They activate the BK channel by increasing sensitivity to intracellular Ca^{2+}. The Ca^{2+} ions are bound to the electron dense of Ca^{2+} bowl

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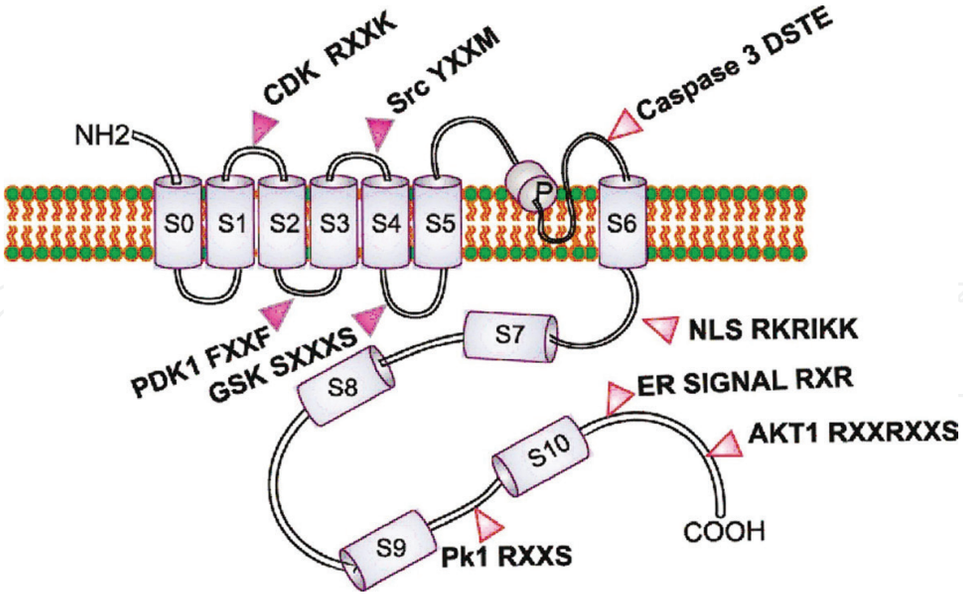

Figure 1. Significant phosphorylation sites in BKα subunit.

and activate cytosolic domain. The cytosolic domains are connected with transmembrane spanning region, S6 by 17 amino acid peptide chain, called linker peptide. A cytosolic domain through linker peptide opens and activates the gate PGD domain. Recently the cryo-EM study illustrates the BK channel structure and gating pore size is 1.7–2.0 ηm resolution [9].

The leucine zipper (LZ) motif is originally described as DNA binding proteins and reported to play an important role in both assemblies of ion channels and interactions of protein kinase and protein phosphatase. The LZ motifs serve to anchor a number of different BK channel associated proteins [10]. The LZ and EF-hand motif containing proteins regulate the mitochondrial swelling leading to apoptosis [11]. Therefore, it can be concluded that BK and other interacting proteins are regulating apoptosis through post-translational modification of phosphorylation or palmitoylation (**Figure 1**) [10].

BKα channels are sensitive to Ca^{2+} regulation through phosphorylation by serine-threonine and tyrosine kinases [12, 13]. Thirty putative phosphorylation sites were identified from seven different BKα splice variants [8]. Among them, the BK-DEC variant has an additional 60 amino acids at the extreme end of the C-terminus which contains 11 serine/threonine and tyrosine residues. The BK channels are directly involved in tyrosine phosphorylation in the presence of c-Src kinase domain in C-terminus of channel. The vital role for c-Src kinase mediating signal transduction on G-protein coupled and integrin receptor activation leads to the regulation of membrane ion channels [12]. The $\alpha5\beta1$ integrin activation leads to increasing activity of BK channel. The BK channel phosphorylation of $\alpha5\beta1$ integrin at Tyr-766 through intracellular signaling pathway involving c-Src kinase [14].

