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Calcium Activated K⁺ Channels in The Electoreceptor of the Skate Confirmed by Cloning. Details of Subunits and Splicing

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Abstract

Elasmobranchs detect small potentials using excitable cells of the ampulla of Lorenzini which have calcium-activated K⁺ channels, first described in 1974. A distinctive feature of the outward current in voltage clamped ampullae is its apparent insensitivity to voltage. The sequence of a B_K channel α isoform expressed in the ampulla of the skate was characterized. A signal peptide is present at the beginning of the gene. When compared to human isoform 1 (the canonical sequence), the largest difference was absence of a 59 amino acid region from the S8-S9 intracellular linker that contains the stx regulatory domain. The ampulla isoform was also compared with the isoform predicted^{*} in late skate embryos where stx was also absent. The B_K voltage sensors were conserved in both skate isoforms. Differences between the skate and human B_K channel included alternative splicing. Alternative splicing occurs at seven previously defined sites that are characteristic for B_K channels in general and hair cells in particular. Skate B_K sequences were highly similar to the Australian ghost shark and several other vertebrate species. Based on alignment of known B_K sequences with the skate genome and transcriptome, there are at least two isoforms of Kcnma1 α expressed in the skate. One of the β subunits (β 4), which is known to decrease voltage sensitivity, was also identified in the skate genome and transcriptome and in the ampulla. These studies advance our knowledge of B_K channels and suggest further studies in the ampulla and other excitable tissues.

Keywords

Electoreception; calcium-activated potassium channel; alternative splicing; genome; Little skate; *Leucoraja erinacea*

1.1 Introduction

Calcium-activated K⁺ channels, which are of fundamental importance [29], were first described in the ampulla of Lorenzini of the skate (*Lecoraja erinacea*) [9,10,13,20] based on voltage clamp of the epithelium with ion substitution [9,10,13]. The original objective of this work was to confirm the presence of such channels in the ampulla by cloning the mRNA and to characterize these channels based on their amino acid sequence. Voltage dependent calcium and calcium-activated K⁺ currents arise from the apical faces of the

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receptor cells, whose basal faces synapse on afferent nerve fibers. It is impractical to record from single receptor cells in the intact epithelium. The amino acid sequence of the large conductance calcium-activated B_K channel was first described in drosophila [3] and then in the mouse [7]. Auditory hair cells, which are homologous to ampullary receptor cells [36,42] have similar channels which show alternative splicing at seven defined sites [5,30]. The little skate genome project [24] which includes the m-RNA transcriptome from embryos, has led to the discovery of contigs with high homology to mouse and human B_K channels. Shark embryos have functioning electroreceptors used in predator avoidance, which suggests that skate embryos also do this [21]. The availability of the skate genome presented an obvious opportunity to clone, identify and characterize the B_K channel in the ampulla of Lorenzini at the amino acid level. Brief communications have appeared [23,25].

2.1 Materials and Methods

2.1.1 Tissue Collection

Adult skates were obtained from the Marine Biological Laboratory in Woods Hole, MA and shipped alive in chilled seawater. They were killed instantly by pithing of the brain and spinal cord. Pithed animals showed no response to dissection of the ampullary capsule and canals. Approximately 100 ampullae were dissected with a short segment of canal and afferent nerve (Fig. 1a) and then homogenized. All procedures were approved by the Stanford laboratory institutional review board. The pithed animals were discarded.

2.1.2 cDNA Cloning and Sequencing

Total RNA was isolated from the homogenized tissues using Trizol reagent (Invitrogen, CA, USA) following manufacturers protocol. cDNA was synthesized using SuperScript II Kit™ First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo (dT)₃₀ primer, following the manufacturer's protocol. PCR was used to amplify *Kcnma1* using primers designed from the 3.4kbp sequence of the embryonic transcriptome assembly. The amplification product was ligated into a PCR2.1 vector (Invitrogen) and sequenced using an Applied Biosystems 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Eight primers were used to make overlapping PCR products to completely sequence the *Kcnma1* cDNA clone (Table I).

2.1.3 Sequence Analysis

An initial predictive assembly of the skate *Kcnma1* protein sequence was performed using data from late stage embryos to obtain the PCR primers. The resulting assembly has a 1210 bp 3' untranslated region (UTR). Additional sequence analysis was performed to compare the cloned ampulla sequence with other species and hair cell types of physiological interest. The human *Kcnma1* protein sequence (SwissProt Q12791) was used to identify an mRNA transcript of *Kcnma1* in the skate embryonic transcriptome and in coding exons of the *L. erinacea* genome assembly (24,43). Standard sequence alignment programs, TBLASTN from NCBI BLAST (1), were used to do this at SkateBase (<http://skatebase.org>), a public repository of skate genomic and transcriptome contigs (43). 100% of the embryo amino acid sequence was analyzed for the ampulla and there was 97% identity. Two identical and complete sequences were obtained for the ampulla based on the fact that there were two

successful clones. The cDNA sequence for the α subunit has been submitted to Genbank (accession number KJ765631). Similar procedures were used to identify the beta subunit, *Kcnmb4*. The open reading frame in *Kcnma1* cDNA was identified using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The signal peptide was predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). Multiple interspecies sequence alignments were analyzed using MUSCLE (15).

3.1 Results

3.1.1 cDNA Sequence of the Skate B_K α -subunit

Presence of the calcium activated K⁺ channel in the ampulla of Lorenzini of the skate is confirmed by the fact that all of the primers designed based on the embryo channel sequence functioned successfully in the PCR of mRNA from the adult ampulla. Fig. 1b shows a gel electrophoresis performed using the start and end primers which yielded a bright band of 3.4 kilobases as predicted. A predicted full length sequence for the α subunit of the B_K channel, as deduced by co-assembly of Illumina mRNA-sequence reads in late stage embryos, is shown in Fig. 2 (subject). This is aligned with the sequence of the cloned α subunit from the Ampulla of Lorenzini (query). Overall identity between the two sequences is 96%. Non-identical amino acids are found primarily at exon-exon junctions of three known alternative splice sites. There are three single amino acid substitutions that are not found at alternative splice sites. The sequences are identical at four of the known alternative splice sites (blue bars). The assembled embryo channel sequence is also shown in Supplemental Figure 2, which illustrates the location of known structural features of the B_K α subunit, including the seven membrane spanning regions (S0-S7). The intracellular structures, S8-S10, are now called RCK1 and RCK2. The 3' end of the α subunit is followed by a 121- nucleotide 3' untranslated region.

Figure 3 shows alignment of the amino acid sequence for the ampulla of Lorenzini (query) against the sequence for isoform 1 of human B_K α (subject). The most significant difference is the absence in the skate of 59 amino acids from the intracellular portion of the channel in a region called the S8-S9 linker (Supplemental Figure 2). Missing amino acids are shown as dashes in Fig. 3. In the human B_K channel (Isoform 1), the intracellular region contains a 59 aa insert at splice site 4 known as “strex” which has regulatory functions [17]. In the skate sequence, the region of Fig. 3 that contains dashes is broken up by three groups of amino acids, but these show very poor homology with the human sequence. Thus it is fair to say that “strex” is absent in the skate ampulla B_K α -subunit, and also in the assembled embryo channel. Nevertheless, a search of the skate genome revealed one contig of the genomic DNA build (contig 43473, residues 1288 to 1462) which contains an exon that encodes a 56 aa sequence that is orthologous to human strex (Fig. 8d).

