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Identification of Ca^{2+}-activated K^+ channel splice variants and their distribution in the turtle cochlea

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Turtle auditory-hair cells are frequency-tuned by the activity of calcium-activated potassium (K_{Ca}) channels, a cell's characteristic frequency being determined by the K_{Ca} channel density and kinetics which both vary systematically along the cochlea. As a first step towards identifying the source of K_{Ca} channel variation, we have isolated, by reverse-transcription polymerase chain reaction on dissociated hair cells, the main cDNAs homologous to the *slo* gene which encodes the channel's α-subunit. A total of six alternatively spliced variants were identified, the smallest of which is 94% identical to a mouse *Slo* sequence. Variation occurs by insertion of exons at only two splice sites, two of these exons encoding novel 31- and 61-amino acid sequences. As we were unable to detect splicing at other potential sites, we infer that the six variants correspond to naturally occurring combinations. The spatial distribution of the variants, defined by isolating hair cells from different regions of the cochlea, indicated that some isoforms were non-uniformly distributed. Those containing large inserts in the first splice site were notably absent from the highest-frequency region. We suggest that alternative splicing of the *slo* gene may contribute to variation in K_{Ca} channel properties.

Keywords: hair cell; electrical tuning; RT-PCR; Ca^{2+}-activated K^+ channels; alternative splicing

1. INTRODUCTION

Electrical tuning of the auditory receptor potentials is one of the main mechanisms by which hair cells extract the frequency components in a sound stimulus (Crawford & Fettiplace 1981). Electrical tuning has been reported in all vertebrate classes except mammals and arises by the combined action of a voltage-sensitive calcium current and a calcium-activated potassium (K_{Ca}) current (Art & Fettiplace 1987; Hudspeth & Lewis 1988; Fuchs *et al.* 1988). In the turtle cochlea, the frequency to which a hair cell is tuned, its characteristic frequency, changes systematically along the cochlea (Crawford & Fettiplace 1980). A cell's characteristic frequency is determined mainly by the density and speed of activation of its K_{Ca} channels (Art & Fettiplace 1987; Wu *et al.* 1995). When characterized in membrane patches from intact hair cells, single K_{Ca} channels in all cells have a similar conductance (approximately 300 pS) and Ca^{2+} sensitivity, but different mean open times (0.35–14 ms) correlated with the cell's characteristic frequency and cochlear location (Art *et al.* 1995). The mechanism by which the channel kinetics are altered is unknown. One plausible hypothesis involves the differential expression of kinetically distinct isoforms of the K_{Ca} channel in different regions of the cochlea (Wu & Fettiplace 1996). To examine this hypothesis, we have identified the main K_{Ca} channel isoforms that exist in turtle hair-cells.

The *Slo* K_{Ca} channel α-subunit was first cloned from *Drosophila* (Atkinson *et al.* 1991) and shown to produce a functional K_{Ca} channel when expressed in *Xenopus* oocytes (Adelman *et al.* 1992). Transcripts encoding α subunits have since been isolated from vertebrates and arise from a single gene that is alternatively spliced (Butler *et al.* 1993; Pallanck & Ganetzky 1994; Tseng-Crank *et al.* 1994). A total of six splice sites have been identified in the cytoplasmic tail of vertebrate *Slo* (Tseng-Crank *et al.* 1994; Vogalis *et al.* 1996) and alternative splicing yields isoforms with different channel properties. Most recently, a full-length α-subunit clone, *cSlo-1* was derived from a chick cochlear library and shown to produce K_{Ca} channels with large conductance similar to native hair-cell channels (Jiang *et al.* 1997).

2. METHODS

(a) *Hair cell RNA isolation*

RNA was obtained from hair cells derived from a single cochlea and a reverse transcriptase polymerase chain reaction (RT-PCR) strategy was employed to isolate cDNAs of interest by using standard methods (Sambrook *et al.* 1989). Turtles (*Trachemys scripta elegans*, carapace length 100–125 mm) were decapitated and the heads split sagitally with a razor blade. This procedure was approved by the Animal Care Committee at the University of Wisconsin. The brains were collected and immediately frozen on dry ice for later use. The cochlear duct was dissected out and hair cells were isolated as described previously (Art & Fettiplace 1987). Briefly, this involved incubation of the cochlea for 30–35 min in a saline (composition in mM: NaCl,

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125; KCl, 4; $CaCl_2$, 0.1; $MgCl_2$, 2.2; NaHEPES, 10; pH 7.6), containing papain (0.5 mg ml^{-1}, Calbiochem, La Jolla, California), bovine serum albumen (0.1 mg ml^{-1}) and L-cysteine (2.5 mM). Cells from the entire length of the cochlea were harvested by aspiration into a glass pipette (internal diameter, 200 μm), which had been pre-coated with tri-N-butylchlorosilane to render it hydrophobic. The tip of the pipette was filled with about 20 μl of RNase-free mineral oil before collection of the cells. The mineral oil and cells were transferred to a centrifuge tube containing 1 μl of RNasin (Promega, Madison, Wisconsin, USA) on ice. For studying the distribution of transcripts, the portion of the papilla situated on the basilar membrane was divided into four regions of equal length using a tungsten needle, and cells from each region were collected separately. Although the basilar papilla contains several cell types (hair cells, supporting cells and nerve terminals), hair cells are the only intact cell type normally isolated (Art & Fettiplace 1987). Supporting cells are disrupted by the isolation procedure and their RNA is therefore most likely to be degraded by endogenous RNases before addition of RNase inhibitor.