The PKC phosphorylation site (S1076) is lying on c-terminus of human BKα channel that influences the regulation of protein kinase on BKα channel activity which may subsequently alter pulmonary smooth muscle tone functions [15, 16]. This reveals the dual role of PKC on BK channel on tracheal smooth muscle. They are phosphorylation of S695 by PKC on BK

channel which is located in between the conductance of two regulators (RCK1 and RCK2) and inhibits the channel open state probability. The second phosphorylation of S1151 by PKC on C- terminus of BK channel and inhibit their channel open state activity.

3. Isolation of protein complexes from MAMs in cochlea

3.1. Maintenance of mouse cochlear hair cell culture

3.1.1. Isolation of the organ of Corti

Decapitate 2 weeks old (CBA/J) mouse at the base of the foramen magnum using scalpel. Briefly rinse the head with 70% ethanol and remove the epidermis using a scalpel blade. Open the cranium along the sagittal suture using a scalpel blade bisect the head equally half and remove the forebrain, cerebellum, and brainstem using blunt dissection. Remove the temporal bones, dip them briefly in 70% ethanol, and transferred into 35 mm dish. Remove the bulla and surrounding tissue from the petrous portion of the temporal bone and identify the conch shaped cochlea and separate it from the vestibular system using forceps. Remove the calcified bony labyrinth of the cochlea carefully removed from basal region to apical end. Spiral ligament and organ of Corti is tightly attached and coiled inside the bony labyrinth. Carefully remove the organ of Corti by securing the spiral ligament at the hook region of the base using forceps and unwinding it as you move apically. Begin at the base and remove the spiral ligament from the organ of Corti using no. 55 fine forceps.

3.1.2. Micro-isolation of hair cells from sensory epithelium of organ of Corti

The isolation of hair cells from mouse cochlea was described [17]. Remove the organ of Corti at the base of hook region by using two ½ cc insulin syringes with the help of U-100 28G½ needles as forceps. The organ of Corti consist of spiral limbus and sensory epithelium (outer and inner hair cells) cells starting from apex to base of organ of Corti. The sensory epithelium was separated from spiral limbus with help of insulin syringes. The sensory epithelium explant was transferred into fresh Petridis (35 mm) containing 1 ml of DMEM with 10% FBS, ampicillin (10 mg/ml), and 400 μl of each poly-L-ornithine (0.01%) and laminin (50 μg/mL). The Petri dish was incubated at 37°C with 5% CO_2. After 48 h carefully change the above fresh medium then the adhesive outer and inner hair cells was started multiplication on the Petri dish.

3.2. Transfection of candidate genes in mouse cochlear hair cells by nucleofector device (Lonza)

The mouse cochlear hair cell culture is washed with pre-incubated PBS buffer and adds 1 mL of trypsin solution then incubated 37°C for 1 min. and harvest (2.5 × 105) cells. The cells were centrifuged at 300 g for 10 min at room temperature and the supernatant removed and

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appropriate nucleofector solution (containing 2 μg of plasmid vector or 100 ηM of SiRNA and 100 μL of P4 primary cell 4D-nucleofector X solution) added into the nucleocuvette. Gently tap the nucleocuvette vessels to make sure the samples were premixed and the cover bottom of the cuvette. Place the nucleocuvette vessels and close the lid into retainer of the 4D-nucleofector X unit and select the appropriate program [18]. After completion of the run carefully remove the nucleocuvette vessels and resuspended cells with pre-warmed culture medium. The gene expression or down-regulation will be observed after 4 h transfection to 4 days.

3.3. Mitochondria and endoplasmic reticulum isolation from mouse hair cell culture

The mitochondria were isolated from mouse cochlear hair cell cultures using a kit per manufacturer's instructions (Qproteome TM Qiagen). The cells were washed with PBS buffer and harvest these cells with 1 ml of disruption buffer containing protease inhibitor cocktail and incubated 10 min at RT. After 10 min the cells were centrifuged at 6000×g for 10 min and collect the pellet and discard the supernatant. The pellet was resuspended in purification buffer followed by spun at 20,800×g for 15 min. Mitochondria and ER were layered on the surface of a density gradient centrifugation. Both mitochondria and endoplasmic reticulum were removed from the respective gradient and diluted in storage buffer, and spun at 8000×g for 10 min. The pellet consisting of purified mitochondria and endoplasmic reticulum were either resuspended in storage buffer and store in −80C or resuspended in protein lysis buffer get mitochondria and endoplasmic reticulum proteins.