3.1.2 Signal Peptide in the 5' Untranslated Region

The first methionine in Fig. 1 and 2 is preceded by 99 nucleic acids. About half of this region codes for a signal peptide that is preceded by a sequence with two methionines (MLAM). The presence of a signal peptide in the 5' untranslated region is clearly indicated by analysis of the sequence with Signal P (Supplemental Fig. 1). The signal peptide causes

the channel to be inserted into the membrane. The 33 nucleic acids (out of 99) preceding the signal peptide may be untranslated, although the reading frame remains open. The methionine that initiates the sequence in Fig. 1 aligns with the beginning of the human α -subunit (see Fig. 2) and also the beginning of the mouse subunit. As shown in Fig. 8a, the amino acids that precede MEPV are identical (with one exception) in the ampulla and embryo sequence. However, the first six amino acids for this region in the ampulla sequence cannot be identified with certainty because they are specified by the start primer. All 99 nucleic acids that code for the amino acids in Fig. 8a that precede *MEPV* are contained in skate genomic contig 13351 (Fig. 8b).

3.1.3 Alternative Splicing in Embryonic vs. Adult Ampullary B_K Channels

The ampulla sequence in Fig. 2 has seven alternative splice regions shown by solid blue lines above the ampulla (Query) sequence. These regions have been described by Miranda-Rottmann et al. [30] in the chick cochlea and are widely accepted. Four of these regions, 1, 4, 5 and 7, show no differences between the ampulla and embryo derived sequences. At site 2, there are two alternative exons, designated 10 and 11, found in the chick cochlea (Fig 4a; [30]). The sequence found in the skate embryo channel is nearly identical to chick cochlea exon 10, except that the sequence SYSAVS in chick cochlea is TYSAVN in skate embryos. Both sequences are 31 amino acids in length. The versions of alternative exon 11 found in the chick cochlea and ampulla are also 31 amino acids long, but differ from each other by one amino acid change. Furthermore, both the predicted skate embryo channel and chick cochlear exon 10 have the sequence IELIG compared to AALIL which is at the same position in the ampulla and chick cochlear exon 11. Site 2 is within the S6-RCK1 linker, which exerts mechanical force on S6 during calcium gating [32]. The IE to AA change (compare exon 10 and skate embryos with cochlea exon 11 and ampulla) is thought to induce flickery gating in a human *Kcnma1* splice variant[40].

Fig. 4b (reading frame 3) shows that exons 10 and 11 are located close together on skate genomic contig 115017 (red lettering). The sequences found are identical to the predicted skate embryo channel (exon 10) and the ampulla channel (exon 11). In this instance, both exons are flanked on both sides by the equivalent of stop codons at the boundary between the two exons and the flanking introns. A genomic contig would be expected to show both of two alternatively spliced exons separated by an intron. The complete sequence of chick B_K channel, cSlo, reported by Jiang et al. [18] agrees with Fig. 4a, exon 10.

At site 3, the exon boundaries have been reported as ESR//ILI or ESS//ILI (Fig. 5, left). For the skate ampulla, the boundaries are ESR and IFI (Fig. 2, Query), and a previously undescribed four amino acid insert, GAKC, is present. No insert is present in the skate embryo predicted channel, where the exon boundaries are ESS//ILI (Fig. 2, Subject). Fig. 5 (left) also shows the 6-7 amino acids preceding the site 3 boundary marker, which are similar in the chick cochlea, skate ampullae and skate embryos. The four amino acid insert, SRKR, which has been found at site 3 in rat and human B_K channels [39] was not found in the assembled skate embryo channel, or in the ampulla. The SRKR insert plays a role in circadian rhythm in the rat and other species.

3.1.4 Evidence in the Skate Genome for an Alternative Exon at Site 7

At splice site 7, the sequence EKQKY is found in both the embryos and the ampulla (Fig. 2) and also the chick cochlea. Splice site 7 in the chick cochlea sometimes contains exon 35, which is not present in human isoform 1 (see Genbank XM_007539391). A search of skate transcriptome contigs revealed perfect alignment with a 46 nucleotide portion of exon 35. The translation is SSLIPPIREVEDECstop, as in the chick cochlea [30]. A corresponding genomic contig (2678105) codes for the last 41 amino acids of exon 35, which are also the same in chick cochlea and the skate. It is unclear what tissue in the skate normally harbors exon 35, or whether the corresponding skate transcript might be present in the ampulla but thus far undetected.

3.1.5 Novel Alternative Splicing at Site Six in the Skate Ampulla

Alternative splice site 6 occurs in the general area between the beginning of exon 28 and the beginning of exon 30, which contains the calcium bowl. Previous descriptions of splice site 6 show that exon 29, which is often skipped, can be inserted between exon boundaries TEL/VND [30]. However comparison of sequences in several species suggests that exon 29 arose from a gene duplication. Amino acids TEL occur twice -- once at the end of exon 28 and again in exon 29 (Fig. 7). As shown in Fig 6a, a sequence of 26-28 homologous amino acids that can be identified as exon 29 is present in the chick cochlea [22] and the skate electroreceptor. A virtually identical sequence was identified as alternative exon B in an early description of the mouse B_K channel [7] and later in the mouse cochlea [38] and the rat [17,35]. Fluorescence imaging studies have shown that exon 29 occurs in inner hair cells of the mouse cochlea, which are the primary auditory receptor cells [5].

The sequence of exon 29 in the ampulla has 20 out of 28 amino acids that are identical to the corresponding amino acid in the chick cochlea [22]. In contrast, the sequence for the skate embryo B_K channel has only 9 amino acids that are found in the chick cochlea. The embryo channel is actually more homologous to exon 28 (see Fig. 7). The nucleotide sequence for exon 29 that was obtained by cloning the B_K channel in the ampulla can be found in the skate genome (genomic contig #2698762) and shows 100% agreement (Table II), but this sequence has not been identified in the transcriptome. These observations suggest that the B_K channel sequences obtained from the ampulla derive from sensory receptor cells, which have strong homology to auditory hair cells (inner hair cells) in several vertebrates.

An exact, and previously unreported alternative splicing scheme for exon 28 and exon 29 is shown in Fig. 6b and 7. As seen in Fig. 7, exon 28 consists of two discrete regions. There is an initial region of 15 amino acids which are highly conserved in five vertebrate species and in both isoforms of the skate B_K channel. The remainder of exon 28 is more variable, and is entirely skipped in the ampulla of Lorenzini, where the first 15 amino acids of exon 28 are followed by exon 29. In Fig. 6b, the red pathway represents the splicing scheme for B_K channels in which exon 28 is present in toto and exon 29 is skipped, while the black pathway shows the splicing scheme for hair cell type channels such as those in the ampulla. Figure 6b might also apply to the chick cochlea, where exon 29 is known to be expressed [22] but the possibility of partial skipping of exon 28 in the chick cochlea has not been studied.

The green line in Fig. 6b represents a predicted sequence for the zebrafish BK channel in which exons 28-30 are reported in sequence, as if they are all transcribed (NCBI Ref. Seq. XM_005156680.1). A rat B_K channel sequence derived from cDNA libraries also shows expression of both exons 28 and 29 [17], as also noted in Fig. 6b. In the study of Ha et al. [17] rat mRNA from several brain regions was amplified by PCR which confirmed that both exons 28 and 29 can be expressed. Accompanying patch clamp studies showed minor differences between B_K channels containing exon 28 and 29 and channels having only 28.

The strongest experimental evidence for the scheme shown in black in Fig. 6b is that in the skate ampulla of Lorenzini, the second portion of exon 28 is absent in the mRNA (Fig. 7 fourth line), and is followed by exon 29, and then exon 30.