The hair cells were lysed by addition of an equal volume (*ca.* 30 μl) of RNase-free water and the solution was extracted with 1 volume of chloroform to remove lipids and proteins. A total of 1 μl of glycogen (10 mg ml^{-1}), one-tenth volume 3 M sodium acetate (pH 5.2), and 2.5 volumes of 100% ethanol were then added, and the mixture incubated at −20 °C overnight. Samples were spun at 14 000 *g* at room temperature for 30 min, washed once with 60 μl of 80% ethanol, and spun for 5 min at 14 000 *g*. Ethanol was decanted and samples were dried *in vacuo*. RNA was isolated from turtle brain by using the guanidinium isothiocyanate–caesium chloride gradient method (Sambrook *et al.* 1989).

(b) *cDNA production, amplification and sequencing*

First-strand cDNA was prepared from each RNA sample by using 50 pmol of random hexamer primers (Boehringer Mannheim, Indianapolis, Indiana, USA) and 200 units of SuperScript RNase H$^-$ reverse transcriptase (GIBCO-BRL, Gaithersburg, Maryland, USA) in a 20-μl volume according to the manufacturer's protocol. After incubation at 23 °C for 15 min, followed by 42 °C for 2 h, 1 μl of 4 M NaOH was added and samples were heated to 65 °C for 5 min to inactivate the enzyme and to hydrolyze RNA. The cDNA was precipitated and collected by centrifugation, rinsed with 80% ethanol, dried and resuspended in 20 μl of distilled water.

A total of six overlapping segments, each between 500 and 1200 base pairs (bp) in length (see figure 1), were amplified by using degenerate primers designed from alignment of the deduced amino acid sequences for human (U11717; Tseng-Crank *et al.* 1994), mouse (L16912; Butler *et al.* 1993), dog (U41001; Vogalis *et al.* 1996), and chicken *Slo* (U23821; Jiang *et al.* 1997). The sequences of the degenerate primers are given in figure 1. Alignment of the vertebrate *Slo* 5′ sequences that included the first four potential start ATG codons were used to design primers. Our forward segment 1 primer is based on the chick *cSlo-1* sequence and includes the third ATG which will be used in the later numbering as the start methionine. At the 3′ end, design of segment-6 reverse primers is not straightforward because of both the divergence of the untranslated regions and the carboxy termini. In the chick, two termini can be seen. A partial sequence, *cSlo-2*, includes a carboxy terminus which is 54 amino acids longer than the equivalent region in *cSlo-1* (Jiang *et al.* 1997). Long forms are also found in *canSlo* and *mSlo* and short forms are seen in *hSlo*. We amplified a turtle long-form sequence by using a downstream primer based on a conserved 3′ untranslated region between *cSlo-2* and the long form of *mSlo*, 23 bp downstream from the stop codon. Repeated amplifications of brain and hair-cell cDNA using primers based on the short form *cSlo-1* failed to generate any product.

PCR was done in a 50 μl solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase (GIBCO BRL, Gaithersburg, Maryland, USA), 200 pmol of each degenerate primer or 50 pmol of each non-degenerate primer, and cDNA from a single papilla reverse transcription or 30–50 ng from brain cells. With degenerate primers, PCR conditions were 35 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 90 s, and extension at 72 °C for 150 s, with a final extension of 10 min. Negative controls with no added template or samples that had not been reverse transcribed were run in parallel in each amplification. PCR product sizes were determined by polyacrylamide gel electrophoresis (Sambrook *et al.* 1989), calibrated with DNA size markers (Life Technologies, Gaithersburg, Maryland, USA), and visualized with ethidium bromide. To determine if a band contains multiple products with different sequences, a restriction enzyme digestion with a common cutter (*Hae* III or *Hinf* I) was done and fragments were size-separated by gel electrophoresis. If a band contains more than one product, the sum of the sizes of the individual fragments after digestion will be greater than the size of the original single PCR band (Buck & Axel 1991).

PCR products were blunt-end subcloned into pCR-Blunt (Invitrogen, San Diego, California, USA) and both strands were sequenced using Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio, USA) or with an ABI automatic sequencer at the University of Wisconsin Biotechnology Center (Madison, Wisconsin). Sequences were analysed using DNASTAR software (DNASTAR Inc., Madison, Wisconsin, USA), and BLAST search protocols on the databases at the National Center of Biotechnology Information (National Institute of Health, Bethesda, Maryland, USA). As a safeguard against potential sequence errors generated by the polymerases, sequences for every segment were obtained from at least three clones, each derived from a different hair-cell preparation.