3.4. Transmission electron microscopic studies

The BKα gene cloned in pCDNA3.1 mammalian expression vector and transfected in mouse cochlear hair cell cultures by using Nucleofector device. After transfection, both control and BK transfected cells were harvested and the cells were fixed with glutaraldehyde. The fixed cells were transferred in to wire gauge. The morphological changes of hair cells with respective of apoptosis such as plasma membrane dissolution; mitochondrial bulging, ER, and nuclear fragmentation were observed under electron microscopy with different concentration of BK transfection in the absence and presence of curcumin loaded silica nanoparticles. The synthesis of silica nanoparticles and encapsulation of curcumin will be carried out using a published procedure. One of the Co-PI is familiar with the synthesis and characterization of silica nanoparticles. The silica nanoparticles will be coated with polymers (polyethylene glycol) (PEG) or polyethylenimine (PEI) to enhance the biocompatibility of the nanoparticles. Initially, the amount of BK with appropriate time intervals is evaluated to activate apoptotic pathways in mouse cochlear cells.

3.5. Proteomics approach

The appropriate BKα gene was transfected with mouse cochlear hair cell cultures. After 48–72 h transfection, the mitochondria were harvested from the control and BK transfected mouse cochlear hair cell cultures. The proteins from mitochondria were isolated from both

control and BK transfected hair cells. The 50 μg of proteins were mixed with sample buffer and loaded in IEF gel strips. IEF will be performed using 7 cm immobilized pH gradient (IPG) gel strip, pH 3–10 (Protean IEF Cell System, Bio-Rad). Proteins were resolved by IEF in the first dimension and SDS-PAGE (12% acrylamide) in the second dimension. Precision Plus (Bio-Rad) molecular weight marker was used to determine relative mobilities. Gels were stained with silver staining and images were captured using the Molecular Imager versa doc MP Imaging System (Bio-Rad). The resolution of the scanning gel was 53 μm, and images were processed with the standard version of PDQUEST software (Bio-Rad), which is used to identify spots by pi and molecular weight with the help of standards. The BKα transfected protein gel is compared with control gel and qualitative differences of appeared (up-regulated proteins) and disappeared (down-regulated proteins) protein spots were excised and subjected to reduction, alkylation, and trypsin digestion as described previously [19]. Peptides were extracted and concentrated under vacuum centrifugation. The peptides of each sample were injected into LC–MS/MS then identification of each protein spots.

4. Structural link between ER and mitochondria

The mitochondria consists of two membranes viz. an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) which have several convoluted foldings called as cristae. The shape of mitochondria is very heterogeneous in living cells from sphere to interconnected tubules [20]. The formation of mitochondrial network is well documented by the continuous movements of mitochondria by motor proteins. During the mitochondrial movements rarely two mitochondria encounter each other to form fuse [20]. Sometimes the mitochondrial tubules can undergo fission to form two or more mitochondrial units. However, both mitochondrial processes are much complicated because of the coordination of fusion and fission of four lipid bilayers. The first mitochondrial fusion protein Fuzzy onions 1 protein (Fzolp) was identified in *Drosophila melanogaster* [21]. Later two Fzolp homologus mitofusin 1 (MFN1) and mitofusin 2 (MFN2) were identified from mammals [22]. The MFN1 and MFN2 have very high 81% homology and both are localized in the outer mitochondrial membrane [23]. These proteins are formed by the docking of two juxtaposed mitochondrial fusion through their transmembrane spanning region [24]. During the mitochondrial fission both Fis1 and dynamin-related protein1 (Drp1) play a vital role in mammals [25].