3.1.6 Locations of Single Amino Acid Changes

As seen in Fig. 2, the ampulla sequence has 3 nucleotide differences that change single amino acids as compared to the embryos, and are not here described as part of recognized alternative splice sites. These are substitutions. All three substitutions were present in both successful clones. The first substitution (P78 for Q) is in the S0-S1 linker (Fig. 2 and Supplemental Figure 2). The other two D385 for N and R429 for K, are in the S6-S7 linker. There were no substitutions nor any mutations in the voltage sensor regions when the ampulla channel was compared to the skate assembled embryo channel (see Fig. 1, green letters). The three substitutions observed are most likely due to normal genetic variations in the animals studied.

3.1.7 Comparison with the Australian Ghost Shark

Efforts to sequence the *L. erinacea* genome have paralleled a similar project in the Australian ghost shark, *Callorhynchus milii* [41]. *C. milii* is a cartilaginous fish like elasmobranchs and has electroreceptors, but belongs to the Holocephali subclass rather than Elasmobranchii. Four full length isoforms predicted for the *C. milii* B_K channel are in GenBank (XM_007898487.1 to 89.1 and 96.1). These sequences are highly similar to those reported here for *L. erinacea*. The *C. milii* B_K channel has 23 amino acids preceding the usual start methionine (including MLAMAGDNASS) that are highly homologous to the initial portion of the predicted embryo BK channel and cloned ampulla channel (Fig. 8a). All four isoforms have this sequence. It is not known if Strex is expressed in *C. milii*, but two of the four isoforms contain a Strex sequence that is highly similar (50 out of 57 amino acid identities) to that found in skate genomic contig 13351 (Fig. 8b). None of the four *C. milii* isoforms contain exon 29. When the nucleic acid sequence for Kcnma1 in *C. milii* is compared to the skate embryo sequence the overall alignment is 85% identical.

3.1.8 Beta Subunits

B_K channels have beta subunits which contain two membrane spanning regions. Up to four beta subunits (one for each alpha chain) may be present in a B_K channel. In order to characterize skate beta subunits, the skate genome contig library was searched for possible alignment with each of the four human beta subunits. A full length skate ortholog of human β4 was found and its amino acid sequence is given in Fig. 9. The skate sequence for β4 shows 77% identity with the human version. The skate sequence was also aligned with the

ghost shark beta 4 gene as shown. The presence of beta 4 subunits, and also $\beta 1$, is known to reduce the voltage-sensitivity of B_K channels. Presence of the skate $\beta 4$ subunit is a potential explanation of for voltage insensitivity (i.e. absence of repolarization) under experimental conditions such as strong depolarization, where calcium entry does not occur. No sequences similar to human beta 1 (*kenmb1*) were found in the current skate or *C. milli* genome sequences. It is possible that orthologs of beta 1 exist in the skate and *C. milli*, but are not yet represented in the genome assemblies.

To further assess the possible role of $\beta 4$ subunits in down-regulating B_K channel function in the ampulla, messenger RNA for the $\beta 4$ subunit was identified in the ampulla through PCR and cloning experiments. Primers were designed to identify the exons of beta 4 individually (Table IV) and the amino acid sequence of the cloned PCR products is shown in Table III. mRNAs that coded for exon one and three were successfully identified, and were virtually identical to that predicted from assembly of the skate $\beta 4$ gene (Fig. 9). The first 20 amino acids of the second exon were also identified and agree with Fig. 9. The sequence for exon one and the partial sequence for exon two were each obtained from one clone in the same experiment. The sequence for exon three was obtained in five clones all of which gave the same sequence. Failure of PCR and cloning to obtain a full length sequence was due to non-specific binding of the primers that were designed for this purpose and possibly to deterioration of the ampulla mRNA sample during multiple re-freezings.

4.1 Discussion

4.1.1 Principal Differences Between Skate and Other B_K Channels

The results reported here are in agreement with prior work by Rohmann et al [37] who sequenced 206 amino acids starting with the pore in brain tissue obtained from skate. In comparing the skate B_K channel with the human isoforms there is generally a high degree of homology throughout the sequence. The most significant difference is the absence in the skate of 58 amino acids from the intracellular region (S8-S9 linker) – shown by dashes in Fig. 3. At the beginning of the discrepant region, 5 amino acids are present which are not contained in the human form (LQDEN in the embryos. RQDEN in the ampulla.) Note that the sequence CKR/LQD corresponds to alternative splice site 4. In the human B_K channel, (Isoform 1) the S8-S9 linker contains a 59 AA insert at site 4 known as “strex” which has regulatory functions [17]. The sequence of human strex is shown in Fig. 8d. In the skate version of the B_K channel (both the assembled embryo channel and the ampulla) all or almost all of strex is absent. In Fig. 2, there are only two amino acids in the ampulla B_K channel that align with the same amino acids in human strex (PSVLS in the ampulla vs. CSCMS in human strex). No homology with human strex occurs in the assembled embryo channel (Fig. 1).

The name strex is a contraction of the phrase “stress axis exon.” The strex exon is mainly found in the brain and involves pathways that mediate stress. In the rat brain, B_K channels that lack strex (called ZERO) coexist with channels that have strex [28]. Since skate electro-receptors lack efferent innervation, there is no obvious reason why an exon required for regulation of B_K channels by cyclic nucleotide protein kinases should be present in the

receptor cells, or how the receptor cells could function as part of a “stress related axis” since they are not postsynaptic and do not secrete adrenalin.

4.1.2 Comparison with Splice Variants in the Chick Cochelea

Analysis of chick cochlear B_K channels by Miranda Rottmann et al. [30] Fig. 6 *op. cit.* showed a total of 48 different splice variants. All but eight of these were extremely rare (less than 0.25% of the total). None of the eight most common splice variants contained strex. 54% of all clones ended with KYVQEDRL (which is similar to the ampulla clone). These were designated “short cSlo”. Most of the “short slo” transcripts were present at low levels. One of the common KYVQEDRL clones, accounting for 1.66% of the total clones, was homologous to the skate ampulla B_K sequence because it contained exon 11 and did not contain strex. The other common “short cSlo” clone accounted for 53% of the total clones and had exon 10 at splice site two and also did not contain strex. In these respects, the most common “short cSlo” clone is similar to the assembled skate “embryo” channel. Hair cells in the ampulla of Lorenzini may be different from those in the chick cochlea because there are no outer hair cells and also no tonotopic gradient.

4.1.3 Three Dimensional Structure of the B_K Channel in Skate

Yuan, et al [44] have recently determined the three dimensional structure of the intracellular portion of the human B_K channel by X-ray crystallography, beginning with the sequence HIVVCGHITLES....etc, which is highly conserved in the skate and begins at amino acid 345 in Fig. 2. What gives structure to the intracellular portion of the molecule are specific groups of amino acids that form either α - helicies or β - pleated sheets. The intracellular structure is organized into two tandem domains, called RCK1 and RCK2. Four pairs of tandem domains form the “gating ring” which has the pore at its center and can open the pore when cytosolic calcium binds to the “calcium bowl” which is in or next to RCK2. Based on the findings of Yuan et al [44] α - helicies designated αA through αJ are in RCK1, while αK through αX are in RCK2. For the β - helicies, βA through βJ are in RCK1 while βK through βT are in RCK2. This is a total of 44 structured regions.