Sets of turtle-specific primers flanking alternate splice sites (SS) SS1, SS2, SS3 and SS4 were used to examine the distribution of splice variants within the cochlea. The PCR conditions were 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 60 s, and extension at 72 °C for 120 s. The forward and reverse primers were:

SS1:	5′AAAGCTCTTAATGATAGCCATAGA;
	5′CACTTGCAATGAAGAATCCTAAAGTA.
SS2:	5′GTCATGATGACATCACAGATCC;
	5′CTCATGCCTCCATTTCGCTGC.
SS3:	5′GAAGATGAACATCCGTCGACAC;
	5′CATGTTGTCAATCTGCTCATTGC.
SS4:	5′CACCAGGAATGGATAGGTCATC;
	5′GTGTCTGGGTCGTCGTCATCG.

To investigate further possible combinations of SS1 and SS2 inserts, a pair of turtle-specific primers, one just downstream of SS2 and the other bridging the splice junction for SS1 = 4 amino acid insert (SRKR) were used. The latter primer included 11 bases upstream of the splice site and the first ten bases of the insert. The primer pair would not amplify fragments with SS1 = 0. The forward and reverse primers were as follows:

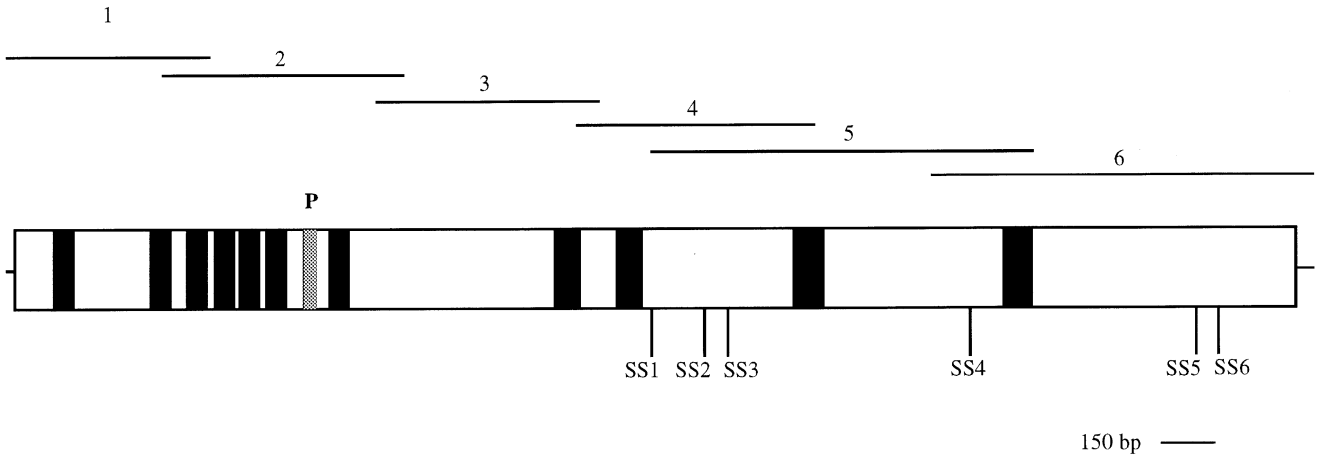


Figure 1. Schematic diagram of the turtle Ca^{2+}-activated K^+ channel *tSlo* cDNA. The box is the open reading frame with the filled sections representing 11 amphipathic-helical domains (S0–S10, see Meera *et al*. 1997) and the pore region (P). The location of sites for alternative splicing identified in other vertebrate *Slo* are denoted as SS1–SS6. The numbered lines indicate the size and position of the six regions amplified with RT-PCR by using degenerate primers based on other vertebrate *Slo* sequences. The forward (5′ to 3′) and reverse (3′ to 5′) primer pairs (where 'I' denotes inosine) were as follows.

1. 5′CCCAAGATGGATGCGCTCATCA;
   5′CCACAGCTTGTCATTGGCTGC.
2. 5′CA(A/G)ATIGA(T/C)ATGGCITT(T/C)AA(T/C)GTITT;
   5′(G/A)TTIAC(A/G)TC(A/G)TCIC(G/T)(A/G)TC(C/T)TT(A/G)TG.
3. 5′AA(A/G)CA(T/C)ATIGTIGTITG(T/C)ACITGGCA(A/G)A;
   5′TACAT(T/C)TC(A/G)TTIGCIACICC(T/C)TC.
4. 5′AA(A/G)ATIGA(A/G)GA(A/G)GA(T/C)ACITGGCA(A/G)A;
   5′CAAAIAT(A/G)CAIACIACIAC(A/G)TGICC.
5. 5′ACGGGAAAGCAGAAGCCGAAAA;
   5′GTICC(A/G)CAIGC(A/G)AAIGG(T/C)TGIGT.
6. 5′GCAGGACAAGGAATGCATCTT;
   5′CATGCACACTATCATACATATGC.