The endoplasmic reticulum is an extensive network of cisternae and microtubules and stretches from the nuclear envelop to the plasma membrane of all eukaryotic cells occupying 10% of the total cell volume [26]. The ribosomes bind to the peripheral of ER and to the nuclear envelope comprising the rough ER and ribosome-free is called as smooth ER. The peripheral ER consists of sheath-like cisternae and the thickness of the sheets and diameter of the tubules ranges from 60–100 nm. ER and microtubule associated proteins play a predominant role in shaping of mammalian cells [27]. The cytoskeleton membrane proteins 63 kDa (CLIMP63), VAP-B/Nir3 couple and p22 are involved with dynamics of ER and microtubule [28]. CLIMP63 is an integral peripheral ER membrane proteins which is anchoring of ER to microtubules and maintains the spatial distribution of ER network. The p22 has myristoylated EF-hand protein binds microtubule in a Ca^{2+} dependent manner and providing a link between

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ER morphology and Ca^{2+} [29]. The ER morphology is still controversial even though reticulons and DP1 proteins are enriched in ER tubule rather than sheets and nuclear envelope [30]. Another protein dynamin-related membrane GTPases atlastins are involved in the control of morphology of ER by promoting the branching of the tubules [31].

5. Protein liaison in tethering to ER and mitochondria

Close contact between the membrane of ER and outer mitochondrial membrane was first identified in late 1960 by several independent groups [32]. ER membranes co-purifying with mitochondrial fractions were observed under electron microscopy which, revealed that direct communication between cisternal space of ER and inner mitochondrial membrane (IMM) space [33]. The 20% of mitochondrial surface were direct contact with ER and each contact appears to vary between 10 to 25 nm in length [34]. The functional importance of these two organelles contact sites is further established by the quasi-synaptic mechanism of transmission of Ca^{2+} crucial function of during apoptosis.

The nature of ER–mitochondrial tethering has remained largely elusive. Szabadkai et al. [35] reported that IP3R is localized on membrane of ER and VDAC is localized on the OMM and both are physically attached through 75 kDa glucose-regulated proteins (GRP 75) (**Figure 2**). IP3R play a major role on the mobilization of calcium from ER to mitochondria as a function of apoptosis [36]. During steady-state transfer of Ca^{2+} molecules from ER to mitochondria, the mitochondrial anti-apoptotic proteins Bcl2 is drastically reduced [37]. The phosphofurin acidic cluster sorting protein-2 (PACS)-2 is also involved in the regulation of apoptosis

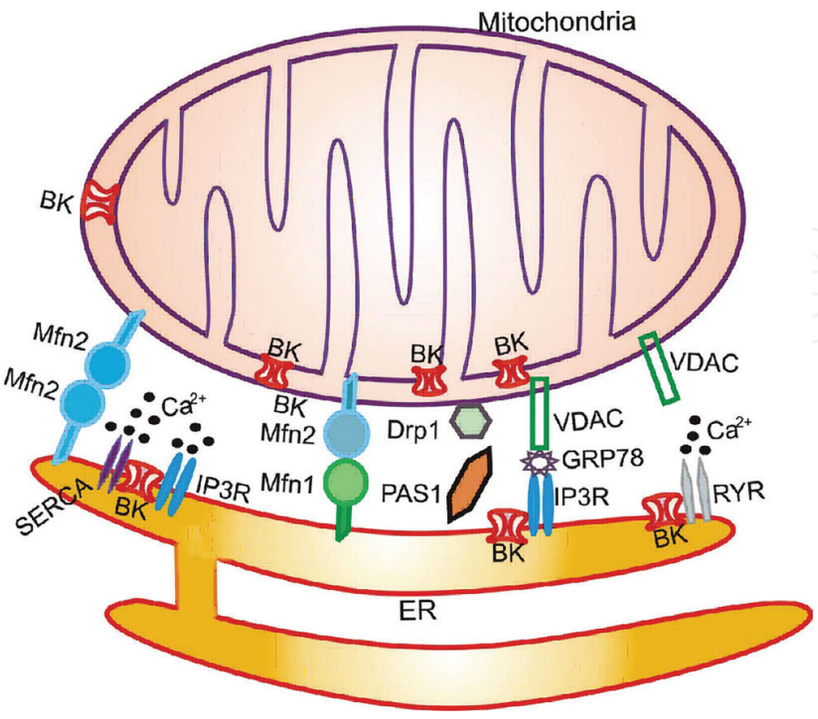

Figure 2. Tethers between ER and mitochondria.