The amino acid sequence of the human and skate B_K channels are sufficiently similar that the structure of the two channels can be assumed to be more or less the same. Other than alternative splicing at sites 3 and 6, there are only five structured regions of the crystalized portion of the B_K channel where the structure present in the human channel might be distorted in the skate by multiple (2-6) differences in constituent amino acids. Three of these regions in the human form are α - helicies (αK , αO and αU) and two are β - pleated sheets (βH and βO). βO has the sequence IITEL in the human, MMTEL in the skate embryo, and MITEL in the ampulla. Note that the βO sequences comprise the c-terminal end of exon 29 which is alternatively spliced in the skate ampulla (Fig. 6). There are 34 structured regions which are identical in the human B_K channel and the skate embryo, and there are 5 regions where only one amino acid differs. Neither of the two substitutions identified in the intracellular region lies within an alpha helix or a beta pleated sheet (Fig. 2 and [44]). The version of hslol found by Yuan et al. does not have strex –a further similarity to the skate B_K channel(s).

4.1.4 Physiological Role of Skate B_K Channels

Important physiological properties of the ampulla of Lorenzini that are likely to be mediated by B_K channels include sensitivity and accommodation [4,12,14, 31]. Receptor cells in the ampulla are tonically active and remarkably sensitive and can detect changes of less than 1 μ V [11, 12, 14]. Spontaneous activity of the receptor cells in vivo produces 20 Hz oscillations in epithelial current that result from interaction between calcium channels in the apical face and voltage dependent K⁺ channels in the basal face [11,12]. Repetitive action potentials have been recorded from dissociated receptor cells using the whole cell patch technique [2]. A principal function of the calcium activated current in the apical face is to hold the receptor cells near threshold in spite of variations in average potential. Onset of an excitatory stimulus increases the activity of the receptor cells and post-synaptic nerve firing, while termination of the stimulus can cause the nerve to stop firing [12,13].

4.1.5 Possible explanations for Voltage Insensitivity of the outward current

Calcium activated currents in the ampulla of Lorenzini do not show voltage dependence and appear to be purely sensitive to calcium [13]. This might suggest that the B_K channels in the ampulla might have special features. The voltage sensor region of S4 is conserved in the ampulla (Fig. 2, green letters and Supplemental Figure 2) and has all four arginines per α chain, 12 per channel. One of these is known to participate in gating according to Ma et al. [27]. In addition, the charged residues in S2 (D154 and R168) and S3 (D187) that are believed to participate in gating [27] are both present in the embryo and ampulla B_K channel. These findings disprove the suggestion that the voltage sensor is defective or nonfunctional in the ampulla of Lorenzini [8].

There are three other mechanisms that could lead to voltage insensitivity of B_K channels in the skate. These are: (i.) Suppression of voltage insensitivity by beta subunits. (ii.) Reduction of voltage insensitivity by alternative splice variants. (iii) Strong preponderance of S_K channels over B_K channels.

- i. Of the four types of beta subunits, beta 1 and beta 4 are known to inhibit voltage sensitivity of the channel, particularly at low calcium concentrations [6,33]. Beta 4 has been found in the skate genome (Fig. 10). The failure to find beta 1 in the genome suggests that the skate version of this subunit may have poor homology to the human ortholog. Expression of beta 4 in the ampulla has been confirmed by successful PCR and cloning experiments using primers for exon one and exon three (Table III). Further efforts are needed to determine whether exon two is present in the skate. Based on Fig. 10, 48 out of 210 amino acids in beta 4 (23%) are different in the skate than in the human. The conjecture that the beta 4 subunits have special properties that prevent the B_K channel from opening in low [Ca²⁺]_i is therefore tenable.
- ii. There is evidence that alternative splice variants, such as those described here, can modify the function of the voltage sensor [32,40]. Niu et al. [32] noted that artificial modification of exon 10 at splice site 2 by arbitrary addition of amino acids produced a shift in voltage sensitivity of the channel so that larger depolarizations were required to open the channel. A large shift in voltage

sensitivity of the B_K channel in the ampulla would explain the results of Clusin and Bennett [9,10] where voltage sensitivity appeared to be absent. Beta subunits have also been shown to shift voltage gating to more positive potentials at low calcium [6].

- iii. Sensory hair cells are known to contain both B_K and S_K channels. It is not certain that B_K channels are sufficiently plentiful in the ampulla or appropriately localized to explain the epithelial outward current. S_K channels are purely calcium-dependent and exhibit no voltage-dependent gating [13,45]. If a voltage-insensitive S_K channel were present in the receptor cells at high levels and B_K channels were comparatively rare, then very strong depolarization to the calcium equilibrium potential would produce no delayed rectification or late outward current under voltage clamp [11,12]. A skate homolog of the S_K3 has been assembled by alignment with human KCNN3, and is present in the skate genome (manuscript in preparation).

4.1.6 Conclusions and Future Directions

Based on the physiologic and comparative data, it has been proposed that the calcium activated potassium channels in the ampullae are localized to the receptor cells and are concentrated in the apical faces at the base of the hair. This could be confirmed using fluorescent antibodies against the B_K channel [36]. Further studies should focus on whether S_K channels are present in the ampulla and whether they are localized to the apical faces of the receptor cells. Additional clones of the B_K channel should also be obtained to determine whether more than one isoform is present in the electroreceptor. Deep sequencing should be performed on mRNA from the ampulla to further characterize the B_K channels, their subunits and also the S_K channels. Additional studies would include expression of the B_K channels in oocytes followed by single channel recordings, or structural characterization of purified B_K channel protein [19,44]. Protein folding and structure are influenced by alternative splicing, and features of the channel's structure might be predicted from the data given here. Similar alternative splicing in the B_K channels of other vertebrates should be investigated, especially splice site 6. Sequences from the Australian ghost shark should be cloned, particularly those from the ampulla of Lorenzini. Finally, in the event that a more detailed version (build) of the skate genome is released, the genome should again be searched for orthologs of human *kcnmb1* which, like *kcnmb4*, might contribute to voltage-insensitivity in the skate B_K channel in the ampulla of Lorenzini.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

aa	amino acid(s)
B_K	large conductance calcium-activated potassium channel
cDNA	complementary DNA
cSlo	a version of the Slo gene sequence (an older term for the B _K channel α subunit) found in the chicken
hSlo	a version of the Slo gene found in humans
Kcnma1	another term for the α subunit of the B _K channel
Kcnmb4	β subunit number 4 of the B _K channel
KCNN3	one of the sequences for small conductance calcium-activated potassium channels
mRNA	messenger RNA
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
S_K	small conductance calcium-activated channel
Slo	an older term for the α subunit of the B _K channel derived from experiments in <i>Drosophila</i> where a Slowpoke mutant with abnormal behavior was found

Highlights

- We characterized the B_K channel in the skate electroreceptor (ampulla of Lorenzini).
- We cloned and characterized cDNA's for the α and $\beta 4$ subunit.
- The presence of B_K channels in the ampulla is consistent with the known physiology.
- $\beta 4$ subunits may explain absence of voltage gating of the B_K current.
- Our results retrospectively confirm the discovery of a new class of K^+ channels.

Fig. 1.

a. Photograph of two dissected skate ampullae of Lorenzini with several millimeters of canal and several mm of afferent myelinated nerve attached. The canal is approximately 1 mm in diameter. b. Polyacrylamide gel electrophoresis of the intact, full length B_K channel for the ampulla of Lorenzini. The electrophoresis was run in duplicate and shows discrete bright bands of 3.4 kilo bases for the left and center lanes. The right lane is the marker lane, for which the test bands are labeled.

