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Big Potassium (BK) ion channels in biology, disease and possible targets for cancer immunotherapy

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Abstract

The Big Potassium (BK) ion channel is commonly known by a variety of names (Maxi-K, KCNMA1, slo, Stretch-activated potassium channels, KCa1.1). Each name reflects a different physical property displayed by this single ion channel. This transmembrane channel is found on nearly every cell type of the body and has its own distinctive roles for that tissue type. The BKα channel contains the pore that releases potassium ions from intracellular stores. This ion channel is found on the cell membrane, endoplasmic reticulum, Golgi and mitochondria. Complex splicing pathways produce different isoforms. The BKα channels can be phosphorylated, palmitoylated and myristylated. BK is composed of a homo-tetramer that interacts with β and γ chains. These accessory proteins provide a further modulating effect on the functions of BKα channels. BK channels play important roles in cell division and migration. In this review, we will focus on the biology of BK channels, especially its role, and that it has in the immune response towards cancer. Recent proteomic studies have linked BK channels with various proteins. Some of these interactions offer further insight into the role that BK channels have with cancers, especially with brain tumors. This review shows that BK channels have a complex interplay with intracellular components of cancer cells and still have plenty of secrets to be discovered.

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1. Introduction

Cells are very dynamic and remarkable products of evolution. They have complex biochemistries dealing with nucleic acids, proteins and lipids, while residing within a complex internal ionic environment that can regularly fluctuate (Figure 1). The cell's internal ion concentrations are usually different from the fluids that surround it [1]. This concept is fully appreciated by most physiologists, pharmacologists and neuroscientists, but can be overlooked by other medical researchers. These differences in ionic concentrations within the cell actually make the cell electrochemically charged or polarized, so they can be considered as a natural battery with stored potential energy. This electropotential makes it possible to regulate cell functions based on the inward or outward flow of specific ions, so that various effector functions can occur as a result of ions changing their positions relative to membranes. Influx or efflux of ions induces rapid changes in membrane potential; these so-called "electrosignals" can drive chemical and cellular functions within the cells [2]. The Nernst equation provides the framework by which various electrical potentials result when the ions cross the cell's membrane. Ions are transferred via various transmembrane proteins, which evolved to transport specific ions. Ions cannot diffuse through lipid bilayers and only pass through specific transmembrane proteins such as ion channels and transporters. Since this phenomenon is selective to different ions, numerous channels specific for different cations or anions have been identified. Over 340 human genes encode for these various types of ion channels.

Ion channels are specialized transmembrane proteins that only allow the flow of a specific ion through a given pore. Exchangers allow the cell to swap one ion or multiple ions or other solutes in opposing directions. Ion channels are found within the cells at different locations; i.e., cell membrane, ER, Golgi, nucleus, mitochondria and may regulate specialized intracellular environments within the organelles [3]. They can be activated and induced to open through a wide range of signals ranging from changes in membrane potential in the case of voltage-gated channels, mechanical membrane stretching in the case of mechanotransducers, or secondary messengers, such as calcium, ATP, oxygen, carbon monoxide, hormones and other biological response modifiers (cytokines, neurotransmitters, etc.).

The first recognition that electricity mediated by ions has biological implications was made by Luigi Galvani in the late 18th century. Galvani showed frog muscles twitched in response to an electrical charge applied to them. This discovery is commonly shown in many elementary science textbooks. Ion channels play important roles in many diverse processes such as nerve impulses, secretion of hormones, cell proliferation, sensory transduction, learning/memory, regulation of blood pressure, salt/water balance, fertilization, and cell death [4]. Pharmacological research targeting these molecules are developing better drugs for many diseases [5]. Ion channels are beginning to attract the interest of cancer researchers

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who see these channels as vulnerabilities by which tumors can be attacked. Several previous reviews have covered ion channels within cancer [6–16]; many reviews have also covered basic BK biology dealing with normal physiology [17–24]. This review will cover BK channels, especially in the context of cancer biology and tumor immunology.

2. Early History and Nomenclatures

Potassium channels contain the largest number of different family members within the ion channels. The International Union of Pharmacology (IUPHAR) recognizes 78 different potassium family members. These channels can either expel or import K^+ into cells. One particular family member is the Big Potassium (BK) ion channels, which under physiological conditions releases K^+ cations from internal stores. BK channels are characterized by the largest conductance of K^+ ranging between 100 to 300 picosiemens (pS) when the channel is opened, as measured by patch-clamping electrophysiology. The BK channels are also known as Maxi-K (maximum-potassium) channels. This electrical property distinguishes these channels from lower conductance measurements displayed by small K^+ (SK; $KCa_{2.1}$) channels (4–20 pS) and intermediate K^+ (IK; $KCa_{3.1}$) channels (20–80 pS). For the sake of picking a name for this review, we will be using the BK nomenclature.

BK channels go by a variety of other descriptive names. Table 1 shows some of their different names given to the BK channels over the years. Various researchers worked on their own aspects of these channels, so each name reflects that original researcher's focus. A mutant fruit fly strain was first established and given the descriptive name "Slowpoke" [25]. These flies responded somewhat slower towards various stimuli usually in the flight wing muscles and nerves, so they had motor disruptions in the muscles and neurons. The Slowpoke channel was cloned from this mutated *Drosophila melanogaster* in 1987 [26]. This gene was later found to have about 50% homology with the equivalent channels from the human *hSlo* or mouse *mSlo-1* genes. Subsequent functional electrophysiological studies identified that these largest K^+ currents were carried by channels produced from these *hSlo* and *mSLO-1* genes [27]. BK channels open at lower voltages when small amounts of intracellular calcium were present and this lead to the name calcium activated potassium channels. This name, $K_{Ca1.1}$, was applied by the IUPHAR [28,29,30]. Stretch-activated K^+ channel-calcium dependent (SAKCA) was also used to designate BK channels when mechanical stress was applied to the cells. The Human Genome Organization's (HUGO) official name for BK is KCNMA1. This diversity in nomenclature used to describe a single channel is confusing to newcomers in the field. It should be understood that much of this early electrophysiological work was done before these genes were cloned and recognized to be the same channel. This work occurred before any standardized nomenclature was universally adopted. Many of these diverse names are still used in the current literature. This historical analysis illustrates that BK channels have a wide variety of electrophysical properties and when these channels are expressed in different tissues/organs they can have somewhat different characteristics.

BK channels are expressed by many but not all cells in the body. Of interest, human embryonic kidney cells (HEK), Chinese hamster ovary (CHO) cells and oocytes don't

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display BK channels when analyzed by standard electrophysiological readings [31,32]. These cell lines have proven invaluable in studying BK and other ion channels as they allow for the easy transfection of ion channel genes for functional assays. The stable genetic knock-down of BK channels in mice does not produce an embryonic lethal phenotype, suggesting these channels are not essential for fetal life. These $BK^{-/-}$ mice have neural defects in motor performance. The knock-down mice also display erectile dysfunction, over-activate bladders and hearing loss [33–36]. In humans, this ion channel has been identified as a playing a role in epilepsy and in paroxysmal dyskinesia by an Asp-to-Gly mutation at position 434 [37,38]. There is also an increased risk factor for cardiovascular disease for patients with distinct BKα channels with four mutated haplotypes identified [39].

3. BK Channel Structure

The BK channel is derived from a single gene found on chromosome 10 (10q22.3) in the human genome and chromosome 14 in the mouse genome. There are 27 exons. The BK channel is composed of 4 alpha (α) subunits which self-assemble into homo-tetramers. BKα are found in the ER, Golgi complex and plasma membrane, as would be expected for the normal synthesis of any transmembrane protein. BKα can be found within the inner membrane of mitochondria [40,41]. The mitochondrial DNA doesn't encode for any BK channel, so the presence of BK within the mitochondria indicates that this protein transmigrates into this organelle. There does appear to be a 50 amino acid sequence located at the C-terminus of the BK channel that allows this isoform to enter into the mitochondria; the last 3 amino acids have a Asp-Glu-Cys (DEC) sequence [42].

Figure 2 illustrates the 7 splice variants that can arise from the single BK channel gene using the UniProtKB/Swiss-Prot database. The National Center for Biotechnology Information (NCBI) database lists 8 transcripts, three transcripts don't appear to form viable BK channels; whereas, the Ensembl database lists 23 different spliced transcripts. Four transcripts fail to translate any proteins, another four transcripts lead to major truncations that do not encode a functional channel since these proteins possess less than 264 amino acids. Finally, there is also another BK protein with 866 amino acids, whether this channel is functional remains to be proven. BK channels display a wide variety of translated proteins due to many altered mRNA splicing pathways of their 27 exons [43,44]. These splicing variants appear to be tissue/organ specific, so some intrinsic tissue-specific genetic elements help the splicing process form the necessary BKα isoforms specifically needed for any given tissue. For the inner ear cochlea almost all forms of BK can be expressed [45,46]. BK channels can be phosphorylated [47], palmitoylated/S-acylated [48–50], and myristylated [51] which can account for more apparent different electrophoretic BK channels when examined by Western blotting. These different post-translational modifications may also give rise to various nuanced functions of this channel.

Each BKα subunit is composed of a seven transmembrane spanning regions. The topography of BK channels has been identified by x-ray crystallography at the 3 Ǻ level [52]. BK channels have been seen by high resolution electron microscopy [53]. Figure 2 shows some of the spliced variants of BK channels. The BKα chain is classified as having 10 domain regions, described as S0–S10. Each domain contains about 21 amino acids is

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responsible for a different function. Near the N-terminus are the S0 region and the terminal amino acid resides on the extracellular region. The S0 region can interact with regulatory BKβ subunits (see below). The S0–S6 regions are various transmembrane spanning regions. The S1–S4 regions contain the voltage sensor that detects charges on the cell surface and accounts for the voltage-gated opening properties of the BK channel. There is some speculation that these intracellular regions between the S1–S4 domains directly interact with other regions of the BK region found with the S7 and S8 domains. The transmembrane S5 and S6 regions of BKα encode the actual pore that allows K^+ to cross the membrane. The remaining BK channel resides in the intracellular region. Within this segment, the S7 and S8 domains form the regulator of conductance of K^+ (RCK1) region [54]. This segment allows the binding of divalent cations, such as Ca^{+2} and Mg^{+2}, which helps stabilize the configuration that allows the channel to open by interacting with the intracellular regions of S1–S4. An increase in the intracellular Ca^{+2} levels reduces the voltage at which the channel opens. The S9 and S10 regions also allow Ca^{+2} to bind in the so-called "calcium bowl" and form anther RCK2 region. Another function proposed for the RCK1 and RCK2 regions is to allow the 4 BKα chains to form a stable functional homo-tetramer.

4. BK Splicing Pathways

According to the current UniProtKB/Swiss-Prot databank, there are at least seven different splice variants of human BK channels. The mechanism by which three different splice versions of BK, hbr5 and glioma BK are generated is shown in Figure 4. Similar splicing pathways occur with the other variants, too. The glioma BK channel (gBK) was originally discovered within human gliomas, hence its descriptive name [55]. All human gliomas and other human tumors that we have tested to date possess the gBK variant at both the mRNA or the protein levels [55,56]. Using the canonical splicing pathways described by Steitz [57,58]; the splicing of the pre-BK mRNA is controlled by various "SNURPs"-small nuclear ribonucleoproteins: U1, U2AF, U5, along with other heterogeneous nuclear ribonucleoproteins (HNRP) within a region of the nucleoli called the splice some. The gBK sequence resides within exon 19 and hbr5 is coded by exon 20. Under normal conditions (Left Panel), exons 19 and 20 are both spliced out, so the low molecular weight species of the basic BK channel is translated and expressed. The left panel shows that snurp U1 binds to the 3′ end of exon 18, while snurp U5 will interact with the 5′ end of exon 21, then the splicing enzymes such as U2AF and other proteins such as the HNRPL now allow the splicing process to occur. A lariat loop is eventually formed where the 3′ end of the intron usually at a guanine residue is cut and then associates with an adenine residue at the end of the looped rope section. This very large lariat containing both exons 19 and 20 is then degraded. The remaining mRNA is then spliced together and translated as the basic BK channel protein, so exon 18 is fused with exon 21 in the final transcript.

For the expression of hbr5 variant (isoform 4)(Middle Panel), U1 still interacts with the 3′ end of exon 18, while the U5 snRNP now binds to the 5′ of exon 20. This occurs when the exon 19 region is spliced out so the mRNA only expresses hbr5. In the next splicing sequence, the intron between exons 20 and 21 is now eliminated, so the C-terminal of the BK channel is then added to the processed final mRNA.

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With gBK (Right Panel), expression only occurs when the hbr5 insert is simultaneously present, so most likely there are multiple sequential splicings, first the intron between 18 and 19 must be eliminated. Next the splicing of the 3′ end of exon 19 is spliced together with the 5′ end of exon 20, resulting in the gBK/hbr5 linkage. Afterwards, exon 21 is added onto the mRNA to completely construct the final BK channel mRNA.