SS1–SS2: 5′ACGGGAAAGCAGAAGCCGAAA;
5′CTCATGCCTCCATTTCGCTGC.

None of the primer pairs employed in these experiments were found to yield products when turtle genomic DNA was used as a PCR template. Unless otherwise stated all reported sequences were derived from hair-cell preparations.

3. RESULTS

(a) *cDNA cloning and the sequence of* tslo

We have identified six alternatively spliced cDNAs homologous to *Slo* that occur in turtle hair cells. Of the isoforms, two contain novel exons. The minimal sequence, *thc1*, is 3560 bp in length and contains a 3498-bp open reading frame encoding a 1166-amino acid protein (figure 2). *Thc1* includes the long form of carboxy terminus seen in the C-terminal partial sequence *cSlo2* but not in *cSlo1* (Jiang *et al*. 1997). *Thc1* is 94.3% identical at the amino acid level to the mouse brain *Slo* form *mbr8* (see figure 2) which also has the long form of the carboxy terminus (Butler *et al*. 1993). The full-length sequence, obtained from PCR products amplified by using degenerate primers, was confirmed by amplification of hair cell and brain cDNA using turtle-specific primers which flanked segment overlaps. Sequences of the resulting PCR products were identical to the corresponding regions in the full-length sequence constructed from segments 1–6. This second series of amplifications revealed no additional splice sites.

The full open reading frame was constructed from RT-PCR products amplified from six overlapping segments (figure 1). Segments 1, 2 and 3 contained only a single variant, whereas segments 4 and 5, which include the presumptive splice sites SS1–SS4 (Tseng-Crank *et al*. 1994), gave variants of different sizes. Segment 6 also includes the splice sites SS4, SS5 and SS6. A total of ten clones of segment 6, each from a different hair-cell preparation, yielded only one variant. No segment 6 clone, when digested with a common cutter, gave fragments indicative of more than a single PCR product (see §2). The inability to detect diverse amplification products in this segment suggests that alternative splicing at SS4, SS5 or SS6 makes little or no contribution in turtle hair cells.

(b) *Alternatively spliced variants*

PCR amplifications of segments 4 and 5, which span the region containing alternate splice sites SS1–SS4 in human *Slo* (Tseng-Crank *et al*. 1994), resulted in numerous products of different size. A total of four sets of splice variants, each from more than four clones from independent PCR reactions, were obtained and sequenced. Sequences and restriction digestion patterns of all variant