Author Manuscript	Query	1	MEPVIIPIDAEMICDSSDQRMWAFGLASSMVTFFGGLFIILVWRTTQYLWAVCCHGKIR	60
	Sbjct	1	MEPVIIPIDAEMICDSSDQRMWAFGLASSMVTFFGGLFIILVWRTTQYLWAVCCHGKIR	60
	Query	61	KDVQKIGGAANQADDAN ¹ DLDEKEGEIPQGEVGMWTSVKDWAGVMISAQTLTGRVLVVLV	120
	Sbjct	61	KDVQKIGGAANQADDAN ¹ DLDEKEGEIPQGEVGMWTSVKDWAGVMISAQTLTGRVLVVLV	120
	Query	121	FALSIGALVIYFIDSSDPIESCQNFYDDFTLQIDMAFNVFLLYFGLR ² FIAANDKLWFWL	180
	Sbjct	121	FALSIGALVIYFIDSSDPIESCQNFYDDFTLQIDMAFNVFLLYFGLR ² FIAANDKLWFWL	180
	Query	181	EVNSVVDFFTVPVFSVYLS ³ RSWLGLRFLRALRLIQFSEILQFLNILKTSNSIKLVNLL	240
	Sbjct	181	EVNSVVDFFTVPVFSVYLS ³ RSWLGLRFLRALRLIQFSEILQFLNILKTSNSIKLVNLL	240
	Query	241	SIFISTWLTGAGFIHLVENSQDPWENFQNSQPLTYWECVYLLMVTMSTVGYGDVYARTTL	300
	Sbjct	241	SIFISTWLTGAGFIHLVENSQDPWENFQNSQPLTYWECVYLLMVTMSTVGYGDVYARTTL	300
	Query	301	GRLFMVFFILGGLAMFAR ⁴ YVPEIAAL ⁵ LNRRKKGTT ⁶ FNATRGRKHIVVCGHITLESVSNF	360
	Sbjct	301	GRLFMVFFILGGLAMFAR ⁴ YVPEI ⁵ LI ⁶ NRKKGTT ⁷ +A ⁸ GRKHIVVCGHITLESVSNF	360
	Query	361	LKDFLHKDRDDVNVEIVFLHNIS ⁹ PLELEALFKRHFTQVEFFQGSVLNPHDLARVKIESS	420
	Sbjct	361	LKDFLHKDRDDVNVEIVFLHNIS ⁹ PLELEALFKRHFTQVEFFQGSVLNPHDLARVKIESS	420
	Query	421	DACLILAN ¹⁰ YCPDPAEDASNIMRVISIKNYPKIRIITQMLQYHNKAHLNIPSWNRE	480
	Sbjct	421	DACLILAN ¹⁰ YCPDPAEDASNIMRVISIKNYPKIRIITQMLQYHNKAHLNIPSWNRE	480
	Query	481	GDDAICLAELKLGFIASQCLAPGLSTMLANLFSMRSYIKIEEDTWQKYYLEGVANEMYTE	540
	Sbjct	481	GDDAICLAELKLGFIASQCLAPGLSTMLANLFSMRSYIKIEEDTWQKYYLEGVANEMYTE	540
	Query	541	YLSSAFIGLSFPAVCELFCVVKLLLLAIEYK-NTRES ¹¹ RGAKC ¹² IF ¹³ INPGNHIKHEGTLG	599
	Sbjct	541	YLSSAFIGLSFPAVCELFCVVKLLLLAIEYK-NTRES ¹¹ I ¹² INPGNHIKHEGTLG	596
	Query	600	FFIASDAKEVKRAFFYCKACHDDITDTKRIKCGCKRLQDENPSVLSPKKKQRNGGMRS	659
	Sbjct	597	FFIASDAKEVKRAFFYCKACHDDITDTKRIKCGCKRLQDENPSVLSPKKKQRNGGMRS	656
	Query	660	PNSSPKLMRHDPLSIPGNDQIEEKDASVKKYDSTGMFHWCPSEMEKVI ¹⁴ LTRESEAMTVL	719
	Sbjct	657	PNSSPKLMRHDPLSIPGNDQIEEKDASVKKYDSTGMFHWCPSEMEKVI ¹⁴ LTRESEAMTVL	716
	Query	720	SGHVVCIFGDLNSALVGLRNLVMP ¹⁵ LRASNFHYHELKHIVFVGTLEYLTREWETLHNFPK	779
	Sbjct	717	SGHVVCIFGDLNSALVGLRNLVMP ¹⁵ LRASNFHYHELKHIVFVGTLEYLTREWETLHNFPK	776
	Query	780	VSILPGTPLCRADLRVNLNLCDCM ¹⁶ CVIIISANQNNIDDCSLQDKECILASLNKSMQFDDS	839
	Sbjct	777	VSILPGTPLCRADLRVNLNLCDCM ¹⁶ CVIIISANQNNIDDCSLQDKECILASLNKSMQFDDS	836
	Query	840	IGVLQANSQGFTPPGMDRTSPDSS ¹⁷ ---G ¹⁸ KLL ¹⁹ PLVSFSQEKNF ²⁰ GANIPMI ²¹ TELVNDSNVQF	896
	Sbjct	837	IGVLQANSQGFTPPGMDRTSPDSS ¹⁷ G+P+ ¹⁸ GANIPM+TELVNDSNVQF	891
	Query	897	LDQDDDDDPDTELYLTQPFACGTAFVSVLDSLMSATYFNDNILT ²² LIRTLVTGGATPELE	956
	Sbjct	892	LDQDDDDDPDTELYLTQPFACGTAFVSVLDSLMSATYFNDNILT ²² LIRTLVTGGATPELE	951
	Query	957	SMLAEENALRGGYSTPQTLANRDRCRVAQLALYDGP ²³ FADLGDGGCYGDLFCKALKTYNML	1016
	Sbjct	952	SMLAEENALRGGYSTPQTLANRDRCRVAQLALYDGP ²³ FADLGDGGCYGDLFCKALKTYNML	1011
	Query	1017	CFGYIYRLDAHLRAQNQCTKRYVIANPHYEFELVSTDVIFCLMQFDHNAGQSRGSVSHSS	1076
	Sbjct	1012	CFGYIYRLDAHLRAQNQCTKRYVIANPHYEFELVSTDVIFCLMQFDHNAGQSRGSVSHSS	1071
	Query	1077	HSSHSSSKSSSVHSPSSTNRSNRVKTRDPREKQKYVVDK ²⁴ KV	1119
	Sbjct	1072	HSSHSSSKSSSVHSPSSTNRSNRVKTRDPREKQKYVVDK ²⁴ KV	1114

Fig. 2.

Alignment of the α -subunit of the B_K channel of the ampulla of Lorenzini of the skate (Query) against the skate sequence assembled from Illumina short reads obtained from a skate embryo (Subject). Bold underlined red letters show amino acids in the ampulla sequence that differ from the embryo sequence. Blue lines over the Query show the location of the seven known alternative splice sites in the α -subunit (see text). The two sequences differ only at splice sites 2, 3 and 6. There are three single amino acid substitutions which are not at alternative splice sites. Green letters show the amino acids believed to comprise or be associated with the voltage gate. These are highly conserved.