The resulting BK proteins developed from the finalized spliced transcripts found in Figure 3 are now shown in Figure 4, the top panel shows the basic BK channel, the middle panel the hbr5 isoform (isoform 5), while the bottom panel illustrates the gBK protein. Hbr5 possesses an additional 29 amino acid residues, while gBK contains 33 amino acids.

The gBK insert contains three different putative phosphorylation sites, which can allow further protein docking interactions with casein kinase-2, calmodulin-dependent protein kinase-2I and protein kinase C to occur [55]. The importance of these additional modulators is that further enhanced signaling pathways could be theoretically imposed upon the cells that possess gBK, such as enhanced tumor migration, which can induce a more aggressive phenotype to be manifest. All three of these genes have been associated with various aspects of glioma biology [59–65]. Using the Repository for Molecular Brain Neoplasia Data (REMBRANDT) (https://caintegrator.nci.nih.gov/rembrandt/) database that correlates microarray data with patient survival; we analyzed these various isoforms of casein kinase-2, calmodulin-dependent protein kinase-2 and protein kinase C to determine if any gene could account for altered glioma patient survival. REMBRANDT allows one to examine the gene expression derived from 577 brain cancer specimens. There was no apparent effect of the casein kinase-2 isoforms: 2A1, 2A2, and K2B. The expression of any of these isoforms failed to show any improved or diminished survival of glioma patients. So casein kinase-2 is unlikely to play a role with glioma pathogenicity in the case of gBK. For calmodulin dependent kinase-2, when this 2D isoform was down-regulated, this genotype forecasts a better survival. So this isoform could have relevance with gBK. When the 2A, 2N2 or 2KG isoforms of calmodulin dependent kinase-2 are down-regulated poor patient survival resulted. With Protein Kinase C, when PKC delta is down-regulated, better survival occurs, while when the PKC epsilon form is down-regulated, a poor survival is seen. So depending upon which isoforms of calmodulin-dependent kinase-2 or protein kinase C is expressed, different patient survival profiles result. So these pathways might be potential targets of therapy for glioma patients in the context of gBK expression.

5. HNRPL acts as a toggle switch between the STREX and gBK/hbr5 variants of BK

The most common variant form of BK channel that has been investigated is called the stress axis-regulated exon (STREX) isoform. STREX is found within isoforms 1 and 3 (Figure 2). This variant is expressed within normal tissues and can be further induced by stimulation with hormones and other stressors [17,18,43,66–68]. This variant probably plays an important role during stressful times such as pregnancy. Both STREX and gBK/hbr5 are spliced into the basic BK channel at the same position, so only one splice variant is permissible. In our work with various human tumor cells, we fail to find any STREX

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expression by qRT-PCR techniques, while gBK/hbr5 is always found at both mRNA and protein levels. In Steitz's work [57,58] that explains how mRNA splicing occurs with snurps, HNRP plays an important role in normal mRNA splicing. A recent report by Liu, *et al.*, 2013 [69] showed that when the HNRP-L isoform was over-expressed, the STREX exon was excluded from the final mRNA splice variant of BK channels. In 2005 Yajima and colleagues [70] demonstrated that human gliomas over-expressed HNRPL. Some of the HNRPL peptides could stimulate human $CD8^+$ immune responses in the context of major histocompatibility complex (MHC) class I molecules. We examined a variety of human brain cancers including adult derived Glioblastoma Multiforme (GBM) and pediatric brain cancers such as GBM, low grade gliomas, eppendymomas and pilocytic astrocytomas for the expression of HNRPL by quantitative real time polymerase chain reaction (qRT-PCR) analysis [71]. We discovered that all of these brain tumors heavily expressed HNRPL, so they could be potential tumor antigens that cytolytic T lymphocytes (CTLs) can recognize. Our serendipitous finding now explains why cancer cells do not express STREX, but do make gBK/hbr5: the over-expression of HNRPL may simply prevent STREX splicing from occurring; thus permitting the gBK/hbr5 splicing isoform to be produced instead.

Besides glioma cells, gBK is found in other cancers [55,56]. We have discovered that small cell lung cancers (SCLC) actually make more gBK than glioma cells do [72](see below). Small cell lung cancers are actually derived from neuro-endocrine precursor cells. When we discovered the relationship with HNRPL and STREX, we tested whether 3 SCLC cell lines and 3 freshly isolated SCLC samples taken from autopsy cases could express HNRPL. In all of the SCLC specimens, the HNRPL mRNAs were all highly expressed. So this provides a confirmation of the glioma results with HNRPL and gBK and suggests a broader expression of this splice variant of BK in various tumors originating from different tissues with high levels of HNRPL.

6. BKβ (KCNMB) Chains

As mentioned above, the pore-forming BKα subunits can assemble with regulatory β subunits. Four different β subunits have been identified to date in mammals: β1–β4 [73–75]. The β1 (KCNMB1) gene is located on human chromosome 5 (5q34), the β2 (KCNMB2) and β3 (KCNMB3) genes are found on chromosome 3, 3q26.32 and 3q26.3–q27, respectively, and β4 (KCNMB4) resides on chromosome 12 (12q15). Each β subunit contains two transmembrane spanning regions with both the N and C terminals being intracellular. The transmembrane segments are connected by an extracellular "loop" that contains putative glycosylation sites. The transmembrane region of BKβ subunit near the C-terminal most likely interacts with the S0 region of the of the BKα subunits. β1 and β2 share major sequence similarities as do β2 and β3. The β4 is more distantly related to the other β subunits and have not been described in either *Drosophila* or *Caenorhabditis elegans*, suggesting they represent a new acquisition during evolution.

In terms of function, one may draw an analogy between the BKα subunit and the on-off switch of a stereo console. The BKβ subunits act as a rheostat that fine tune the actions of the BKα chains, as one would do for the "bass" control on the stereo. Controlling the stereo's bass now allows the listener to fine tune the music, so that the person can fully

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appreciate the sound quality to his/her own taste. BKβ subunits may delay the opening of the BK subunit, enhance the sensitivity to voltage, and hasten the closing of the channel, thus, allowing the cell to fully fine tune the BK channel to meet that tissue's requirement for proper responses mediated by the BKα subunit. Figure 5 illustrates the fine tuning process that the BKα and BKβ display once the BK channels are activated. Under physiological conditions, in which the concentration of K^+ is higher inside than outside the cell, the BK channel carries a large efflux of this ion, resulting in a large outward current (Top Panel). This type of current is produced when HEK, CHO cells or oocytes are transfected/ transduced with only the BKα subunit. If BKβ subunits are expressed without any BKα, nothing will happen, since only the BKα subunit forms the functional channel. When the proper conditions are reached (intracellular Ca^{+2}, changes in membrane potential and a proper gradient of K^+ across the membrane) patch clamp recordings on cells transfected with the BKα display a very fast rise in conductance (activation), due to a large efflux of the K^+ as the channels open. This rise in conductance continues until a stable plateau is reached. The plateau lasts for a limited time and is then followed by a sharp fall off (repolarization), when the channel closes. There is an over-recovery as the deactivation phase resolves the impulse. Finally, the BK returns to its initial resting state.

When the BKβ1 subunit is co-transfected with the BKα, it induces a slight leftward shift of the open probability of the channel versus voltage. It increases the apparent internal [Ca^{+2}] and voltage sensitivity of the subunit; this enhances the stability of the channel's open state. Furthermore, it slows the activation and deactivation kinetics of the channel. In contrast, co-expression of the BKβ2 and some BKβ3 subtypes produce faster inactivating currents. Four different splice variants of the BKβ3 subunits have been identified. The BKβ3a and BKβ3c produce inactivating currents when co-expressed with BKα, while BKβ3b induce a faster and incomplete inactivation only evident at a large depolarization. Co-transfection of the BKβ3d does not produce any viable changes in the kinetics of BKα, which raises the question as to its regulatory interaction with the subunit. Finally, the BKβ4 subunit decreases the apparent Ca^{+2} sensitivity of the channel. Its effects on channel activation kinetics are similar to those with BKβ1, however it induces very fast deactivation kinetics. Thus, the various BKβ subunits produces a wide range of different electrophysiological properties of the BKα that best suit a variety of the different tissues that it is expressed.

Figure 6 shows the various interactions of the BKβ chains with BKα and BKγ chains. The β1 and β4 subunits bind to 17β-estradiol [76,77] and activate the BK channel. Besides interacting with the BKα subunit, the BKβ1 chain also interacts with the calcium-activated chloride channel-1 (CLCA-1) chloride channel [78]. Expression of β subunits also confers differential sensitivity of BK channels to various venom-derived peptides blockers and activators. A number of small peptides are known to block BK channels, the most studied being iberiotoxin (selective for BK channels) and charybdotoxin (blocker of BK, KCa3.1 and Kv1.3 channels). Both toxins/peptides are inactive with BK channels containing the β4 subunit [79–82]. Slotoxin (αKTx1.11) also can discriminate between the β subunits of the BK channel; this toxin activates the channels composed of α + β1, while it is inactive on α + β4 channels [83]. Martentoxin, derived from the venom of the scorpion, *Buthus martensi Karsch*, blocks BK channels composed of α + β4 [84]. This peptide however opens gBK and BK channels formed of α + β1 [85]; it was however not tested on channels composed of

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α + β2 or α + β3. Using a crude extract from this scorpion venom, U251 cells were killed *in vitro* via apoptosis. In the same paper when a subcutaneous U251 tumor grown in immunodeficient mice were treated with this extract, the U251 tumor growth was significantly inhibited [86]. So crude extracts of these toxins can be safely used in animal models. The conopeptide Vt3.1 preferentially inhibited the BK channels containing the α + β4 subunits [87].

The BKβ1 and BKβ4 proteins share 22% homology which can explain some of the toxin interactions with those 2 subunits described above, because they have conserved sequences which can bind to the venom-derived toxins. The BKβ4 subunit also has interesting modulatory properties for the BKα chains. When HEK-293FT cells are transduced with the BKβ4 chain [88,89], the cell surface trafficking pathway of BKα is altered, allowing most of the BKα to be retained intracellular in either the Golgi or ER compartments. In contrast, when BKβ1 chain is co-expressed, more BKα shuttles to the cell's surface [89]. When the BKβ2 gene is co-transfected with BKα chain into HEK cells, less BKα chain manages to translocate to the plasma membrane [90]. Currently there is little information concerning the expression of β subunits with various cancers. So this field is wide open for future investigations and may offer future therapeutic opportunities.

The β2 and β3 subunits are the largest molecular weight species of these BKβ family members; these proteins share 43% homology to each other. The BKβ3 subunits in turn can have 4 variants, BKβ3a-d [91,92]. The Illumina, Inc. database suggests there are another 2 variants of the BKβ3 subunit that can also exist. The N-termini of the BKβ3a and BKβ3c subunits have a "ball and chain" motif. The "ball" region is composed of a cluster of charged amino acids that when bent properly by the "chain" region now allows this region of the BKβ subunit to interact with the intracellular pore region of the BKα subunit. Once the "ball" closes up the pore, this prevents further K^+ efflux and inactivates the BKα chains. The BKβ3d and 3b variant lacks the "ball" motif, and behaves like BKβ1 [91]. There are patients who manifest an idiopathic generalized epilepsy (IGE) [93]. This is caused by a truncation of the terminal 21 amino acids within the BKβ3b-isoform by a base pair deletion within Exon 4.

7. BKγ chains

BKα subunits can also interact with BKγ subunits, also known as leucine-rich repeats (LRR). Currently there are 4 subtypes known to associate with the BKβ chains. LRRC26, LRRC52, LRRC55 and LRRC38 are also known as BKγ1–4, respectively [94]. Just as with the various BKα and BKβ subunits, these BKγ accessory proteins show a differential organ-specific distribution. By the stereo analogy, the BKγ subunits can be "treble" control knobs. Thus, an incredible complex and diverse physiology can be produced by the various combinations of BKα, BKβ and BKγ subunits. Early reports indicate that BKγ1 seems to preferably associates with BKα + BKβ1, while BKγ3 and BKγ4 associate with BKα + BKβ4 [94].

The majority of the BKγ chain primarily resides on the extracellular side of the cell. These chains display very little intracellular regions. Rotterlin/mallotoxin activates BK channel

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activation only within BKγ1-positive (LRRC26) found in parotid cells(Figure 6) [95]. So even though the BKγ channels do not possess much of an intracellular region, the binding of this toxin, somehow modifies the extracellular protein conformation and activated the BK channel. LNCap prostate cancer displays BKγ1/LRRC26 [95]; other than that very little is known about differential tumor distribution of these subunits.

8. BK channels play key roles in cancer cell function

BK channels have been thought to play several roles in cancer biology. The two key functions have traditionally been cell proliferation/cell division and migration.

