ELSEVIER

Hearing Research 125 (1998) 120–130

HEARING RESEARCH

Expression of novel potassium channels in the chick basilar papilla

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Received 10 March 1998; received in revised form 11 July 1998; accepted 13 July 1998

Abstract

Ionic currents are critical for the functioning of the inner ear auditory sensory epithelium. We set out to identify and molecularly clone the genes encoding the channels responsible for several currents in the chick basilar papilla. Here we describe an inward-rectifying K^+ channel, cKir2.3, present in both hair cells and support cells in the apical end of the chick basilar papilla. The biophysical properties of the human ortholog, hKir2.3, are similar to those of an inward-rectifying channel found in the apical end of the chick basilar papilla, suggesting that this channel may contribute to the corresponding current. Additionally, we describe two new members of the Kv6 subfamily of putative regulatory voltage-gated K^+ channels, cKv6.2 and cKv6.3. Both are expressed in hair cells in the apical end of the chick basilar papilla; cKv6.2 is also strongly expressed in support cells and in the brain.

Key words: Potassium channel; Inner ear; Cochlea; Hair cell

1. Introduction

The avian basilar papilla is the auditory organ responsible for the mechanoelectrical transduction of sound waves into frequency information. The basilar papilla is composed of a sickle-shaped sensory epithelium containing a precise array of approximately 11 000 signal-transducing hair cells (Tilney et al., 1986; Janas et al., 1995) each of which is surrounded by support cells. Hair cells have graded morphological features. Two extremes in morphology are the 'tall' and 'short' hair cells, with variable features including cell shape, stereocilia number and height, and innervation patterns (Takasaka and Smith, 1971; Hirokawa, 1978; Tanaka and Smith, 1978; Manley, 1990; Fischer, 1992; Tilney et al., 1992; Tilney and Tilney, 1992).

The basilar papilla is organized in a tonotopic fashion along its length with a high-to-low frequency gradient of sensitivity from base to apex. Ionic channels determine the electrophysiological properties of each hair cell. The interplay of several ionic channels contributes to an electrical tuning mechanism in avian, amphibian, and reptilian cochleae (Fettiplace, 1987; Fuchs et al., 1988; Hudspeth, 1989; Wu et al., 1995; Wu and Fettiplace, 1996), which sharpens frequency selectivity for hair cells.

While the morphological and electrophysiological features of the basilar papilla are well-described, very little is known of the biochemical bases underlying these properties. The physical and functional gradients detected within the basilar papilla should parallel graded changes in the expression of ion channels and structural proteins. As emerging evidence of this notion, recent work has shown differential expression of many splice variants of cSlo (Ca^{2+}-activated K^+ channels) along the length of the chick basilar papilla (Navaratnam et al., 1997; Rosenblatt et al., 1997). Two additional K^+ channel currents that have been directly measured in the chick basilar papilla are due to an inward-rectifying

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0378-5955/98/$19.00
PII: S0378-5955(98)00141-5

K^+ channel and a voltage-gated K^+ channel (Fuchs et al., 1988; Fuchs and Evans, 1990; Murrow, 1994; Pantelias et al., 1998). Both currents are located predominantly in the apical end of the basilar papilla. Two channels which could account for these currents have been molecularly cloned from the chick basilar papilla, and PCR analysis indicates they are present in the same region of this organ (Navaratnam et al., 1995; Navaratnam et al., 1997). Their cellular localization has not been determined.

As a further step towards understanding the dynamics of how the remarkably complex basilar papilla works, we set out to identify and clone genes encoding components of both the inward-rectifying and the voltage-gated K^+ channel currents, using a single-sided PCR technique developed in our laboratory (Peale et al., 1998). Here, we describe an inward-rectifying K^+ channel, cKir2.3, and show that it is expressed in both hair cells and support cells in the apical end of the basilar papilla. This channel could therefore account for all or part of the corresponding current measured by Fuchs and Evans (1990).

We also describe two new putative voltage-gated K^+ channels, cKv6.2 and cKv6.3, which are most closely related to rat Kv6.1 (Drewe et al., 1992). A functional role for Kv6.1 in vivo has not been determined, although when coexpressed with Kv2.1 and Kv2.2 it can coassemble and the resulting complexes exhibit modified kinetics (Salinas et al., 1997). cKv6.2 is expressed at similar levels in both hair cells and support cells in the chick basilar papilla. cKv6.3 is expressed at much higher levels in hair cells. Expression of Kv6.1 has not been described in the inner ear of any species.