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Query	1	MEPVIIIPIDAEMICDSSDQRMWAFGLASSMVTFFGGFLFIILVWRTTQYLVWAVCCHCGIKR	60
Sbjct	1180	M+ +IIP+ E+ CDS QRMWAFGLASSMVTFFGGFLFIIL+WRT +YLW VCCHCG K MDALIIPTMEVPCDSRGQRMWAFGLASSMVTFFGGFLFIILLWRTLKYLWTVCCCHCGKT	1239
Query	61	KDVQKIGGAANQADDANPDLDEKEGEIPOGEVGMWTSVKDWAGVMISAQTLTGRVLVVLV	120
Sbjct	1240	K+ QKI ++QAD +DEKE E EVGMWTSVKDWAGVMISAQTLTGRVLVVLV KEAQKINNGSSQADGTLKPVDEKE-EAFAAEVGMWTSVKDWAGVMISAQTLTGRVLVVLV	1298
Query	121	FALSIGALVIYFIDSSDPIESCQNFYDFTLQIDMAFNVFLLYFGLRFAIANDKLWFWL	180
Sbjct	1299	FALSIGALVIYFIDSS+PIESCQNFY DFTLQIDMAFNVFLLYFGLRFAIANDKLWFWL FALSIGALVIYFIDSSNPIESCQNFYKFTLQIDMAFNVFLLYFGLRFAIANDKLWFWL	1358
Query	181	EVNSVVDFTTVPFVSVYLSRSLGLRFLRALRLIQFSEILQFLNLIKTSNSIKLVNLL	240
Sbjct	1359	EVNSVVDFTTVPFVSVYL+RSWLGLRFLRALRLIQFSEILQFLNLIKTSNSIKLVNLL EVNSVVDFTTVPFVSVYLNRSWLGLRFLRALRLIQFSEILQFLNLIKTSNSIKLVNLL	1418
Query	241	SIFISTWLTGAGFIHLVENSQDPWENFQNSOPLTYWECVYLLMTMSTVGYGDVYARTTL	300
Sbjct	1419	SIFISTWLT AGFIHLVENSQDPWENFQ+NQ LTYWECVYLLMTMSTVGYGDVYA+TTL SIFISTWLTAAAGFIHLVENSQDPWENFQNNQALTYWECVYLLMTMSTVGYGDVYAKTTL	1478
Query	301	GRLFMVFFILGGLAMFARYVPEIAALILNRKKYGGTFNATRGRKHIVVCGHITLESVSNF	360
Sbjct	1479	GRLFMVFFILGGLAMFA YVPEI LI NRKKYGG++A GRKHIVVCGHITLESVSNF GRLFMVFFILGGLAMFASYVPEI IELIGNRKKYGGSYSAVSGRKHIVVCGHITLESVSNF	1538
Query	361	LKDFLHKDRDDVNVEIVFLHNISPDELEALFKRHFTQVEFFQGSVLNPHDLARVKIES	420
Sbjct	1539	LKDFLHKDRDDVNVEIVFLHNISP+LELEALFKRHFTQVEF+QGSVLNPHDLARVKIES+ LKDFLHKDRDDVNVEIVFLHNISP+LELEALFKRHFTQVEF+QGSVLNPHDLARVKIESA	1598
Query	421	DACLILANRYCPDPAEDASNIMRVISIKNYNPKIRIITQMLQYHNKAHLNIPSWNRE	480
Sbjct	1599	DACLILAN+YC DPDAEDASNIMRVISIKNY+PKIRIITQMLQYHNKAHLNIPSWN+E DACLILANKYCADPAEDASNIMRVISIKNYHPKIRIITQMLQYHNKAHLNIPSWNKE	1658
Query	481	GDDAICLAELKLGFIASQCLAPGLSTMLANLFSMRSYKIEEDTWQKYYLEGVANEMYTE	540
Sbjct	1659	GDDAICLAELKLGFIASQCLA GLSTMLANLFSMRS+KIEEDTWQKYYLEGV+NEMYTE GDDAICLAELKLGFIASQCLAQGLSTMLANLFSMRSFKIEEDTWQKYYLEGVNEMYTE	1718
Query	541	YLSSAFIGLSFPVAVCELCFVKLKLKLLLAIEYKNT-RESRGAKEIFINPGNHKIKEHETLG	599
Sbjct	1719	YLSSAF+GLSFP VCELCFVKLKL++AIEYK+ RESR I INPGNH+KI EGTLG YLSSAFVGLSFPTVCELCFVKLKLMLAIEYKSANRESR----ILINPGNHLKIQEGTLG	1774
Query	600	FFIASDAKEVKRAFFYCKACHDDITDTKRIKCGCK-----RLQDEN	641
Sbjct	1775	FFIASDAKEVKRAFFYCKACHDDITD KRIKCGCK + + FFIASDAKEVKRAFFYCKACHDDITDPKRIKCGCKRPKMSIYKMRACCFDCGRSERD	1834
Query	642	PSVLSPKKKQRNGGMRNS-----PNS-SPKL-----MRHDP-	671
Sbjct	1835	S +S + + + + P++ SPK MR+ P CSCMSGRVGRNVDTLERAFPLSSSVND CSTSFRAFEDEQPSTLSPKKQRNGGMRNSPN	1894
Query	672	-----LSIPGNDQIEEKDASVKKYDSTGMFHCPSKEMEKVILTRSEAMTVLSG	721
Sbjct	1895	L IPGNDQI+ D++VKKYDSTGMFHC KE+EKVILTRSEAMTVLSG TSPKLMRHDPLIPGNDQIDNMDSNVKKYDSTGMFHCAPKEIEKVILTRSEAMTVLSG	1954
Query	722	HVVVICIFGDLSALVGLRNLVMPLRASNFHYHELKHIVFVGTLEYLTREWETLHNFPKVS	781
Sbjct	1955	HVVVICIFGD++SAL+GLRNLVMPLRASNFHYHELKHIVFVG++EYL REWETLHNFPKVS HVVVICIFGDVSSALIGLRNLVMPLRASNFHYHELKHIVFVGSEYELKREWETLHNFPKVS	2014
Query	782	ILPGTPLCRADLRAVNINLCDMCVIISANQNNIDDSLQDKECILASLNKISMQFDDSIG	841
Sbjct	2015	ILPGTPL RADLRAVNINLCDMCVI+SANQNNID SLQDKECILASLNKISMQFDDSIG ILPGTPLSRADLRAVNINLCDMCVILSANQNNIDTSLQDKECILASLNKISMQFDDSIG	2074

Query	842	VLQANSQGFTPPGMDR T SPD SSGKLLPLVSFSQEKNF --G ANIP M ITELVND S NVQFLDQ	899
		VLQANSQGFTPPGMDR+SPD+S P+ ++ + G NIP+ITELVND+NVQFLDQ	
Sbjct	2075	VLQANSQGFTPPGMDRSSPDNS----PVHGLMRQPSITTGVNIPIITELVNDTNVQFLDQ	2130
Query	900	DDDDDPDTELYLTQPFACGTAFVSVLDSLMSATYFNDNILTIRTLVTGGATPELE SML	959
		DDDDDPDTELYLTQPFACGTAFVSVLDSLMSATYFNDNILTIRTLVTGGATPELE+++	
Sbjct	2131	DDDDDPDTELYLTQPFACGTAFVSVLDSLMSATYFNDNILTIRTLVTGGATPELEALI	2190
Query	960	AEENALRGYSTPQTLANRDRCRVAQLAL Y DGPFADLGDGGCYGDLFCKALKTYNMLCFG	1019
		AEENALRGYSTPQTLANRDRCRVAQLAL DGPFADLGDGGCYGDLFCKALKTYNMLCFG	
Sbjct	2191	AEENALRGYSTPQTLANRDRCRVAQLALLDGPFADLGDGGCYGDLFCKALKTYNMLCFG	2250
Query	1020	IYRLRDAHL RAQN QCTKRYVI ANP H YEFELV STDV IFCLMQFDHNAGQSR G SVSHSSHSS	1079
		IYRLRDAHL +QCTKRYVI NP YEFELV TD+IFCLMQFDHNAGQSR S+SHSSHSS	
Sbjct	2251	IYRLRDAHLSTPSQCTKRYVITNPPYEFELVPTDLIFCLMQFDHNAGQSRASLSSHSSHSS	2310
Query	1080	H SSSKKSSSVHS VPS ST NR SNR VKT RD PRE KQKYV VDKKV 1119	
		SSSKKSSSVHS+PS+ NR NR K+R+ R+KQKYV +++++	
Sbjct	2311	QSSSKKSSSVHSIPSTANRQNRPKSRESRDQKQYVQEERL 2350	

Fig. 3.