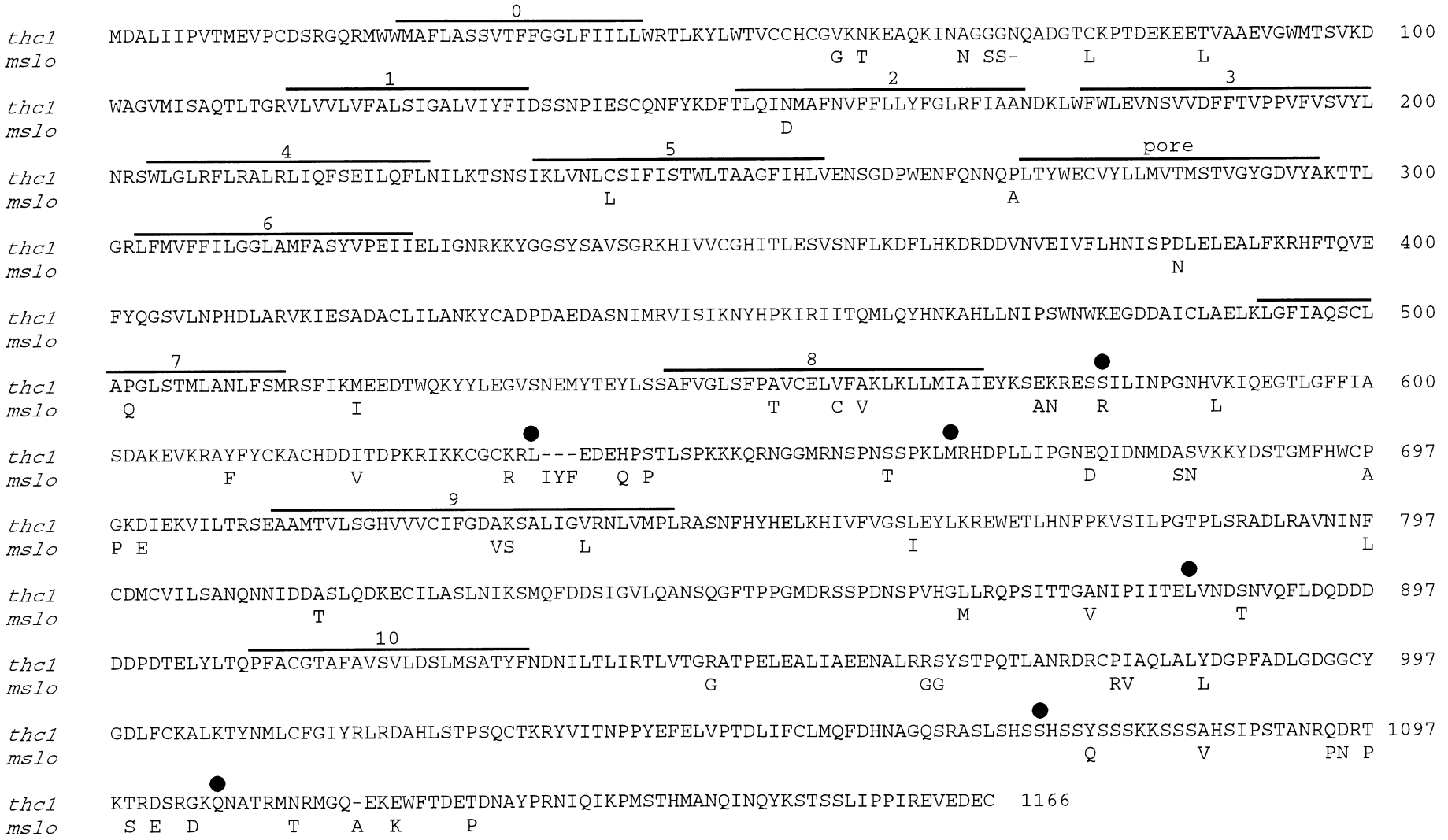
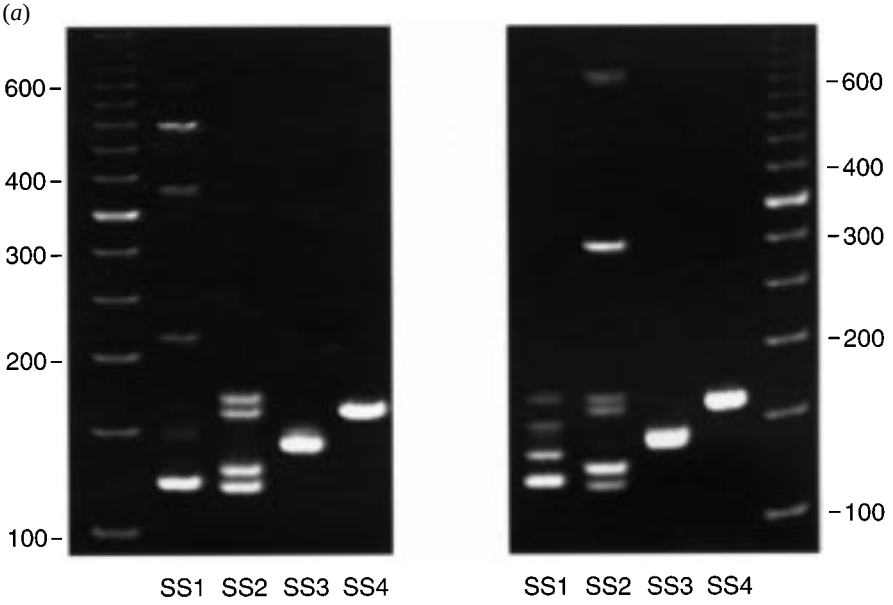


Figure 2. Amino acid sequence of the minimal hair cell isoform of the *tslo* α-subunit. Predicted amphipathic-helical domains (S0–S10, Meera *et al*. 1997) and the pore region are denoted by lines above the sequence. Filled circles indicate sites of alternative splicing (SS1–SS6) identified in other vertebrate *Slo* sequences. Amino acid differences in the mouse *Slo* (L16912; Butler *et al*. 1993) are shown below the turtle sequence. The *mslo* includes at SS2 a 3-amino-acid insert, IYF, which is also found in turtle.

(*a*)



(*b*)

SS1 EXONS

SRKR

SRKRHRAQKKEAMPMSYKRCAAGIHTDPTRC

SS2 EXONS

IYF

IYSKMSIYKRMKLACCFDCGRSERDCSCMSGSVHSNMDTLERAFPLSSVSVNDCSTSLRAF

(*c*)

	SS1	SS2	SS3	SS4
thc1	0	0	0	0
thc2	4	0	0	0
thc3	4	3	0	0
thc4	4	61	0	0
thc5	31	3	0	0
thc6	31	0	0	0