8.1 Proliferation

Cell proliferation is a complex pathway, where the DNA first replicates (S Phase) and then the process begins where the cell actually divides (mitosis). Many ion channels play active roles in cell proliferation [96–100]. When the cell begins the actual division process, the cells do get bigger, and this is where the BK and other ion channels may be playing the key role with helping cell divide and perhaps helps the cell to expand. BK channels assist breast, prostate and gliomas in tumor growth and spreading [101–108]. There are reports that BK channels don't play a role in glioma cell division [109] and that genetic knock-down of BKα promotes osteosarcoma development [110]. So the role of BK channels in human cancer is a very complex one and may not be a universal one.

In our initial work with BK channels, when we used siRNA to genetically knock-down BK channels in U251 glioma cells, we saw an interesting empirical observation, we couldn't fully explain at the time. BK siRNA transfected U251 cells were knocked down by 92–98% as determined by qRT-PCR from Days 2–4 and this was confirmed by the lack of protein as detected by intracellular flow cytometry during that time [111]. These treated cells just didn't expand their number and appeared to be in stasis. These cells were still alive since they resisted trypan blue uptake. This was contrasted with the siRNA scrambled control U251 cells, which continued to proliferate over that time. In our more recent work with U251 glioma, when gBK was knocked down by shRNA, our best stable clone that was knocked down by 70% as evidenced by qRT-PCR had a doubling time of 28 hours, when compared to the cell doubling time of shRNA-control U251 cells of 18 hours. Our empirical evidence suggests that BK does influence cell division in some unknown way. Finally, when we were working with small cell lung cancer cells (SCLC), HTB180 and H1436, we noticed that the ATCC recommended adding 17β-estradiol to those cultures. As we described above (Section 6), 17β-estradiol binds to the BKβ1and β4 subunits and activates the BKα channels. So to encourage SCLC growth, one might need to stimulate BK channels by using 17β-estradiol.

8.2 Migration

The scariest aspect of cancer is its ability to metastasize into distant sites away from the primary tumor. Glioma cancer is not described by being metastatic, *per se*. But gliomas are very invasive within the brain. Back in the 1920's surgeons even removed whole hemispheres in an attempt to treat these very invasive gliomas, without much success [112].

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Migration is a very dynamic process occurring over time and in three-dimensional space. BK channels are firmly linked with glioma migration [113–116]. After extensive surgery and followed by localized irradiation of the tumor margin, the glioma will eventually return in a location where some cancer cells were not eliminated. Migration is a complex process with molecules being turned on and then sequentially getting turned off. For glioma cells, it has been proposed that a series of ion and water channels can cause cell migration [117,118]. Harald Sontheimer's lab has evidence that glioma cells shrink by about 35%, which is very close to their lowest possible volume without risking cell death [119]. These cells can fit into defined pore sizes. Afterwards, the glioma cells swell and return to their normal size. This model is called the "hydrodynamic cell volume" process [119]. Ionizing radiation given to U87 and T98G glioma cells immediately activated their BK channels [120]. More importantly these irradiated cells also had an improved transmigratory ability to penetrate through 8 μm pores. BK channels have been described as mechanosensitive [121,122], meaning when the membrane is stretched the BK channel is activated. BK channel activation has been associated with areas of cells where active actin dissociation occurs [123,124,125]. So when membranes are physically stretched, breaks in the internal actin filaments could occur, and hence this can activate BK channels. Actin filaments are actively assembled at the leading edges of cells, while cells are dissociating their actin cytoskeleton at their trailing edge, where the cell is leaving. So theoretically there are two locations where mechanotransduction of BK could be occurring.

Glioma cells do possess microvilli and filopodia both *in vitro* [126] and *in vivo* [127,128]. One speculation for these structures is that they are used by the cancer cell to probe for weak spots in the normal brain, so when weak spots are found the tumor cells can transmigrate into that tissue and invade a virgin territory [129]. Since BK channels become activated upon exposure to ionizing radiation and increase their migratory capacity [120], glioma cells not killed directly by irradiation begin to become activated, and their infiltrative nature is enhanced. Thus, the glioma cell's invasive nature can be better understood. We have observed that various cytokines, chemokine and other membrane receptors like CD44 or gBK channels are found on these probing structures (Figure 7, Panel A). U251 cells do seem to show polarity of the glioma cells growing on 2D surfaces. Figure 7B shows a close-up of the U251 that shows the gBK channels are heavily expressed on one side of the cell and then there is a paucity of gBK on the opposite side of the cell. When the U251 glioma cells settle on transwell chambers, these probing invadopodia pokes through the 1.2 μm pores and can be found on the lead edges of the U251 cells. Glioma BK staining is found on these protruding tips. We interpret this to be the leading edge of the cell.

Glioma-BK or BK channels are not an oncogene per se, but instead assist the cancer cell in cell migration and invasion and acts like a facilitator. Just as the vascular endothelial growth factor is not an oncogene, but it induces angiogenesis, which assists cancer growth. We postulated that ion channels like gBK are playing a similar type role with SCLC as they supposedly do with glioma cells. When SCLC is loosely packed in a tumor-rich region, cell polarity with respects to gBK staining was also seen [72].

Glioma cells migrate along nerve tracks and along endothelial cells [130–134]. We examined some human primary GBM specimens by electron microscopy and found

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representative examples where the glioma cells interact with either nerve axons (Figure 8, Panel A) or endothelial cells (Panel B). In both cases we saw caveolae, which are structures believed to play roles in receptor-mediated endocytosis [parton + simons 135–137]. We speculate that the receptors are binding to various extracellular matrix proteins or cytokines and start the invasive process [129]. Interesting ion channels (including BK) and other cytokine/chemokine receptors within lipid rafts do interact with caveolin-1 [138–146] not only in cancer cells but with normal cells, such as myometrial cells [146], too. Caveolin-1 and caveolin-2 are proteins that help form the caveolae and play important complex roles in cancer biology and cell signaling, including ion channel regulation [135,136,140–142]. Using REMBRANDT caveolin-1 and -2 both significantly correlated with patient survival; i.e., over-expression (>2.0 fold expression) lead to poor patient survival, and conversely under-expression (<2.0 fold) lead to a better prognosis [129]. However, caveolin-3 failed to show any relationship with brain cancer survivorship. BK channels associate with both caveolin-1 and caveolin-2, but not with caveolin-3 [18]. The most interesting aspect of caveolin-1 is that when it physically interacts with either BK channels or the epidermal growth factor receptor (EGFR) those transmembrane proteins were inhibited [142–146 wang abulrob]. Thus, the interaction between these various molecules is a very dynamic process and requires much more effort to fully understand this biology.

9. Do BK channels play a role within immune cells?

There were a couple of early reports that inhibition of BK channels prevented granulocytes from killing phagocytized bacteria [147,148]. Two studies later refuted this work [149,150]. This in turn, leads to one of the original papers being retracted, since some of that work was not reproducible. So as it stands today, BK channels do not appear to be involved with the killing process of engulfed microbes. However immature THP-1 monocytes which are differentiated into adherent macrophages can produce BK channels [151]. BK channel activation within macrophages does occur after stimulation with various biological response modifiers [152,153].

Lymphocytes have been extensively analyzed for expression of potassium channels over the last three decades. All subsets tested (T, B and regulatory and NK lymphocytes) expressed Kv1.3 and KCa3.1 channels, but BK channels were never identified in these cells [154, 155,156]. Thus, BK channels only seem to play a role in myeloid cells.

10. Activated BK channels can lead to cell death

Actively dying cells can exhibit distinct morphologies, reflecting autophagy, apoptosis and necrosis/paraptosis/necropolis [157–160]. Autophagy consists of cells self-digesting internal organelles due to a lack of vital nutrients. Apoptosis is identified by cell shrinkage, nuclear condensation, DNA cleavage, membrane blabbing and with the high-mobility group box-1 (HMGB1) translocation into the nucleus from its normal pronuclear location [161,162]. Ion channels play active roles in these dying processes [159–160,163–168]. It is currently thought that K^+ efflux occurs via other K^+ channels and Cl^- ions effluxes, but prolonged Ca^{+2} levels are maintained so that pre-existing apoptotic proteases known as caspases are activated. Elevated intracellular levels of K^+ appear to prevent caspases from working

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properly [168,169]. Low intracellular K^+ levels culminate in apoptotic body formation, which restricts the release of various intracellular contents thereby limiting healthy tissue damage. Necrotic cells produce more inflammation, and the chronic state of inflammation can give rise to cancer [170].

We showed that BK channels play an important role in the way that mononuclear phagocytes killed glioma cells displaying the membrane form of macrophage colony stimulating factor (mM-CSF) via respiratory burst, which resembles paraptosis [171]. Our proposed mechanism by which monocytes/macrophages kill mM-CSF-transduced cells was via prolonged BK channel activation. Macrophages after binding to the mM-CSF via M-CSF receptors generate a respiratory burst. Microarray analysis revealed that hemoxygenase-2 and NADPH P450 reductase mRNA was produced by 4 hours [111]. Together these enzymes generate CO that opens the BK channels. Using CO-saturated tissue culture media, T9 glioma cells rapidly swelled within 15–30 minutes. BK channels are activated by CO at a certain intracellular region [172–174] Thus, the BK channel activation is occurring on the tumor target cells [171,175]. Using BK channel openers such as pimaric acid or phloretin, the cell releases K^+, while Na^+ enter, preserving the electroneutrality of the cell. When Na^+ enters, most likely Cl^- anions and water follows, producing the observed cellular swelling. Vacuolization results from the ER and mitochondrial swelling due to the presence of extra Na^+ and water. Cellular homeostatic mechanisms are activated to expel the excess intracellular Na^+ through the ATP dependent Na^+/H^+ anti-porter or other Na^+/K^+ ATPase exchangers [176,177]. The cell expends more ATP to expel the excess Na^+ ions. Physical disruption of the mitochondria reduces its ability to generate sufficient requirements of ATP to maintain ionic homeostasis. We show that as the T9 cells die in response to various BK channel activators or H_2O_2, intracellular levels of ATP are depleted, just prior to cell death. The inhibition of the Na^+/H^+ anti-porter produced paraptosis in cerebellar neurons [177]. The loss of intracellular ATP then resulted in the catastrophic cell lysis within our paraptotic cells [171].

Three SCLC cell lines which display BK channels were killed much faster (5 hours) when activated by BK channel activators (0.01 μM pimaric acid or 1.0 mM phloretin) [72] than human or rat gliomas, where it took 18–21 hours [111,171]. This is most likely due to the fact that gliomas have more internal stores of BK channels (ER, Golgi, mitochondria) than SCLC, which are largely devoid of such internal organelles, so it might take longer to kill these larger cells because all the stored K^+ ions needed to be fully released.

11. Cancer Immunotherapy

Prolonged BK channel activation has important ramifications for tumor vaccines. Dying paraptotic cells produced a number of danger signals [111,171,178]. These killed tumor cells spontaneously activated dendritic cells that stimulated effective anti-tumor immune responses, via heat shock proteins and HMGB1 through a variety of possible pathways [179–183]. This vaccine didn't require any genetic modification with cytokines or co-stimulatory molecules. Any freshly isolated tumor cells could theoretically be killed with BK channel activators. These cells can be irradiated to meet the safety concerns of various regulatory agencies. Allogeneic cell lines could then be used if no autologous tumor cells

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could be readily isolated. In a preliminary experiment, we vaccinated F344 rats with either BK channel activated/killed syngeneic T9 glioma cells with the RT-1^{lv1} haplotype or with BT4C cells which are allogeneic (RT-1^{dv1}). One week later, when the animals were challenged with syngeneic T9 glioma cells. The tumor failed to grow (Figure 9) due to protective immunity that was generated due to the vaccine. The allogeneic BT4c cells proved to be slightly more effective in generating immunity against the syngeneic T9 cells. Therefore allogeneic glioma cells killed by prolonged BK channel activation can be directly translated into a clinical trial.

Another way to target cancer cells is using the gBK variant. The gBK contains an additional 33 amino acid insert contained within the intracellular region and this region only appears in cancer cells [55,56]. Antibodies towards the gBK region therefore will not have any clinical relevance. Most toxins or other drugs that specifically target this gBK region would need to reach the intracellular regions and would have to be chemically engineered to meet that stipulation. So a major financial effort would have to be mounted to develop drugs that could specifically target this region. A more cost-effect approach would be a simple T cell response. Cells replace old proteins daily via an ubiquitination-dependent process. Ubiquitinated proteins are quickly degraded by the proteasome. Degraded peptides (6–18 amino acids) can eventually get processed in the major histocompatibility complex antigens (MHC)[184]. Once complexed with the MHC class I or class II proteins, T cells can now recognize these antigens via their T cell receptors (TCR) via CD8+ or CD4+ T cells, respectively.