Alignment of the cloned α -subunit of the B_K channel of the ampulla of Lorenzini (Query) against human isoform 1 of the B_K α -subunit (Subject). Bold underlined red letters show amino acids in the skate sequence that differ from the human form. Dashes show amino acids present in the human sequence that are missing from the skate sequence. The figure chiefly demonstrates that the “strex” insert at alternative splice site 4 in the human is not present in the skate ampulla of Lorenzini. Based on Fig.2, “strex” is also absent in the embryo sequence.

a

CHICK COCHLEA VS. SKATE SEQUENCES AT SPLICE SITE 2

GGL/AMFASYVPEIIEIIGNRKKYGGYSYSAVSGRK/HIV *CHICK COCHLEA EXON 10*GGL/AMFASYVPEIIEIIGNRKKYGGTYSAVNGRK/HIV *SKATE EMBRYOS*GGL/AMFARYVPEIAALILNRKKYGGTFNSTRGRK/HIV *CHICK COCHLEA EXON 11*GGL/AMFARYVPEIAALILNRKKYGGTFNATRGRK/HIV *SKATE AMPULLA*GGL/AMFA * YVPEI ** LI * NRKKYGGT ***** GRK/HIV *EXON 10 vs. 11*

b

READING FRAMES 1-3 OF SKATE GENOMIC CONTIG 115017 CONTAINING EXONS 10 AND 11

YSSVSyllLRPCLQATSLKS.N..GTARNMVVPIVR.MEESKYLMLCCLFYSRPLSACHSLFLFHACRQCLLATYQKLLSSLTGRNMEGLLMRPEGESKS
 .SILLSPIFFLGHVCKLRP.NHRINREOQEIWWYL.CGKKVSI.WGS^{SAV}SFTDHLHAILFFCSMHVGNVCSLRTRNCCSHP.PEEIWRDDF.CDQREKV
 FFCLLSSS.AMFASYPEIIEIIGNRKKYGGTYSAVNGRK.VSDGALLSLQ.TTFCMPFSFSVPCM.AMFARYPEIAALILNRKKYGGTFNATRGRK.VGG

Fig. 4.

Alternative splicing of the $B_K(\alpha)$ gene at splice site 2. a. Shows alignment of mutually exclusive exons 10 and 11 of the chick cochlea (first and third lines) with sequences for the B_K channel in skate embryos (line 2) and ampulla (line 4). All four sequences have 31 amino acids. The version of the B_K channel in the skate embryos is most similar to exon 10, with only two amino acid changes. The version in the ampulla is most similar to exon 11 with one amino acid change. The bottom line compares exon 10 and exon 11 in the chick cochlea. *'s indicate nine residues that differ in the two exons. b. shows a translation of a section of skate genomic contig 11501 in three different reading frames. Exon 10 and 11 are shown in red at the bottom line. These sequences are identical to those shown in a for the skate. Black and blue letters show intronic sequences or wrong frame translations that seem to indicate actual amino acids. Red dots indicate when the translated sequence gave a stop codon. The blue letters are similar to a B_K amino acid sequence reported in Gen Bank that terminates prematurely, evidently when the reading frame changes (ADD16631.1).

CHICK COCHLEA VS. SKATE AT SPLICE SITE 3 & 4

3	4
<i>Chick Cochlea</i>	<i>Chick Cochlea</i>
ESR//ILI	CKR//LED
Or	Or
EYKSE <u>K</u> RESS//ILI*	CLR//FED
<i>Skate Electoreceptor**</i>	<i>Skate Electoreceptor</i>
EKY NTRESR/GAKC/ <u>I</u> FI	CKR//L <u>Q</u> D
<i>Skate Embryo</i>	<i>Skate Embryo</i>
EKYSNTESS//ILI	CKR//L <u>Q</u> D
<i>Human</i>	
ESR/SRK <u>R</u> /ILI	

*Jiang et al., 1997. Bold **EK** is vs. skate forms. ESS is vs. ESR form.

**Novel four amino acid insert GAKC

Fig. 5. Left column shows alternative splicing of the B_K channel at site 3 in the chick cochlea, the skate and human. Right column shows variations at alternative splice site 4. There is a novel four amino acid insert (GAKC) at site 3 in the skate ampulla, and a known four amino insert (SRKR) in humans. No inserts are present at splice site 4 but there are minor variations in amino acids at exon boundaries. The strex insert is present at splice site 4 in the human (not illustrated).

a

SPLICE SITE 6 -- EXON 29

*Chick Cochlea**

TEL/AKPGKLLPLVSISQEKNSGTHILMITEL/VND (28)

*Mouse Alternative Exon B; Mouse Cochlear Variant;
Rat Alcorex Exon (Exon 29)*

TEL/AKPGKL PLVSVNQEKNSGTHILMITEL/VND (27)

Skate Electrorceptor

TSP/DSSGKLLPLVSFSQEKNFGANIPMITEL/VND (28)

Skate Embryo Channel (Best Fit To Exon 28)

TSP/DSSPVHGLVRPTSVITGANIPMMTEL/VND (26)

*Standard of comparison. Bold means identical.

Fig. 6.
a. Variations at alternative splice site 6 in the chick cochlea, skate, and other species. Skate embryo and ampulla data are repeated in Fig. 7 with preceding sequences. A 28 amino acid insert called alternative exon 29 is present in the chick cochlea [22]. A nearly identical insert (27 amino acids) has been reported in the mouse and designated alternative exon B [7]. Mouse cochlea [22] and rat [17] also have this sequence. The skate electrorceptor contains a 28 amino acid insert at site 6, of which 20 amino acids are identical to those in the chick cochlea. The sequence found in the skate embryo has 26 amino acids of which 9 are identical to those found in the chick cochlea. b. A diagram of the observed alternative splicing in the skate ampulla and embryo-derived B_K channels involving exons 28, 29, 30, which shows partial exon skipping. Includes data from Figs. 6a and 7. The pattern shown by black lines and arrowheads is found in the skate ampulla. The pattern shown by red lines and arrowheads is found in the skate embryo predicted B_K channel. The green lines represent

sequences for *D. rerio*, and also a rat B_K channel [17]. The *D. rerio* sequence is computer-predicted and shows all three exons as they occur in chromosomal DNA. The rat sequence is supported by PCR experiments with cDNA derived from rat brain mRNA [17].