through induces Bid translocation to mitochondria [38]. The first direct ER-mitochondrial tethering proteins are Mfn1 and Mfn2 [39]. They are localized on both organelles membrane and close contact point between the organelles. In addition, they are involved in maintaining the shape of both organelles. Moreover the regular juxtaposition of ER and mitochondria is the cytoskeletal network [40]. Both the organelles were bound with microtubules (MTs) and actin cytoskeleton that provide a scaffold that stabilizes the contact points between the compartments [41]. Recently identified mitostatin protein is binds with keratin and intermediate filaments that inhibits the juxtaposition of ER and mitochondria.

6. The role of bk channel role in mitochondria and ER interactions

Earlier, the molecular mechanism of BK channel function in mouse cochlea we studied, through the system biology approach aided by the sensitivity of coimmunoprecipitation, shotgun mass spectrometry methods to identify 174 BK channel associated proteins (BKAPs). Based on BKAPs developed transient BKα interaction networks are enriched with functional attributes of metabolism, trafficking and scaffolding, development and differentiation, signal transduction, and transport [2]. We, for the first time, employed a large number of ion channel associating proteins in the cochlea. The BKAPs were analyzed through subcellular localization. The majority of the BKAPs nearly 30 and 15% were localized in mitochondria (both membrane and matrix) and endoplasmic reticulum. Comprehensive understanding of the BK channel role in mitochondria and endoplasmic reticulum function has remained unclear. The past decade has concealed an number of unexpected protein-protein interactions that fundamentally changed our view of the localization and functional interactions of proteins inside cells. The functional role of BK channel in mitochondria is no exception.

One of our recent bioinformatics studies has revealed that the proteomics data (BKAPs) with functional attributes remarkably identified nearly 20 and 10% of novel mitochondrial and endoplasmic reticulum BKAPs involved in pro-apoptotic and anti-apoptotic properties which have not been looked in to, so far. Based on these results the preliminary experiments of tunnel assay for the BK overexpressed CHO cells that confirmed 65% cell death when compared with control. Till now, the exact mechanism behind the mitoBK and ER-BK channel role in pro-apoptosis is not known. It is expected that proposed project will give a new dimension in the biomedical field, which in turn may be useful for understanding the mechanism of hearing loss and noise-induced hearing loss (NIHL).

The Ca^{2+} activated potassium channel is playing a predominant role in mitochondria and endoplasmic reticulum-associated proteins. These proteins were contributing either K^+ influx or K^+ efflux of cells through the Ca^{2+} ion binds with Ca^{2+} bowl of c-terminus region [2]. Superoxide dismutase, glutathione S transferase μ, GAPDH, VDAC, and peroxidase are involved in ROS pathway as well as the candidate for BK channel associated proteins. The endoplasmic reticulum proteins calreticulin, GRP78, inositol triphosphate receptor (InsP3R), protein SET, VCP, HSP70, and protein disulfide isomerase are involved in the regulation of calcium-induced calcium release (CICR), protein folding and clearance. These proteins are known to BK channel associated proteins [2]. These two groups of proteins are involved in

the mitochondrial associated-ER membrane (MAMs) to regulate calcium signaling of CICR and cell death. However, we are only beginning to understand the spatial organization and interorganellar signaling in between ER and mitochondria and their functional regulations.

Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondrial subunit. We found that BKα subunits [2] localized in the inner mitochondrial membrane directly interact with other BKAPs like IP3R, calreticulin at the ER face of the MAMs. The molecular chaperone, glucose-regulated-protein 78 (grp78), is linked with inositol 1,4,5 triphosphate receptor-1 (InsP3R-1) and voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (**Figure 2**) [42]. Both Ca^{2+} overload and depletion of the ER Ca^{2+} pool can result in changes of two signaling pathways of unfolding protein response (UPR) and ER overload response (EOR) [43]. Latter induces mitochondrial membrane permeabilization (MMP), opening of the permeability transition pore (PTP), and release of cytochrome c and subsequent engagement of the mitochondrial apoptotic pathway [44]. The Ca^{2+}-sensitive dehydrogenases of the Krebs cycle [45] are stimulated as increased mitochondrial Ca^{2+} boosts ATP production. Increasing ATP production leads to more leakage of free electrons, causing the formation of superoxides. The resulting oxygen ions, free radicals, and peroxides are collectively called reactive oxygen species (ROS), which are effective to damage DNA and apoptosis.

ER membrane BK channels play a predominant role in ER retention, retrieval and normal trafficking [46]. InsP3R and BK channels are localized in the same hotspot region of glioma cells of lipid raft however; they are not directly interacting with each other [47]. The InsP3R are tightly linked with BK channel through continuous releasing of Ca^{2+} molecules in organelles and they are bind with EF-hand motif of Ca^{2+} bowl and activate BK channel to release the K^+ ion (K^+ efflux).

The cellular Ca^{2+} ion are transported through the plasma membrane by receptor and voltage-sensitive channels. Once inside the cell, Ca^{2+} can either interact with Ca^{2+} binding proteins or sequestered into the ER or mitochondria. Both Ca^{2+} overload and depletion of the ER Ca^{2+} pool can result in changes of protein folding and an increase of ER stress by either unfolding protein response (UPR) or ER overload response (EOR) [42]. From these two responses, both incorrectly folded and accumulated proteins are eliminated through apoptosis [48]. Ca^{2+} depleted from the ER is then taken up by the mitochondria, leading to Ca^{2+} overload in this organelle. The latter induces mitochondrial membrane permeabilization (MMP), opening of the permeability transition pore (PTP), and release of cytochrome c and subsequent engagement of the mitochondrial apoptotic pathway [44]. The increasing mitochondrial Ca^{2+} boots ATP production leading to more leakage of free electrons, which results in the formation of superoxides. These oxygen ions, free radicals, and peroxides, collectively called ROS, damage DNA and leads to apoptosis. Reactive oxygen species (ROS) play a key role in mechanism for induction of cochlear damage under various patho-physiological conditions [2]. Caspase-12 is localized in ER and activated by ER stress, including the accumulation of excess proteins that leads to cell death.

7. Conclusions

BK channel is involved in the regulation of pro-apoptosis in mouse cochlear hair cells. A group of proteins is involved in the regulation of mitochondria-associated ER membrane

(MAMs) and in the maintenance of intracellular Ca^{2+} level in cells. The MAMs associated proteins are known to be BK channel associated proteins. However, the details of interactions of MAMS associated proteins with BK channel and their mechanisms of post-translational modification such as phosphorylation and palmitoylation remain elusive. It is proposed that the present study will clearly depict how BKα is involved in apoptosis through K^+ efflux and regulation of BKAPs by phosphorylation. The phosphorylation of activation or inactivation of specific kinases and phosphatase binding motifs mainly regulate the channel gating and different signaling pathways. From these studies, we gain the knowledge of the mechanism of BK channel mediated apoptosis and identification of novel therapeutics to inhibit intracellular calcium induced stress-related apoptosis in cochlear hair cells. Therefore, through this study it is expected to find novel therapeutics for deafness and noise-induced hearing loss population in our society.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Author details

Ackuvinas Swakin Annammal Vargheese, Krishnan Sundar and Thandavarayan Kathiresan*

*Address all correspondence to: t.kathiresan@klu.ac.in

Department of Biotechnology, School of Bio and Chemical Engineering, Kalasalingam University, Krishnankoil, Virudhunagar District, Tamil Nadu, India

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