Two peptides derived from the gBK: SLWRLESKG ($gBK_{704–712}$ or gBK1 peptide) and GQQTFSVKV ($gBK_{722–730}$ or gBK2 peptide), elicited CTL responses that were HLA-A*0201 restricted and killed those glioma cells [56]. Human CTLs were elicited by two different gBK peptides and could kill human HLA-A2+ glioma cells such as U251, LN-18, T98G and U87, but failed to kill HLA-A2-negative cells such as D54 (HLA-A01/A03) or LNZ-308 (HLA-A24). Thus glioma cells can properly process the gBK so that these peptides can be loaded onto the HLA-A*0201 molecule. By SYFPEITHI database analysis which predicts possible peptide interactions with MHC alleles (http://www.syfpeithi.de/) gBK peptides are predicted to bind to other human MHC alleles. The HLA-A03 allele had the ability to bind six different gBK-specific peptides. At this time, we have not validated whether those 6 peptides will work with the HLA-A3 phenotypes. Thus, using CD8-MHC class I pathways may therefore be somewhat limited for CTL responses. The gBK region is also predicted to bind at least six different 15-mers to HLA-DRB1 alleles (*0101, *0301, *0401, *0701 and *1501). Using CD4+ T cells might be a more versatile vaccination pathway to vaccinate patients towards gBK.

We have recently reported that gBK can be found within human small cell lung cancers (SCLC), too [72]. SCLC are derived from neuro-endrocrine precursor cells [185] possesses similarly poor relapse rates [186,187] as high grade gliomas. The expression of gBK by reverse transcriptase quantitative real time polymerase chain reaction was very minimal within freshly resected SCLC samples initially diagnosed with this cancer. When SCLC specimens were obtained from SCLC autopsy cases, the expression of gBK mRNA was extremely high, even higher than glioma samples that we tested. Examination of archival

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paraffin-embedded tissue confirmed the presence of gBK protein was much higher than from specimens taken at their initial diagnosis. Glioma-BK might be a "death signature" that forecasts the immediate future demise of the patient. Three SCLC cell lines which were HLA-A2-positive were also killed by the HLA-A2 restricted CTLs that were generated towards gBK1 and gBK2 peptides. The two SCLC cell lines (HTB-119 and HTB-180) that had the highest expression of gBK were isolated from autopsy samples; whereas, the H-1436 cell line originated from an initial SCLC sample prior to the patient receiving any chemo or radiation therapy [187,188]. Whether chemotherapy or radiation induces altered gBK expression is a topic of future studies. Thus, immunotherapy targeting gBK could be proposed for SCLC patients to prevent their terminal stages.

When we cultured the SCLC cells in the presence of interferon-γ (IFN-γ), MHC class I expression was enhanced; those IFN-treated SCLC also became better targets for the gBK-specific CTL, as normally expected. An unexpected finding was that gBK protein was significantly reduced by IFN-γ treatment within the SCLC cells, as determined by intracellular flow cytometry after 24 hrs. When we performed *in vitro* quantitative proliferation assays, recombinant IFN-γ significantly inhibited the growth of the SCLC cells. If ion channels play a role in cell division then this may provide a mechanism by which IFN-γ can slow the growth of cancer cells. The mechanism by which IFN-γ inhibits cancer cell growth has been an enigma for many years [189,190]. We do know that this process is independent of direct cytotoxicity. IFN-γ along with tumor necrosis factor-α (TNF-α) induces tumor cell senescence, including the Hop-62 SCLC cell line [191]. Interfering with BK channel transcription does inhibit the proliferation of some tumor cells [104–108]. Activated T cells release cytokines after recognition of gBK peptides in the context of the MHC, thereby providing an additional anti-tumor mechanism used against many tumor types, since almost all tumor cells express gBK [55,56]. Iberiotoxin directly prevented SCLC from passing through 5 μm pores as did both IFN-γ and TNF-α. Additional studies do confirm these possibilities. IFN-γ does inhibit human A172 glioma cell transmigration [192]; A172 cells do express gBK (Ge, Hoa and Jadus, unpublished results). B16 melanoma [193] and thyroid cancer cells, likewise are inhibited by IFN-γ in *in vitro* invasion assays, too [194].

12. Are there any other ion channels that have the same vaccinating opportunities like gBK?

One may ask whether there are other ion channels that have additional splice variants displayed only within tumors like gBK, which make them unique tumor antigens? These tumor derived types of channels provide additional opportunities for immunotherapy to be developed against other cancers, since these variants are truly tumor-specific. There is one report that type T calcium channels called $Cav_{3.1}$ channels are found within gliomas [195]. One $Cav_{3.1}$ channel isoform does appear to be a glioma-specific novel T-type variant, Cav3.1ac. This variant isoform 17 arose to the differential splicing of exons 25 and 26, resulting in an 18 amino acid insert occurring [196]. Using the SYFPEITHI database (http://www.syfpeithi.de/) to predict human MHC peptide epitopes, there are some possible MHC class I alleles (A*0201, A*0301, A*1101, A*26 A*6801 and B*37) and class II

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(DRB*0101, *0401, *0701) epitopes that could be explored for T cell vaccine usage. Just like the BK channel, the $Cav_{3.1}$ gene also has multiple aliases, too. Another name of this channel is called calcium channel, voltage-dependent, T type, alpha 1G Subunit (CACNA1G). When one examines the literature using this name, this gene is regulated by methylation status and has been associated with microsatellite instability, which has immediate implications for colorectal cancer [197–199]. Therefore, this ion channel might have possible target implications for colon cancer immunotherapy, too. Colorectal cancer can be stratified into patients who have a high content of Th1 or CTLs [200,201]. These patients tend to have better prognoses, so this antigen might be possible target of these T cells or immunotherapy could be desired.

Whether there are additional variant ion channels that exist in cancers is the million dollar question and certainly has major implications for developing other immunotherapy. Investigators should actively look for this possibility, when they examine their ion channels with various cancers. This may therefore be a very productive multidisciplinary endeavor for future studies.

13. An apparent paradox with ion channels using the current microarray data

Many different labs concluded that BK channels are present or over-expressed in brain cancers and these channels are also functional within glioma cell lines [99,108,111,113–116]. When one searches the currently available microarray data for independent confirmatory evidence that this BK mRNA is over-expressed in clinical samples, this data is surprisingly absent [202]. REMBRANDT identified that only 13 patients had over-expression (>2.0 fold) expression of KCNMA1/BKα (Figure 10, Panel A). This data failed to achieve any statistical significance and the trend for those over-expressing glioma patients was for a better survival. When under-expression of BKα (<2.0 fold) was detected, 75 patients had a significantly worse prognosis (P=0.008) than those patients without any KCNMA1 expression alterations. If the copy-number of the BKα/KCNMA1 gene was examined by REMBRANDT using 552 specimens (Figure 10, Panel B) only 1 glioma patient had an amplified copy number. This patient seemed to survive for a longer time, but then died around 2,000 days, which beat the majority of other patients. In contrast, 84 patients had deletions of the KCNMA1 gene and these patients had a poorer survival, which was statistically significant ($p=2.03\times10^{-7}$). Hence we seem to have a major apparent discordance with the data coming from the microarray data with that reported in the current literature.

So how can these microarray results be reconciled with the published works of many labs? Only speculations are currently available. gBK was not specifically included in the probe set used in the microarrays, so the evidence of this isoform is therefore lacking. The probe for KCNMA1/BKα is included in most microarray chips. The microarray probe sets are usually designed to detect all isoforms of the gene. Early Affymetrix chips were designed for the 3′ ends, so any fine dissection as to which specific gene was up-regulated could be lost. As we show in Figure 2, there are many different BK isoforms. Some BK channels variants with

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different 3′ ends might not have been discovered when these microarrays were initially designed back in the early-mid 1990's. The microarray data is based upon using the mRNA taken from surgical material taken from where the cancer is detected and then comparing it with tissue that is supposed to be cancer free. So there could be loss of information from this procedure. It is likewise possible, that some microarray samples were only done once from any given patient sample and it was not reproduced. Tumors including gliomas are notoriously heterogeneous [203,204], so this can further complicate the analysis. Gliomas can have different subtypes [205]. Gliomas with a mesenchymal subtype phenotype have a different microarray profile from a neural/proneural subtype of glioma. REMBRANDT does not easily allow for stratified searches of these subtypes. Glioblastoma Multiforme stem cells can be CD133+ [206,207] or CD133- [208], so the majority of the tumor (>99%) could be displaying different markers or genes that are different from the rare glioma stem cells (<1%) that are present within a tumor. Additionally, tumor samples include the mRNA taken from host derived cells, such as endothelial cells, pericytes, monocyte/microglial cells and perhaps lymphocytes. The mRNA coming from the cancer stem cells, might be sufficiently diluted so that no major differences in microarray data could be discerned. In contrast, the majority of the papers doing BK channel work use glioma cell lines which are not stem cells or are contaminated with non-tumorous host cells. So there are reasonable explanations that could explain this divergence of results.

Human gliomas do have multiple genetic amplifications and deletions [209,210]. U251N cells actually have a deletion in chromosome 10 [211], so there is usually only 1 copy of the BKα, but BK and gBK does seem to be present (unpublished data). The tumor suppressor gene, phosphatase and tensin homolog (PTEN) loss (10q23) is a common deletion (10q11.2–q26.3) found in gliomas [209,210], and this deletion occurs in the same genomic region where the BKα chain (10q22.3) also resides. When PTEN copy-number was analyzed there were 89 patients with this deletion, which is consistent with a dual gene deletion hypothesis. As we concluded earlier, gBK or BK channels are not true oncogenes and the loss of a tumor suppressor gene probably has a higher cause of malignancy than the effect of BK channel activation associated with tumor migration.

Two of the BKβ subunits (BKβ2/KCNMB2 and BKβ3/KCMB3) do have predictive outcomes with the glioma patients using REMBRANDT (Figure 10, Panels C and D). Sofar, BKβ subunits are known to only interact with the BKα and BKγ proteins. For BKβ2 (KCNMB2), there were only 2 patients that over-expressed this BKβ subunit; 126 patients were down-regulated. The latter group had a statistically shorter survival ($p=2.2\times10^{-4}$). The prognostic value of KCNMB3 also showed a worse survival when this gene was down-regulated. One hundred and fifty-nine patients had a poor prognosis, which was statistically significant ($p=0.006$) when this gene was up-regulated. Twenty-eight patients which possessed lowered KCNMB3/BKβ3 transcripts had a trend towards improved survival but this was not significant. The BKβ3b and d subunits can possibly suppress full BK channel activation (Figure 5); whereas, the BKβa and c subunits do not display this effect. The microarray data doesn't tell us which subunit was actually being detected, so we have an inherent gap of some vital knowledge that allows us to fully explain this effect. Fortunately, we don't have any ambiguous constraints with the KCNMB2/BKβ2 gene. BKβ2 tends to inhibit the action potential of BK channels (Figure 5), so this subunit could be considered

the brake on the BK channels, which limits the full actions of the BKα to be displayed. By inhibiting the brakes on the regulatory BKβ2, this may therefore allow BK channels to fully operate. A similar observation is seen with two other genes which inhibit the BKα chains. Caveolin-1 and -2 can inhibit the functions of the BKα channels can be turned off (ref). The over-expression of caveolin-1 and -2 does correlate very well with poor patient survival and seem to parallel the results with the BKβ2 data. It could be possible that perhaps glioma cells don't have to over-express the BK or gBK gene, but this channel just needs to be judicially used at only a bare minimal level to mediate its full effects for gliomas. More effort will be needed here to fully understand this basic paradox of results, perhaps microarrays that specifically detect the various BK isoforms (α, β, and γ) will solve this mystery.

We likewise analyzed the other ion channels that were highlighted by the reviews of Ding et al., [13] + Arcangeli et al. [7] and determined whether a similar effect could be seen as we saw with the BK channels in relation to glioma patient survival. Many other ion channels reported to be over-expressed with glioma cell lines also fail to show this correlation with REMBRANDT. Table 2 summarizes this lack of statistical of survival. So it is just not that BK channels which show this type of anomaly between microarray analysis and published work. Only the chloride channel-1 (Clc-1) correlated with poor patient survival, too [129,202], sofar no one has reported the significance of this ion channel in glioma cell lines. This work *in toto* suggests we have an interesting phenomenon involving ion channels and glioma that needs to be resolved with microarray data, before we can fully understand this cancer in terms of ion channel function.

14. Proteins that can associate with BK channels: maybe there are other mitigating proteins that regulate the BK function?

Its common thought that if you hang out with the "wrong crowd" you might be more likely to get into trouble. Perhaps other proteins can exert such an effect upon the BK channels that lead the BK channels astray when applied to glioma patients. Proteomic studies done with normal tissue indicate many proteins can physically associate with BK channels [18,212–214]. These techniques include co-immunoprecipitation, yeast two-hybrid system, fluorescence resonance energy transfer (FRET), co-localization studies using confocal microscopy. Thus, BK channels do not operate by themselves but can physically interact with other proteins. We analyzed those genes from those three proteomic studies using the REMBRANDT to determine if any insight could be gleamed from those interactions (Table 3).