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B_K CHANNEL SEQUENCE AT OR NEAR SPLICE SITE 6**EXON 28**

D. RERIO* • GFTPPGMDRSPDNS[PVHGLVRQ ASV TTGSNIPITEL
 GHOST SHARK • GFTPPGMDRA~~SP~~DNS[PVHGIVR **TS** ITTG~~AN~~IPITEL/VND
 SKATE EMBRYO • GFTPPGMDRT~~SPD~~**SS**[PVHGLVR **PTSV** ITG~~AN~~IP**MM**TEL/VND
 SKATE AMPULLA • GFTPPGMDRT~~SPD~~**SS** ABSENT
 CHICK (G.GALLUS) • GFTPPGMDRSPDNS[PVHGLLRQ**P** S **ITTG**~~AN~~IPITEL/VND
 RAT (rSlo) • GFTPPGMDRSPDNS[PVHG**MLRQ****P** S **ITTG**~~VN~~IPITEL
 HUMAN ISOFORM 1 • GFTPPGMDRSPDNS PVHG**MLRQ****P** S **ITTG**~~VN~~IPITEL/VND
 (hSlo1) -----
 βO

EXON 29

Δ

D RERIO (CONTINUED)* TEL[AKPGKLLPLVSFSQEKNSGTNIQMTEL/VND
 AMPULLA **TSPDSS**[GKLLPLVSFSQEKNF**GA**NI**P**MITEL/VND
 CHICK COCHLEA TEL[AKPGKLLPLVS**IS**QEKNSGT**HI**LMITEL/VND
 RAT (rSlo) TEL[AKPGKL PLVS**VN**QEKNSGT**HI**LMITEL/VND

Δ RESIDUES BEFORE THE BRACKET ARE REPEATED FROM EXON 28 ABOVE. TEL IS THE END OF EXONS 28 AND 29. VND IS START OF EXON 30, WHICH HAS CALCIUM BOWL. TSPDSS PRECEDES EXON 29 IN THE AMPULLA.

• INDICATES START OF EXON 28.

/ INDICATES START OR END OF AN INSERT AT A KNOWN EXON BOUNDARY WHICH IS ALSO A KNOWN SPLICE SITE.

[INDICATES START OF ALTERNATIVE SPLICING AT A SITE WHICH IS NEITHER AN EXON BOUNDARY NOR ONE OF SEVEN KNOWN SPLICE SITES.

* THE DANIO RERIO SEQUENCE WAS PREDICTED BY A COMPUTER ALIGNMENT WHICH REMOVED INTRONS FROM A GENOMIC SEQUENCE. EXONS 28-30 ARE SHOWN IN ORDER ON THE CHROMOSOME. NCBI REFERENCE SEQUENCE XM_005156680 (LABELLED Kcnmala). BOLD MEANS DIFFERENT FROM D.RERIO SEQUENCE JUST ABOVE.

βO IS FROM DATA IN YUAN ET AL., 2010. CHICK COCHLEA SEQUENCE FROM MIRANDA-ROTTMANN ET AL., 2010. RAT SEQUENCE IS FROM HA ET AL., 2000 AND IS BASED ON A cDNA LIBRARY, AND BRAIN PCR EXPERIMENTS.

Fig. 7.

Actual or predicted sequences of Exon 28 and Exon 29 of the α subunit of the B_K channel in the skate and five other species (zebrafish, ghost shark, chick, rat and human isoform 1). The sequence for exon 29 labelled chick cochlea is from Miranda-Rottman, et al. [30] and Kim et al. [22] (which agree). The sequence of exon 28 for chick (G. Gallus) is from Jiang et al. [19], where exon 29 was not found. The zebrafish (*D. rerio*) sequence is a computer predicted sequence with introns automatically removed, which gives the amino acids for exon 28, 29 and 30. The skate ampulla sequence is remarkable because the second half of exon 28 is absent, and is followed by exon 29. The predicted skate embryo channel contains both parts of exon 28. Exon 29 is skipped. The first 15 amino acids in exon 28 are highly conserved, and may be present in B_K channels of all higher animals. Alternative splicing that affects a portion of an exon is known to occur, but has not been reported for splice site 6

of the B_K channel. The letters βO represent beta pleated sheet O in the structural study of Yuan et al. [44].

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Fig. 8.

a. Amino acids preceding the conventional initiation site of the B_K (α) subunit, which is MEPV in the skate (aa 1-4 of Fig. 2 and 3). Triangles indicate amino acids not present in the ampulla PCR product because they precede those encoded by the start primer (horizontal bar). Most of the nucleotides in this region code for a signal peptide (see Data Supplement, Fig. 1). b. Translation of skate genomic contig 13351. Non-underlined letters show amino acids at the beginning of the B_K channel. The first 31 non-underlined amino acids preceding *MEPV* agree with those in a. Bold underlined letters are intronic sequences translated as if they were amino acids. Amino acids near the end of the contig agree with the human exon 1 boundary. c. 3' End of the sequence shown in text Fig. 2. The end primer (horizontal bar) begins where there are two consecutive stop codons. Amino acids in italics are encoded in both the ampulla and the predicted skate embryo channel. It is not known if the amino acids CCDHD, which precede the two stop codons, are expressed in the channel protein. d.

Sequence of the Strex exon found in isoform 1 of the human B_K channel and the ortholog of Strex found in the skate genome. Strex was not found in the transcriptome. The skate version of Strex would contain 12 variant amino acids (out of 57) if it were expressed in the ampulla or other skate tissue.

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Table I

Start Primer:	1+	ctgcctctcgcctgtctgtcctcatg
	2+	ccagaactccaaccactgacctactggg
	1-	ccgtaccgaccgtcgacatgggtgacc
	3+	ccagtgccttcacggcttgctctccc
	2-	gcagcttcagcttcacaaagcatagctc
	4+	gcagtccagtccatggcttggtgcg
End Primer	3-	gggatatggctcctgtgatgacgg
	4-	gggaattgtcaaacattcttgtcatca

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Table II

Nucleic Acid Sequence: Ampulla Exon 29 (Spice Site 6) Skate Genome Contig #2698762, Nucleotides 183-260 (Plus/Plus)	
GGCAAATTGCTCCCTTTGGTTTCATTTCAGTAGGAAAAAACTTTGGAGCCAACATCCCAATGATAACTGAGTT	
Corresponding Amino Acids	
GKLLPLVSFSQKFNFGANIPMITELVND	

Table III

<u>EXON ONE OF β4 SUBUNIT</u>	
<u>AK</u> ARFAYEYSEAEDKSIRGLFFMVAGIISLFILGFCWLNPTLNNMQCKAANCTVVSLRQIGEMSECTFTCGADCKGTSLYPCIQIFVNN	
<u>EXON TWO OF β4 SUBUNIT (PARTIAL):</u>	
SQSNSRALL <u>HYDEQQLLNP</u>	
<u>EXON THREE OF β4 SUBUNIT:</u>	
<u>PDDVLLKRT</u> <u>RD</u> <u>ENVLLHCVLWPLL</u> TLLVGV LIVLLTVCGKSLAIRA <u>AELQKMKYS</u> * *	

Bold underlined **E** differs from skate genome (D) but agrees with human form.

** = consecutive stop codons. Solid line is above amino acids specified by a primer (Table IV).

Table IV

Start Primer	A+	5' cgcagcgagggtatatctctcatggcgaaggc
	A-	5' gggatttagaagtagttgctgctcgtcgtaatgg
	B+	5' cctgatgacgtgctgctgaaggagacc
End of Sequence	B -	5' cactaggagtacttcatttctgcaaggcttctgctc
End Primer (3'UTR)		5' cgtgtcatcatgggcatgctgcctgattgtcac*

Exon 1 & 2 (partial): A+ to A-

Exon 3: B+ to B-

* Not experimentally confirmed