Figure 3. (*a*) Polyacrylamide gels of PCR products obtained from hair cells collected from the entire papilla by using turtle-specific primer pairs flanking splice sites SS1, SS2, SS3 and SS4. Shown here are two different preparations. Outer lanes are 50-bp-size markers. For SS1 the zero insert was 118 bp, the bands corresponding to approximate product sizes of 0, 4, 9, 13, 31, 90 and more than 120 amino acids. For SS2 the zero-insert size was 114 bp, the bands corresponding approximately to product sizes of 0, 3, 14, 17, 61 and more than 160 amino acids. For SS3 and SS4, the zero-insert sizes were 137 and 159 bp respectively. (*b*) Amino acid sequences of those alternatively spliced exons isolated from SS1 and SS2. No clones were obtained with inserts in SS3, SS4 and SS5. (*c*) Combinations of alternate exons found in the six variants of *tSlo*. GenBank accession numbers: *thc1*, AF036625; *thc3*, AF036626; *thc4*, AF036627; *thc5*, AF036628.

clones indicate that the SS3 and SS4 splice sites do not contain inserts. By using the labelling system of Tseng-Crank *et al.* (1994), variants are named according to the number of amino acids inserted at the SS1, SS2, SS3 and SS4 splice sites (see figure 3). The 0, 0, 0, 0 variant, *thc1*, constituted roughly half of the total number of clones obtained from hair cells and brain. A 4, 0, 0, 0 (*thc2*) variant was a minor hair cell component and was not found in the brain. In addition, a 4, 3, 0, 0 variant (*thc3*) and a 4, 61, 0, 0 variant (*thc4*) were isolated from both hair cells and brain. A 4, 58, 0, 0 variant, mentioned below, was identified in the brain but not in hair cells. The different distribution of alternative transcripts in the ear and brain suggests that splicing of the *slo* gene is tissue specific.

Differential splicing was also studied by amplification of hair cell cDNA with turtle-specific primers flanking each of the four sites, SS1, SS2, SS3 and SS4. These experiments confirmed that variation in the *Slo* transcript is achieved by alternative splicing at SS1 and SS2, and never revealed any inserts at SS3 or SS4 (figure 3*a*). In seven preparations, PCR products with approximate insert sizes of 4, 9, 13 and 31 amino acids were commonly detected at SS1 and 3, 14, 17 and 61 amino acids at SS2 (see figures 3 and 4).

Bands of larger size at SS1 and SS2 were consistently observed, but we were unable to subclone and characterize these products and therefore cannot comment on their identity. To investigate possible combinations of SS1 and SS2 inserts, hair cell cDNA was amplified with a pair of turtle-specific primers, one just downstream of SS2 and the other bridging the splice junction for SS1 = 4 (SRKR). Use of these primers produced two of the variants already characterized (SS1 = 4, SS2 = 3; SS1 = 4, SS2 = 61) and also two new isoforms with the combination SS1 = 31, SS2 = 3 and SS1 = 31, SS2 = 0 (see figure 3). The new SS1 exon starts with SRKR and can be decomposed as 4 + 27 amino acids, suggesting that SS1 inserts are constructed by concatenation of exons. A common source of confusion stems from the fact that the splice site lies within the codon. Insertion of an exon can therefore also change the identity of the residues flanking the splice site. Thus at SS1, the minimal sequence is '..S**S**IL..' (figure 2) and splicing occurs after the first base of the '**S**' codon, AGT. Insertion of the 12-bp exon encoding the **SRKR** insert alters the second 'S' to 'R' but leaves the 'I' unchanged, resulting in the sequence '..SR**SRKR**IL..'.

The novel SS2 = 61-amino acid insert may also be constructed by exon concatenation. Although possessing a different start triad (IYS as opposed to IYF), the 61-residue insert is most likely built by addition of the 3- and 58-amino acid segments. Inspection of the nucleotide sequence indicates that splicing at SS2 occurs within the CTT codon corresponding to L634 in the *thc1* sequence. Insertion of the 9 bp sequence, TG ATC TAT T, replaces L634 with LIYF. The equivalent region of the 183 bp sequence, TG ATC TAT TCC ....T replaces L634 with LIYS...F, where the dots denote the intermediate 57 residues. We could not find the 58-insert alone in the hair cell clones but it was present in turtle brain, where L634 is replaced by P ... F, the dots again denoting the intermediate 57 residues. Our turtle brain 58-insert is 88% identical at the amino acid level to a *Slo* variant identified in rat adrenal chromaffin cells and PC12 cells (Saito *et al.* 1997), and is 100% identical to a recently reported insert in the chick cochlea (Rosenblatt *et al.* 1997).