Most of these proteins (20) that have been associated with BK channels didn't seem to have any relationship with glioma patient survival. This lack of association could due to that these proteomic studies were done with normal tissues and not cancer cells. So we could be comparing apples to oranges here. When over-expressed genes were examined, we failed to find any gene that had a good prognosis. But eight genes like actin G2, AKT, annexin A5, caveolin-1, caveolin-2, lamin A/C, prohibitin and 14-3-3γ associated with a poor survival. These genes could therefore be candidates that could modify BK channel functions in some

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undefined ways. When under-expressed genes were examined only spleen tyrosine kinase/SYK showed a favorable response within glioma patients. Another gene that was close to being statistically significant (p=0.054) for a favorable response was the proline-rich tyrosine kinase 2/PYK2. Seven genes, ApoA2, FAK/PTK, GRINA, MAGI1, MAP1A and stathmin-2 and -3, showed a bad prognosis for the patients when they were down-regulated. Doing BK-associated proteomic studies using glioma cells would be most useful to confirm any possible proteomic links and will undoubtedly advance the field of how BK channels fully mediate their effects with cancer growth and spread.

Proteomic studies have been done with fresh glioma tissue and glioma cell lines to determine if over-expression occurred when compared to be normal brain tissue is present [215–220]. When we cross-checked their studies to those reported in Table 3, we do have some overlap with those genes. Those genes were highlighted in the Table 3 with asterisks along with the references to those reports. So when cancer biologists want to design molecular theories with BK channels they should take these proteins into account when building their models.

15. Summary/Conclusions

The BK channels are a series of spliced channel variants derived from a single gene that play many roles in basic biology. As a result of complicating splicing pathways, various post-translational modifications and interaction with accessary proteins, the BK channels have altered physiological responses that match the tissue types where it is being expressed. BK channels play active roles in tumor biology providing mechanisms by which cell division and migration can occur. We described a mechanism by which tumor vaccines can be created by inducing a novel cell death pathway that induces immunogenic vaccine by prolonged BK channel activation. Additionally, a novel splicing isoform only found in cancer cells is called the glioma BK channel. This version has important ramifications for cancer immunology by providing another pathway by which tumor vaccines can be created. MHC specific peptides can be processed from the gBK region and T cells can respond to. As with many new fields and paradigms, there are so many more questions to be answered than there are known answers. Although the BK or gBK channel may play a role in cancer migration, this channel is not considered to be a classic oncogene and is probably dominated by other genes and proteins that can associate with it. Therefore, there is still plenty of work to be done with BK before we will have a complete understanding of BK channels in tumor biology.

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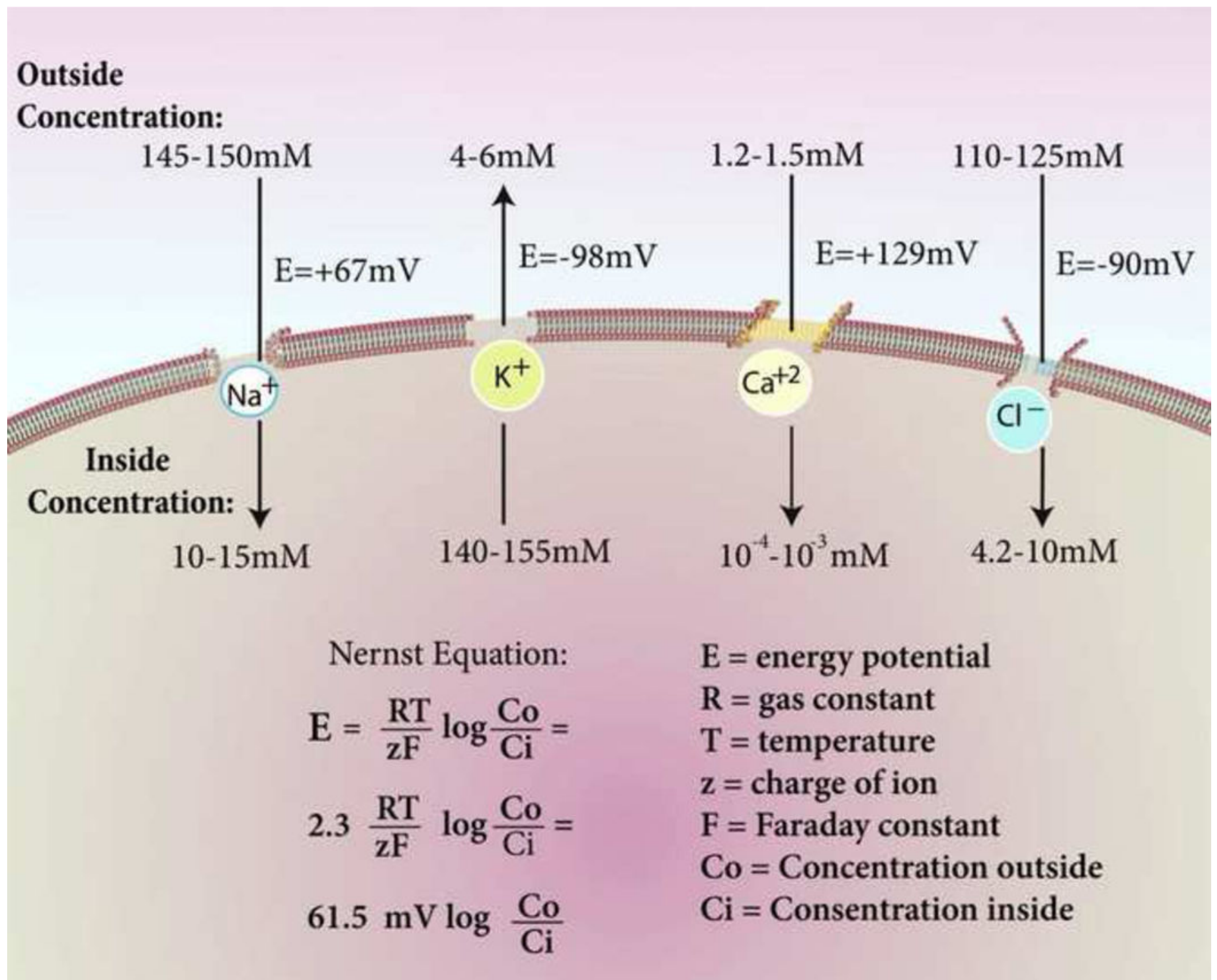

Figure 1.
Cells residing in differential ionic concentrations can act like batteries. The inside and outside concentrations are shown along with the electropotentials that can be generated upon using the Nernst equation using those specific ions.

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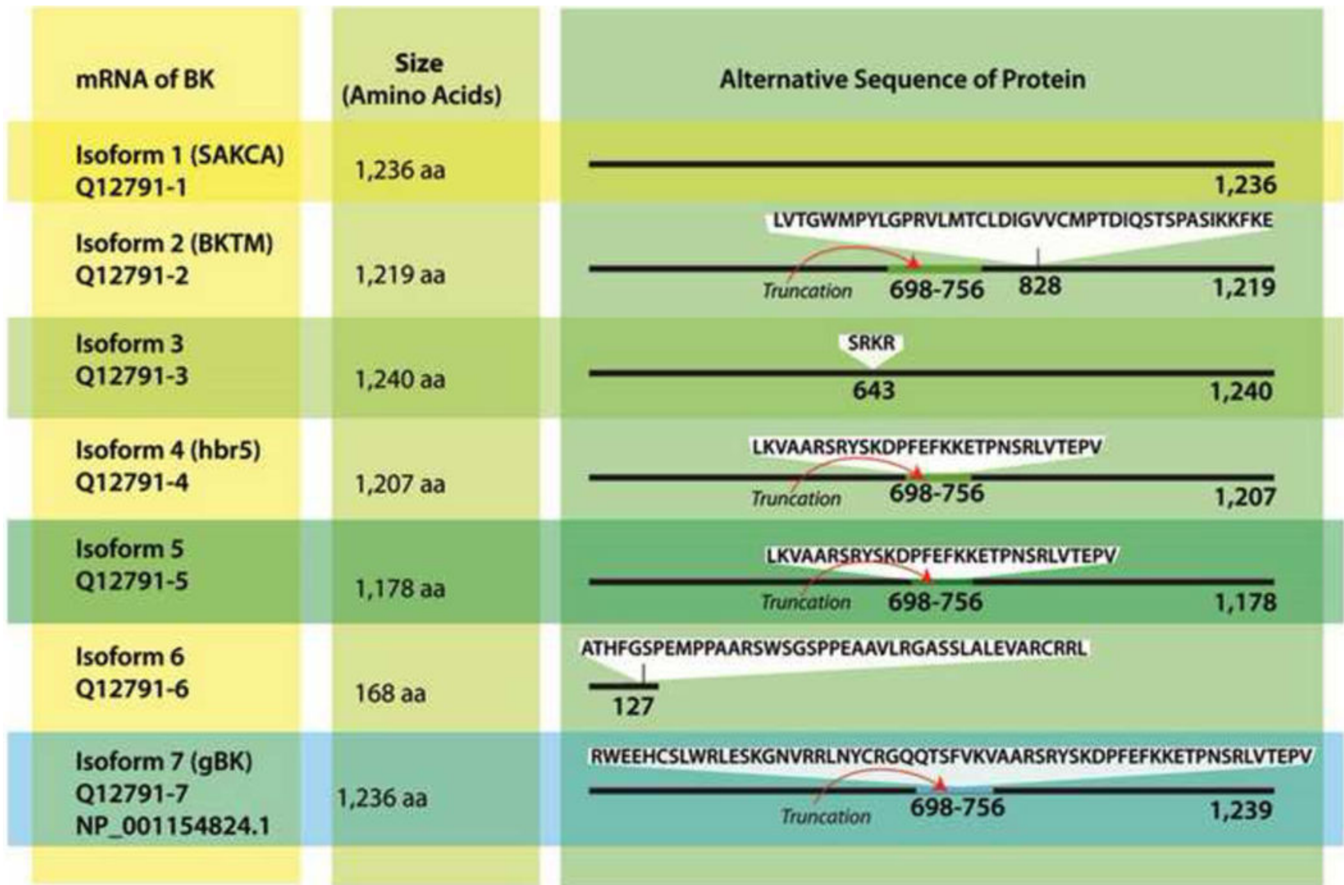

Figure 2.

Different isoforms of the human BKα channels. Using the UniProtKB/Swiss-Prot database there are 7 different isoforms of BKα chains. The sequence number of the BK channel is provided in the first column. The number of amino acids is displayed in column 2 and the key sequences are shown in the right column. The whitened areas indicate where new amino acids are inserted, while deletions of sequences are shown in the green or teal areas, along with the amino acid number where such changes are.

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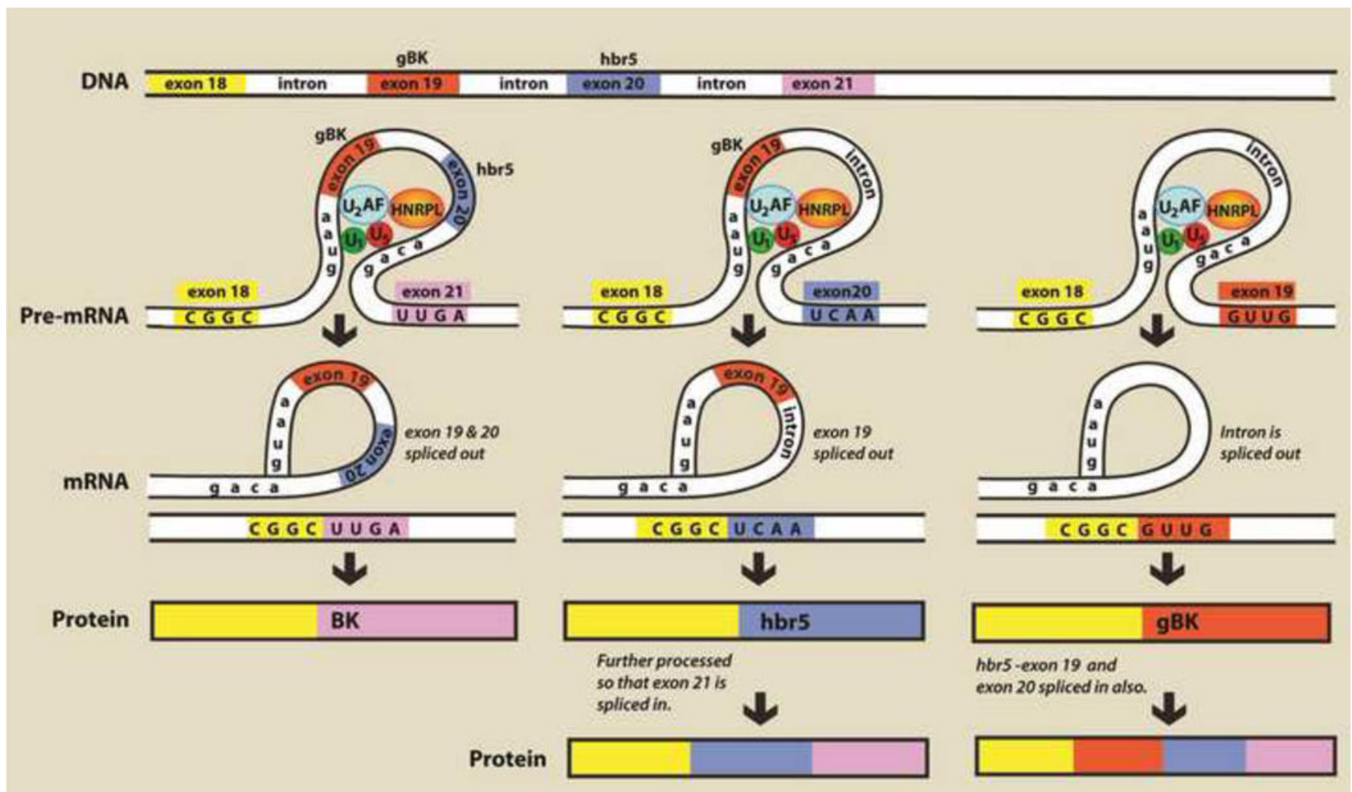

Figure 3.

The mechanism of mRNA splicing for BKα, hbr5 and gBK channels. The top panel illustrates the DNA organization of the BK channel from exon 18 to exon 21. At the pre-mRNA level. The various snurps (U1, U5, U2AF and HNRPL) will interact with the appropriate nucleotides to form the correct loop structures so that splicing will occur. For the basic BKα channel, exons 19 and 20 are spliced out. For hbr5, exon 19 is first spliced out, then exon 21 is then spliced in. To produce gBK, the intron between exons 18 and 19 is removed. Next the hbr5 exon 19 and exon 20 are spliced in to produce the final gBKα chain.

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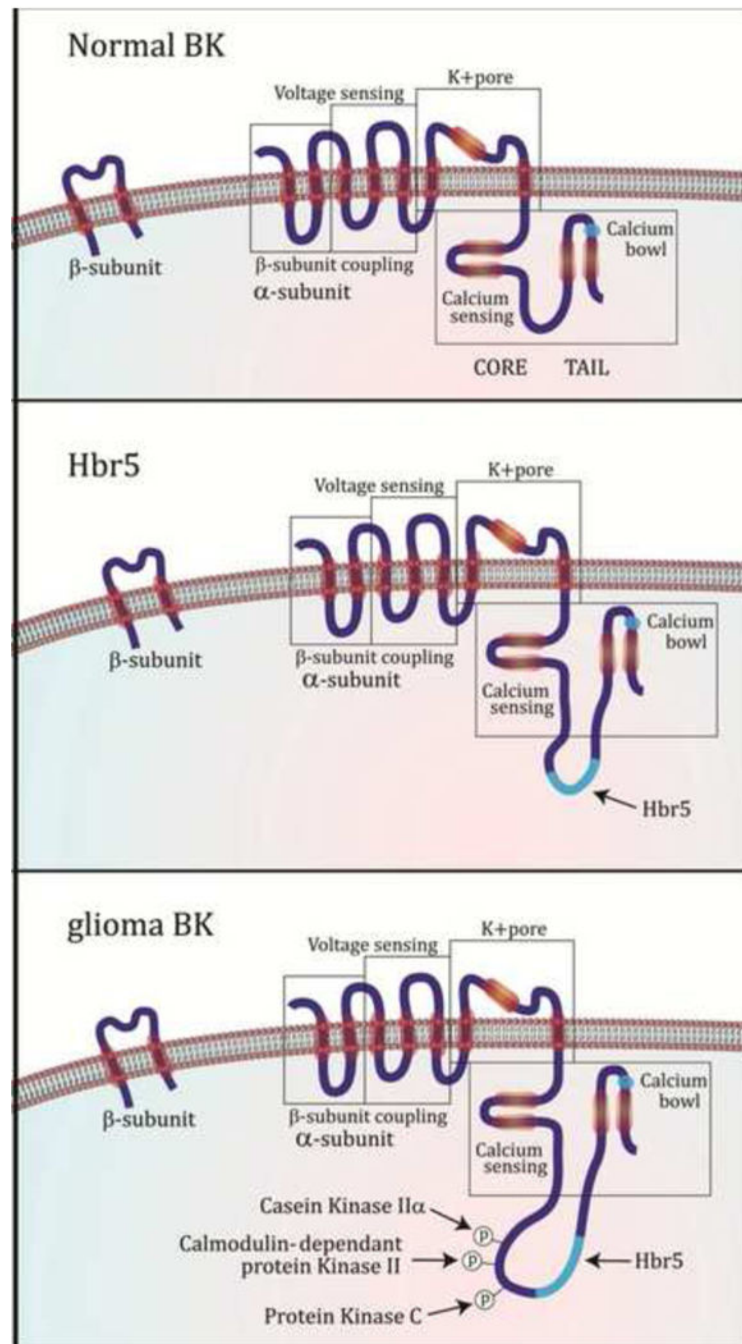

Figure 4.

The final BK, hbr5 and gBK proteins. The top panel shows the basic BKα chain. The BKβ chain will interact with the N-terminal part of BKα chain. In the regions S1–S4 show the voltage sensing region. The S5 and S6 domains illustrate the pore region, while the remaining regions demonstrate the core tail where the calcium sensing and “calcium bowl” are located. The middle panel represents the hbr5 form where an additional 29 amino acids is inserted. The bottom panel shows the gBK heavy chain where an additional 33 amino

acids in inserted prior to the hbr5 insert. There are three putative sites which could allow interactions with protein kinase C, calmodulin dependent kinase II or casein kinase IIα.

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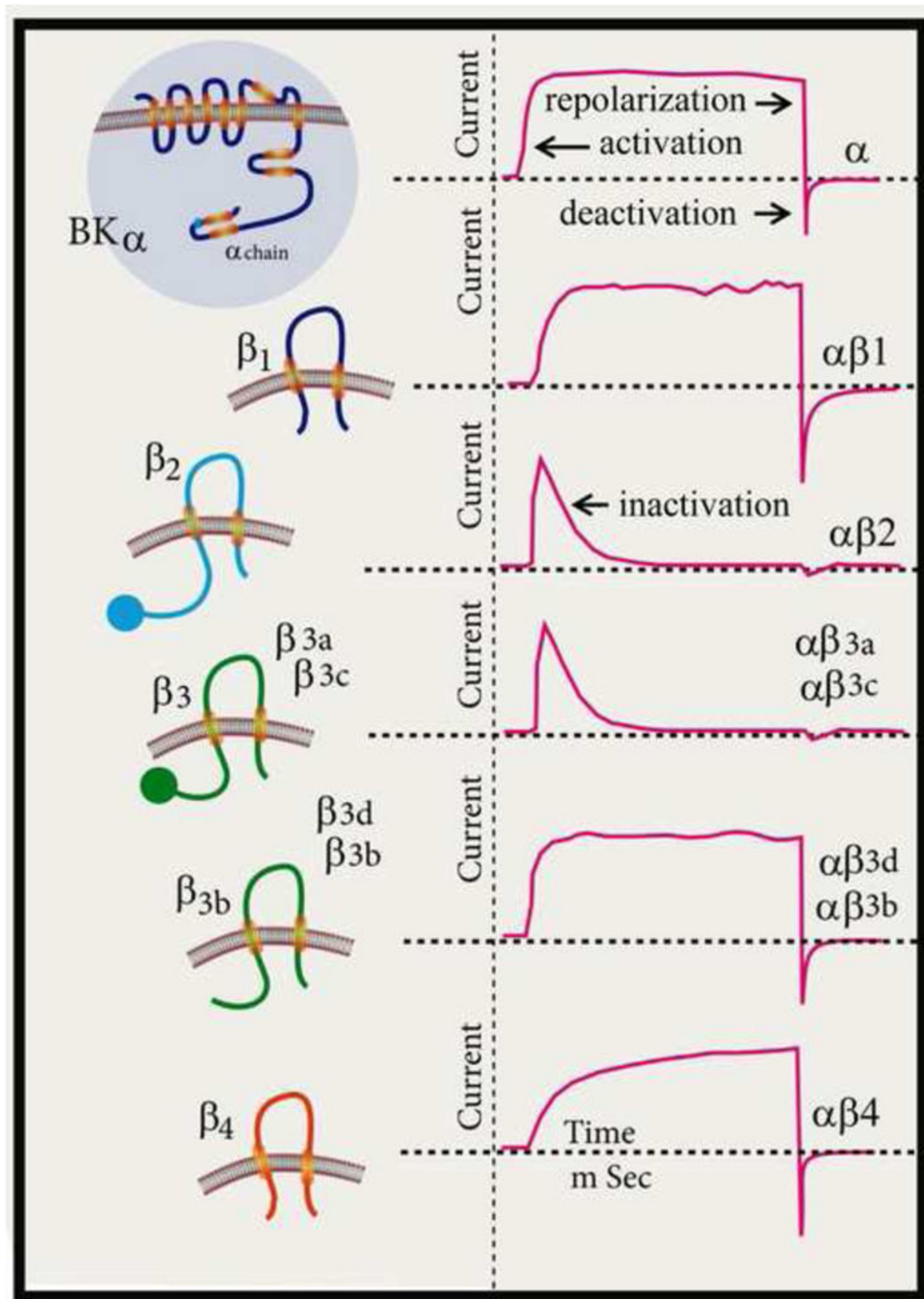

Figure 5.

The regulatory effects of BKβ subunits on the action potentials of BKα. The top panel shows a stylized action potential when the BKα is activated over time on a milliseconds scale. There is an initial activation phase due to the rapid transit of K ions across the membrane. After a period of stable conductance (plateau) it is followed by a repolarization of the channel as it closes. Then there is a deactivation phase. When BKα+BKβ1 are co-expressed (second panel), there is a slower activation phase followed by an enhanced repolarization phase. When BKα+BKβ2 (third panel) and BKα+BKβ3a or c subunits

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(fourth panel) are co-expressed, there is a rapid activation, followed by a very fast inactivation phase, with a very small deactivation phase. In the fifth panel, when BKα +BKβ3b or d subunits are displayed, there is a slightly slower activation phase, while a relatively normal BKα response occurs. In the bottom panel, when BKα+BKβ4 interact, there is a slower activation phase followed by a normal repolarization/deactivation phases.

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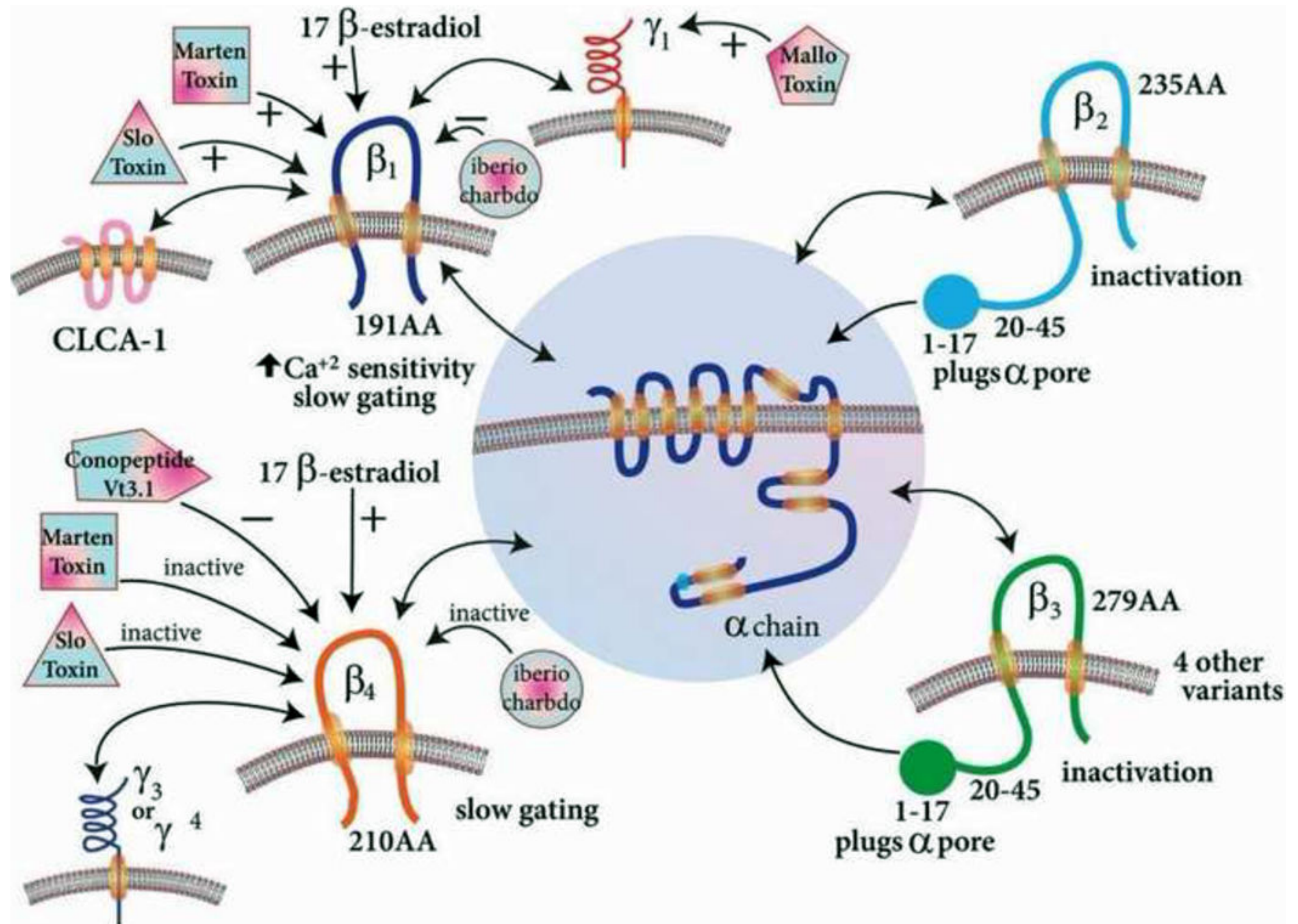

Figure 6.