(c) *Distribution of spliced variants along the cochlea*

Hair cells in the turtle basilar papilla are mapped tonotopically with the characteristic frequency (and the K_{Ca} channel kinetics) increasing over tenfold from one end of the organ to the other (Crawford & Fettiplace 1980). We have previously shown that it is possible to subdivide the papilla into quarters and isolate hair cells tuned to different frequencies from different regions (Art & Fettiplace 1987, fig. 2). In five preparations, amplification with the SS1–SS2 primer pair showed that the inserts SS1 = 4, SS2 = 3 and SS1 = 31, SS2 = 3 were present in the low-frequency portion of the papilla but absent from the high-frequency quarter (figure 4*b*). In addition, two hair-cell preparations amplified with primers flanking SS1 (figure 4*c*) indicated that multiple isoforms with inserts in SS1, including the SS1 = 31 variant, were confined to the low-frequency end of the papilla. Only SS1 = 0 and SS1 = 13 were uniformly distributed. The intensities of other products relative to SS1 = 0 declined progressively from the low- to the high-frequency end. One interpretation of these results is that the smallest sequence, *thc1*, occurs throughout the length of the papilla, but that in hair cells tuned to low frequencies, a variety of other subunits are present with inserts in splice site 1. In contrast, the results with inserts in splice site 2 were less definitive. The isoform, SS1 = 4, SS2 = 61, was always in the high-frequency half (figure 4*b*), but its presence in the low-frequency half was somewhat variable, and in some preparations it was uniformly distributed. That the band in figure 4*b* did correspond to SS2 = 61, rather than to larger inserts in SS1 was verified by digestion with BsrGI, which gave products of the number and size expected for the SS2 insert.

4. DISCUSSION

By using a PCR-based strategy, we have cloned multiple variants of the K_{Ca} channel α-subunit that are expressed in turtle auditory hair cells. The turtle α-subunit is an orthologue of the mouse, human, canine and chicken cDNAs previously described. There are six known alternative splice sites in the open reading frame of vertebrate K_{Ca} transcripts, the sixth determining which carboxy terminus is used. We were able to isolate a single long form of the carboxy terminus and found inserts at only two other sites: SS1 and SS2. If additional sites are used in turtle cochlear hair cells, the resulting transcripts are likely to be of little importance. Assuming that the main variants contain no additional inserts other than those at SS1 and SS2, the six variants that we have characterized specify the naturally occurring combinations of exons. These combinations contain amino acid inserts at SS1 and SS2 of 0, 0; 4, 0; 4, 3; 31, 3; 31, 0 and 4, 61. We have evidence that isoforms containing at least four other inserts are also present (SS1 = 9 and 13; SS2 = 14 and 17). The novel 31- and 61-amino acid inserts are both notably hydrophilic, but we have no data yet on the effects of these inserts on channel properties. K_{Ca} channel mRNA has been shown to be present in neurons during development in the absence of a measurable K_{Ca} current in the cells (Subramony *et al.* 1996). Thus the occurrence of the message does not necessarily reflect the extent of channel incorporation into the membrane. It will be important to verify, by using other techniques such as antibody labelling, that the protein isoforms encoded by the different transcripts are expressed in the hair cell plasma membrane.

Navaratnam *et al.* (1997) and Rosenblatt *et al.* (1997) have both recently reported the presence of numerous isoforms of the K_{Ca} channel in chick cochlear hair cells and shown that variants are differentially distributed along the cochlea. Their results indicate that the isoforms arise by alternative splicing at multiple sites, but the naturally occurring combinations of exons at these sites in the chick, unlike the turtle, have not yet been identified. However, it is worth noting that the chick hair cell variants include a 4-amino acid insert (SRKR) at the equivalent of our splice site 1 and a 3-amino-acid insert (IYF) at our splice site 2. It is conceivable therefore that the combinations occurring in the turtle cochlea constitute a subset of those found in the chick cochlea.

The different isoforms may, in theory, influence either the K_{Ca} channel properties (its Ca^{2+} sensitivity and kinetics), or the channel's interaction with other proteins