The various associations of BKα and BKβ chains. The BKα chain (center) forms a tetramer and can associate with 4 possible β subunits (1–4). The BKβ1 chain can interact with the chloride channel-1 (CLCA-1). When BKα interacts with BKβ1, there is increased Ca^{+2} sensitivity with a slow gating process. 17β-estradiol can activate the BKα + BKβ1 and BKα + BKβ4 combinations. Iberiotoxin and charybdotoxin (circles) can inhibit (negative sign) this BKα and BKβ1 combination, while martenotoxin (square) activates (positive sign) this combination of subunit. The BKγ1 chain will interact with this BKα + BKβ1 combination. When BKα + BKβ2 and BKα and BKβ3 combinations occur there are a rapid inactivation of the potassium action potential due the "ball" motif and plug the BKα pore. There are 4 variants of the BKβ3 subunit. Two forms of BKβ3b and BKβd do not have the "ball and chain" motif and do not display the rapid inactivation. When BKα interacts with BKβ4, iberiotoxin can inactivate the BK pore, while charybdotoxin does not. Slotoxin (triangle) can activate this combination, while conopeptide Vt3.1 (polygon) and martenotoxin will inhibit this combination. The BKγ1 subunit interacts only with BKβ1 subunit; the BKγ1subuniy is activated by mallotoxin (pentagon). The BKγ3 or BKγ4 subunits will only interact with BKα + BKβ4 combination. The exact sequential positions where the various venom toxin and hormones interact with the β or γ subunits are not being implied by this figure.

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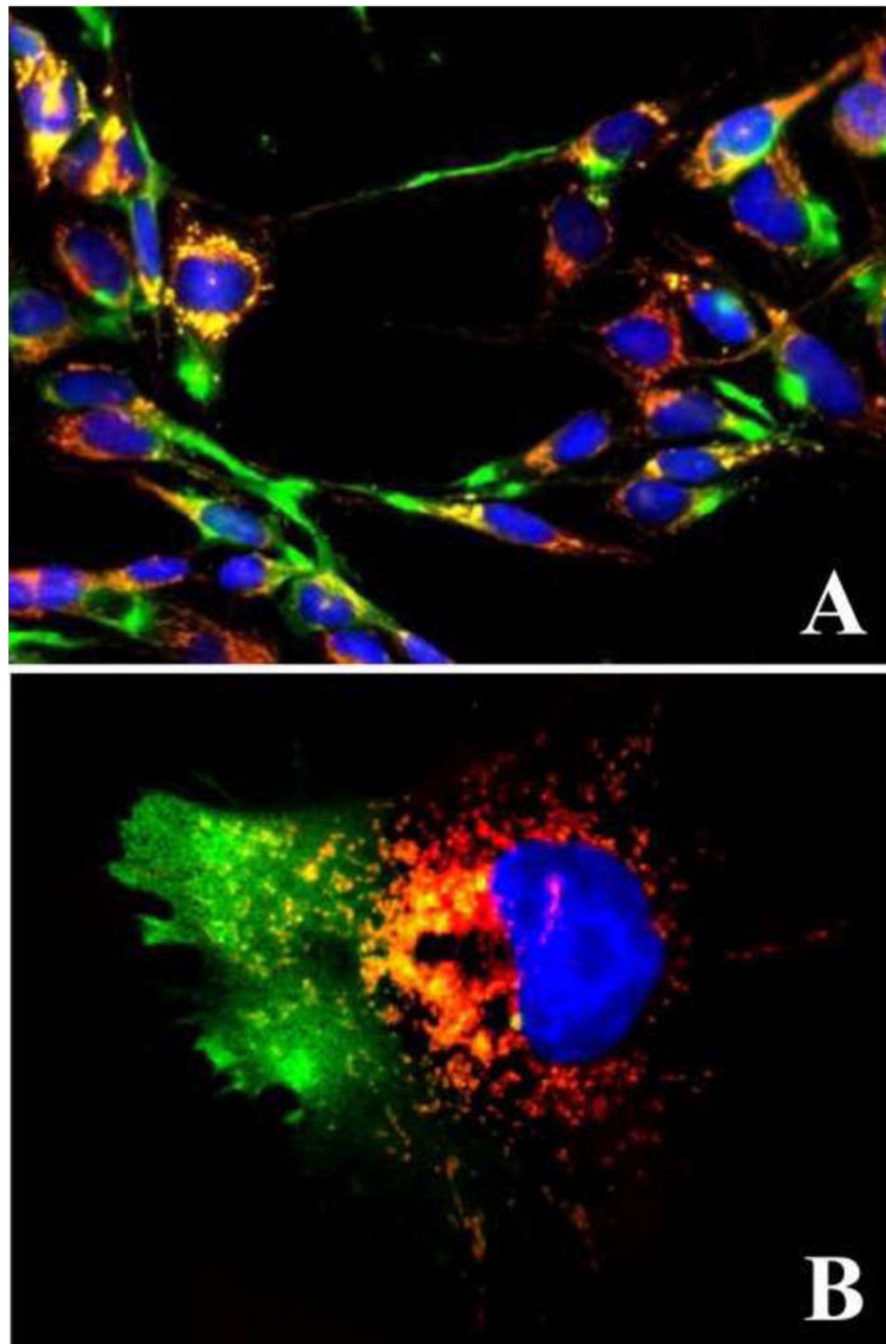

Figure 7.
gBK shows polarity within gliomas. U251 gliomas were allowed to adhere overnight, then fixed, permeabilized and stained with anti-gBK antibody (FITC-green), mitochondria are stained with Mito-tracker (red) and nucleus is stained with DAPI (blue). The left panel (40× mag) shows cells demonstrated polarity. As the cells stretch out there are more green gBK fluorescence along the invadopodia. The right panel (100×) slows more green gBK staining in the leading edge of the cells (left side), while the red mitochondria are distributed relatively uniform in the cells.

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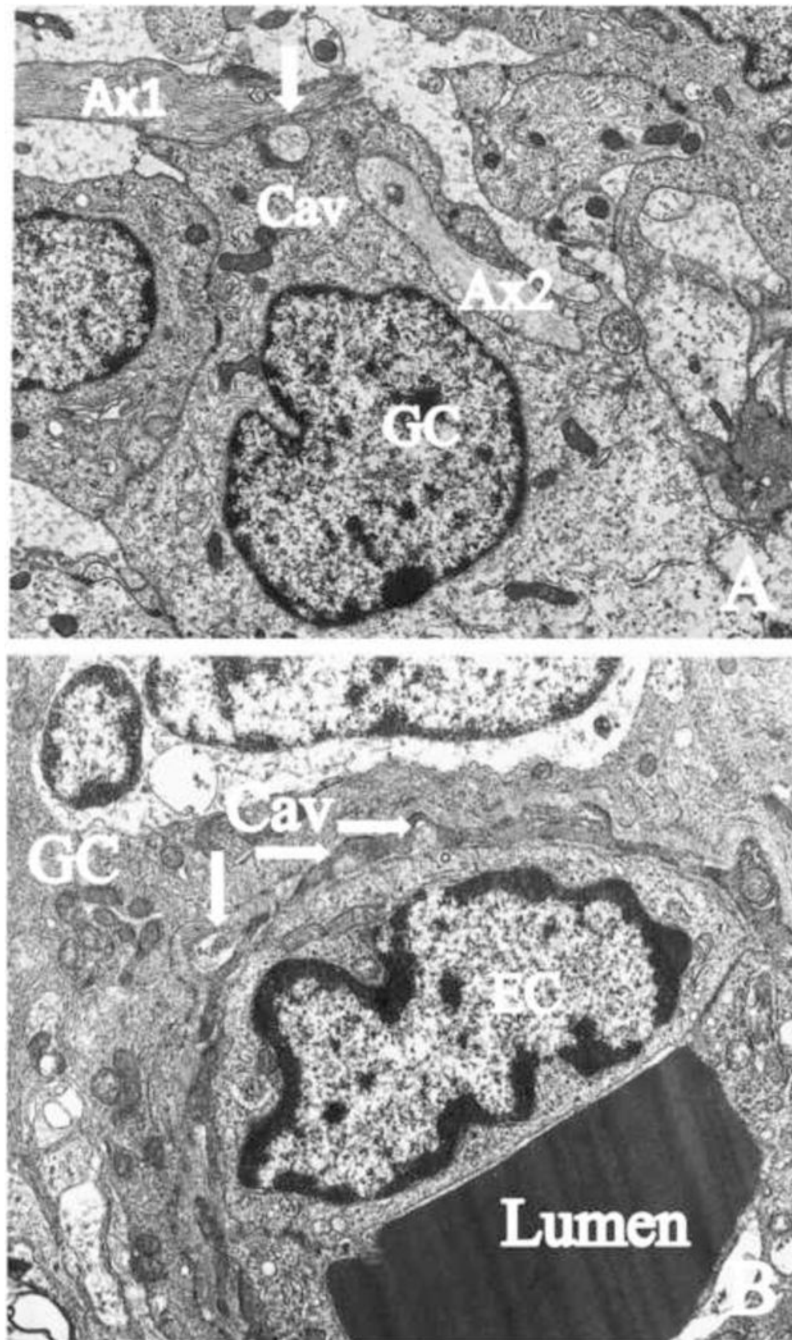

Figure 8.

Electron micrographs show caveolae within human gliomas. Panel A shows glioma cell (GC) involvement with nerves. Two amyelinic axons (Ax1 and Ax2) are in contact with the same glioma cell. Panel B illustrates glioma cell interaction with endothelial cells. L: lumen vessel; EC: endothelial cells. Caveolae (cav) are show by the arrows.

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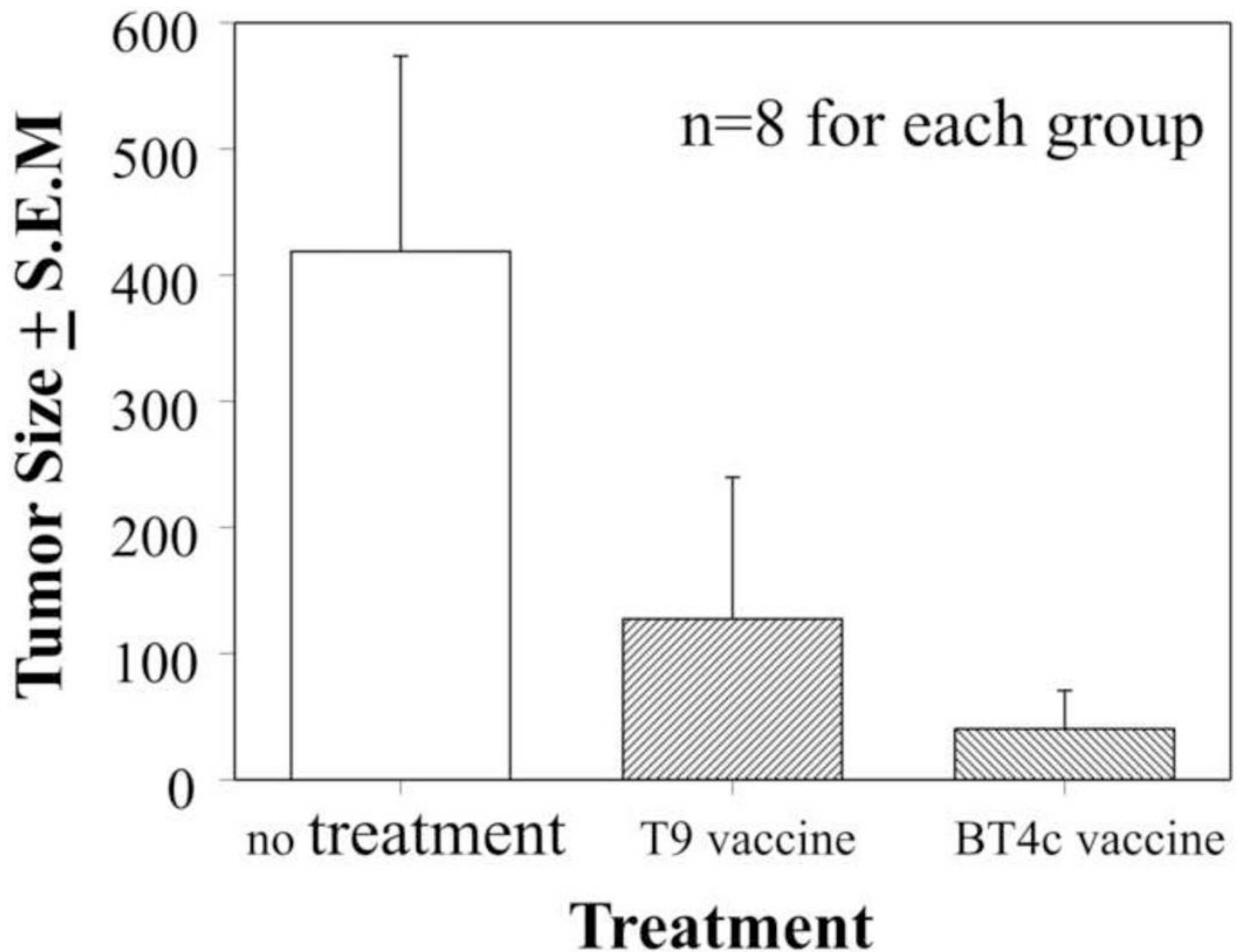

Figure 9.
Vaccination with BK activated/killed allogeneic BT4c cells provides cross-protection immunity against T9 glioma cells. Rats were immunized subcutaneously with one million T9 or BT4c glioma that were activated and killed with phloretin overnight. One week later the rats were challenged with one million T9 cells injected subcutaneously. The tumors were measured on Day 23. Each group contained 8 rats.

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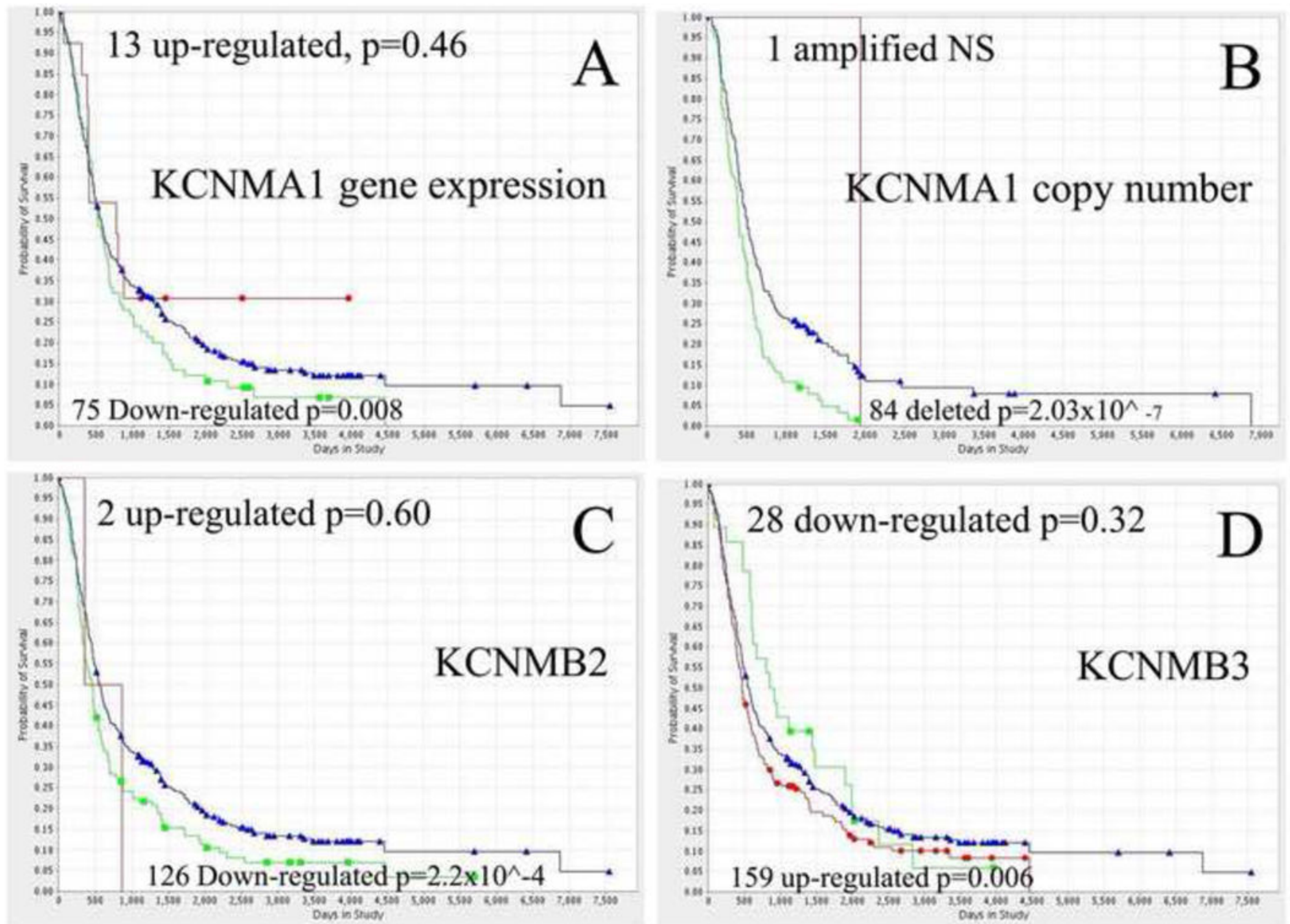

Figure 10.

REMBRANDT analysis of BK genes shows a complex relationship with glioma survival. All glioma patients within the REMBRANDT database were analyzed for the over- or under-expression of KCNMA1 (BK, Panel A). Thirteen patients (illustrated by red circles) had over-expression while 75 had a lowered level of KCNMA1 transcripts (green squares). The blue triangle/line denotes the total profile of all patients. Analysis of gene copy number was also done (Panel B); only 1 patient had an amplified copy number above 2, while 84 had low copy numbers. Panels C and D show the up-regulation or down-regulated of KCMB2/BKβ2 (Panel C) or KCMB3/BKβ3 (Panel D).

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Table 1

Various names and aliases that the BK channels can be called.

1)	Potassium Large Conductance Calcium-Activated Channel, Subfamily M, Alpha Member 1, Calcium-Activated Potassium Channel, Subfamily M Subunit Alpha-1, BKCA Alpha, BK_{CA} Alpha Subunit, Calcium-Activated Potassium Channel Subunit Alpha-1, KCa1, K(VCA)alpha,
2)	SLO1, Slo Homolog, Slowpoke Homolog, hSlo, Slo-alpha, Slo1, SLO-ALPHA, SLO1, mSLO, HSLO
3)	k(VCA)alpha
4)	Stretch-Activated Kca Channel, SAKCA
5)	MaxiK, Maxi K Channel, BK Channel, BKTM, BK Channel Alpha Subunit1
6)	KCNMA1
7)	bA205K10.1
8)	Variants:

STREX: stress induced expression

gBK: glioma BK channel

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Table 2

Discordance between published reports of ion channels within gliomas and REMBRANDT microarray-survival data.

Ion channel	# over-express	# under-express	Comments
Potassium Channels			
KCNMA1 (BKα)	13	75	trends for inverse relationship with survival
KCNMB1 (BKβ1)	45	2	no correlation with survival
KCNMB2 (BKβ2)	2	126, $p=2.21\times10^{-4}$	less expression the poorer the survival
KCNMB3 (BKβ3)	159, $p=0.006$	28	more expression the poorer the survival
KCNMB4 (BKβ4)	10	171	no correlation with survival
KCNH3 (Kv12.2)	0	287	no difference in survival
KCNH2 (Kv11.1)	48	7	trend for both with poorer survival
KCNJ2 (Kir2.1)	3	137, $p=0.028$	less express the better the survival
KCNJ5 (K_{ATP})	12	15, $p=0.058$	trend for less expression with better survival.
KCNK9 (TASK3)	3	133	trend for over-express with better survival.
Sodium Channels			
SCN2A	0	301	no difference in survival
SCN1A	9	93, $p=0.037$	down-regulation the poorer the survival
Chloride channels			
CLCN5 (CLC5)	7	0	no correlation with survival
CLCN3 (CLC3)	0	16, $p=0.023$	less express with poorer survival
CLCN2 (CLC2)	1	5	no correlation with survival
Acid sensing channels			
ACCN2 (ASIC1)	42, $p=1.14\times10^{-6}$	49, $p=0.045$	over-expression and less-expression gave reverse survival
Transient receptor potential channels			
TRPV1	5	15	no correlation with survival
TRPV2	9	31, $p=1.88\times10^{-4}$	less expression the better survival
TRPM8	210, $p=3.16\times10^{-8}$	30	more expression the worse survival
TRPC6	10	21	no correlation with survival
TRPC1	0	73	no correlation with survival
TRPM2	3	53	trend for under-express with better survival
Calcium channels			
CACNA1G (Cav3.1)	0	199	no correlation with survival

The ion channels that were discussed in Arcabeli, et al [7] and Ding, et al. [13] dealing with gliomas were examined by REMBRANDT analysis of gene expression and survival for all gliomas (522 patients). The number of patients that had either over-expression (>2.0 fold) or under-expression (<2.0 fold) are reported. If those gene expression/survival profiles do show statistical significance then it is reported along with the correlation towards better or worse survival.

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Table 3

REMBRANDT analysis of previously reported proteins that associate with BK channels and whether they can correlate with glioma patient survival.

Up-regulated	Down-regulated	Neither Up or Down Regulated
Good prognosis:	Good prognosis:	No Significant relationship:
No genes reported	spleen tyrosine kinase/SYK, Proline-Rich Tyrosine Kinase 2/ PYK2/protein tyrosine kinase 2 beta/PTK2B p=0.054	A kinase (PRKA) anchor protein 5/AKAP5/AKAP79/150; ankyrin repeat, family A2/ANKRA2; β-actin/ACTB; Calmodulin-1; Calmodulin 2; Calmodulin 3: Cofilin-1/CFL1; cereblon/CRBN; caveolin-3; cadherin-associated protein-beta 1/CTNNB1/β-catenin; cortactin/CTTN; glycogen synthase kinase 3 beta/ GSK3B; 3-phosphoinositide dependent protein kinase-1/PDPK1; GSF/ pancreatic and duodenal homeobox 1/PDX1; hippocalcin-like 1/HPCAL1; lin-7 homolog C/Lin7C; myeloperoxidase/MPO; SLOB/PX domain containing serine/threonine kinase PXK; superoxide dismutase 1/SOD1; stathmin 1/ STMN1; syntaxin 1A/STX1A; thromboxane A2 receptor/TBXAR2
Bad prognosis:	Bad prognosis:	
Actin G2/ACTG2; AKT-1/V-Akt Murine Thymoma Viral Oncogene Homolog 1/ RACα218; Annexin A5/ANXA5215; caveolin-1; caveolin-2; Lamin AC/LMNA219; prohibitin/PHB 216,220; 14-3-3γ/ YWHAG215	apolipoprotein A-I/ ApoA1;215,216,220 Focal adhesion kinase/Protein tyrosine kinase/FAK/PTK; glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)/GRINA; membrane associated guanylate kinase, WW and PDZ domain containing 1/MagI1; Microtubule associated protein 1A/MAP1A; stathmin 2/STMN2; stathmin 3/ STMN3;	

Proteins that were described in Lu, *et al.*, [18], Sokolowski, *et al.*, [212], Kathiresan, *et al.*, [213] and Kundu, *et al.*, [214] that reported proteins that directly associate with BK via proteomic methods were analyzed by REMBRANDT to determine whether that gene's up-regulation or down-regulation could be predictive of glioma patient outcomes for either a good prognosis or a bad prognosis. Significance denotes whether a positive or negative correlation occurred (P <0.05). The yellow highlights indicate that these proteins were detected by separate proteomic studies done with either freshly isolated glioma tissue or with glioma cell lines.