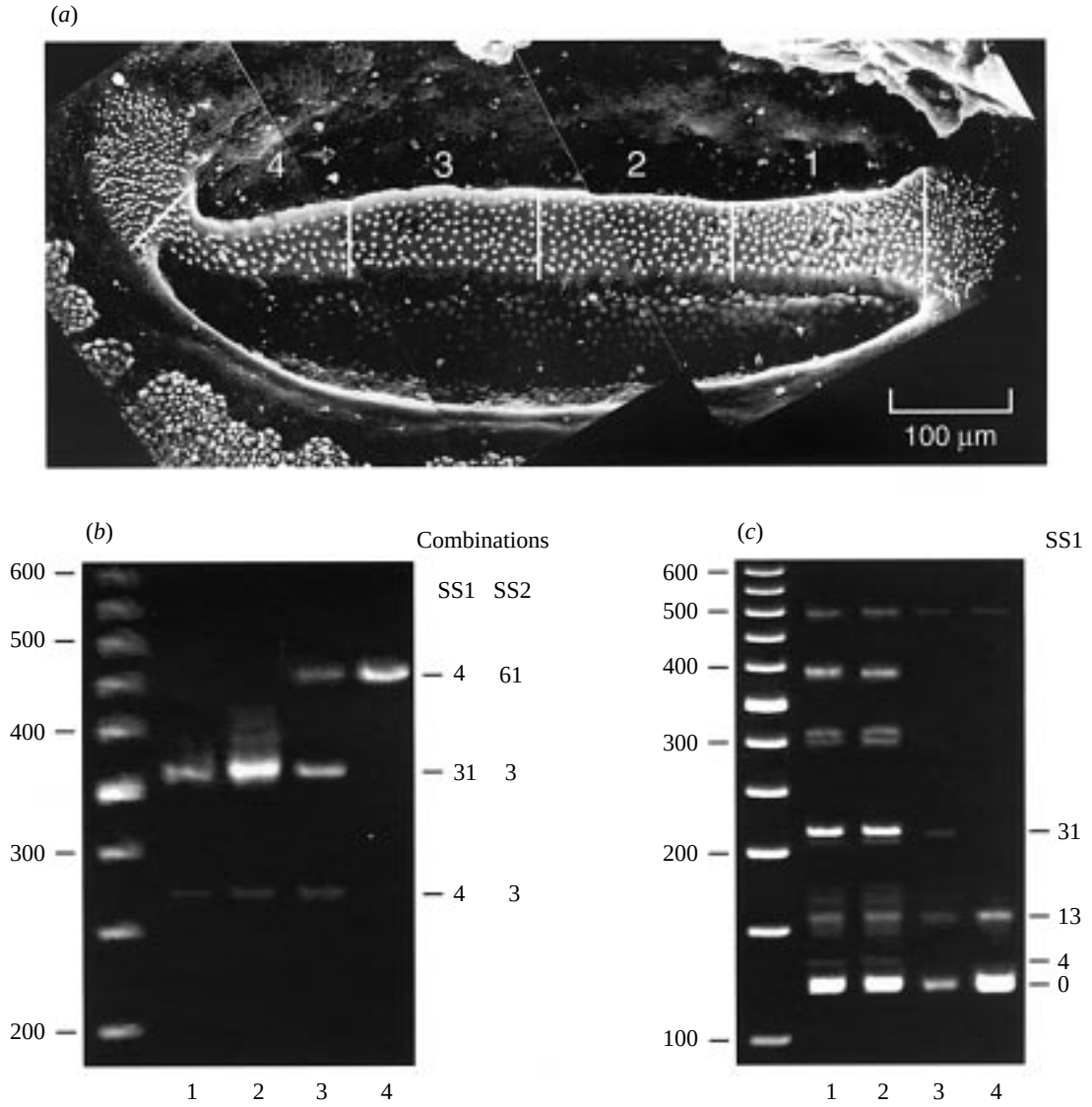


Figure 4. (*a*) Scanning electron micrograph of the turtle basilar papilla oriented with the saccular high-frequency end to the left. Hair cells were collected from four papillar regions (1–4) on the basilar membrane, but not from the extremities where the cells lie on the limbal shelf. The approximate frequency ranges for hair cells in the four quarters are: 1, 40–70 Hz; 2, 70–150 Hz; 3, 150–300 Hz; 4, 300–600 Hz. (*b*) A 4% polyacrylamide gel of PCR products from hair cells isolated from each cochlear region by using a pair of turtle-specific primers, one just downstream of SS2 and the other bridging the splice junction for SS1 = 4 (SRKR). These primers would not amplify fragments with SS1 = 0. Numbers to the left of the gel give base pair sizes obtained from marker lanes, numbers to the right of gel give the deduced SS1, SS2 amino-acid combinations of the visible bands. Note that the '31, 3′ and '4, 3′ combinations are absent from the high-frequency quarter whereas the '4, 61' is absent from the low-frequency region. (*c*) Polyacrylamide gel of PCR products from hair cells isolated from each cochlear region using turtle-specific primers flanking SS1. Note that SS1 = 0 and SS1 = 13 are uniformly distributed but variants containing other inserts are confined to the low-frequency end.

and its targeting of the plasma membrane. These could affect both the density and kinetics of the K_{Ca} channels, the combined variation in which can account for the range of tuning frequencies in turtle hair cells. A previous model employed opposing cochlear gradients in two α-subunit species to regulate the assembly of heteromultimeric channels of variable kinetics and density (Wu & Fettiplace 1996). However, a gradient in one variant coupled with a uniform distribution of the other may be adequate provided the channel density is dictated by the subunit type. Our present results indicate that the mRNA coding for the 4, 3, and '31,3 isoforms were less prominent or absent at the basal high-frequency end of the cochlea, but without a more quantitative approach (see, for example, Gilliland *et al.* 1990) it is not possible to infer a concentration gradient in transcript or channel protein. The existence of at least ten isoforms, six of which have been sequenced, argues that multiple species of K_{Ca} channel exist in turtle hair cells. This complexity was unexpected, but it would support the notion that alternative splicing of the *Slo* gene contributes to variation in channel properties. Heterologous expression of the alternatively spliced K_{Ca}-channel isoforms should allow us to elucidate the role of subunit variation in the tonotopic organization of the turtle cochlea.

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