2. Materials and methods

2.1. Sensory hair cell damage, tissue collection and RNA isolation

White Leghorn chicks were purchased from H & N International (Redmond, WA, USA) as eggs or P1 hatchlings. Eggs were kept in a humidified chamber at 38°C until hatching. For tissue collection, chicks were deeply anesthetized and then euthanized by rapid decapitation. All animals were handled according to the guidelines set by the Animal Care Committee at the University of Washington.

RNA purification for the PCR analysis was as follows. Cochlear ducts from 33 normal chicks at postnatal day 8 (P8) were triturated in a solution of 3 M LiCl and 6 M urea (Auffray and Rougeon, 1980). DNA was sheared by drawing the solution repeatedly through a fine-gauge needle. RNA was pelleted after an overnight incubation at −20°C, and resuspended in NETS buffer (100 mM NaCl, 20 mM EDTA, 20 mM Tris, pH 7.5, 1% sodium dodecylsulfate, SDS). RNA was then extracted with phenol and chloroform, and precipitated with isopropanol in the presence of 0.3 M sodium acetate. Purified RNA was treated with DNase (Promega, Madison, WI, USA) to remove any contaminating genomic DNA. mRNA was isolated using the PolyATract kit from Promega. cDNA was synthesized using Superscript II (Gibco-BRL, Grand Island, NY, USA) and oligo-dT primers; reverse transcriptase was omitted in parallel negative control reactions.

For the construction of a cDNA library, RNA was collected from cochlear ducts from 24 chicks whose inner ear sensory epithelia had been damaged with aminoglycosides. Damage was performed on P7 chicks as described (Stone et al., 1996) with a single high dose of gentamicin (400 mg/kg). This procedure results in complete loss of hair cells in the basal one-third of the basilar papilla. In the remaining two-thirds of the basilar papilla the hair cells appear normal. These animals were euthanized 3 days after the aminoglycoside treatment and cochlear ducts were immediately dissected for RNA purification and cDNA synthesis as described above.

2.2. Multiplex display PCR

A single-sided PCR technique, multiplex display PCR (Peale et al., 1998), was used to identify fragments of K^+ channel genes within coding regions, and in known reading frames. Briefly, single-stranded chick cochlear cDNA was cleaved with *Hha*I (New England Biolabs, Beverly, MA, USA) and ligated to an oligonucleotide containing an upstream PCR primer site. Degenerate downstream PCR primers were specific for either voltage-gated or inward-rectifying K^+ channels. Upstream and dowstream primer sequences are as described in Peale et al. (1998). PCR products were resolved on a 6% acrylamide sequencing style gel. Bands were extracted from the gel and subjected to a second round of non-radioactive PCR, gel-purified, and cloned into pGEM 3Z f(+) (Promega). As a positive control for the PCR reaction, we used a downstream primer for cytoplasmic β-actin (Peale et al., 1998). This yielded a PCR product of 264 bp (including the primer sequence), corresponding to nucleotides 21–261 of chick β-actin. We used this actin fragment in several of the experiments described below, including the probing of a cDNA library, as a control for levels of RNA in a Northern blot, and for in situ hybridization experiments.

2.3. Synthesis and probing of chick cochlear cDNA library

A cDNA library was constructed with mRNA from whole chick cochlear ducts three days after gentamicin

treatment. cDNA was synthesized using oligo-dT primers as described above. We used the lambda ZAP-cDNA Gigapack II Gold Cloning kit from Stratagene (La Jolla, CA, USA). The once-amplified library contains 800 000 independent recombinants, in which β-actin is present at a frequency of 1/1250.

The library was probed with digoxigenin-labeled cRNA probes made from the voltage-gated K^+ channel PCR products cKv6.2 and cKv6.3 listed in Fig. 1A. Sequencing of the entire coding region of cKv6.2 was performed in both directions using a series of Exonuclease III (New England Biolabs) deletions in conjunction with overlapping sets of sequencing primers. Sequencing was performed either manually with Sequenase 2.0 (United States Biochemical, Cleveland, OH, USA) or with the ABI Prism d-Rhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and analyzed in the University of Washington Department of Biochemistry DNA Sequencing Facility.

2.4. Sequence analysis

PCR-derived sequences were analyzed for homology to genes in the combined NCBI databases using BLAST (Altschul et al., 1990). Sequences were aligned using MultAlin (Corpet, 1988) and the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI, USA. Possible sites for *O*-glycosylation were identified using NetOGlyc 2.0 (Hansen et al., 1998).

2.5. Northern analysis

Fifteen micrograms of total RNA isolated from cochlea, liver, muscle, brain, and heart of P8 chicks were separated on a 1% agarose gel with formaldehyde and transferred to a nitrocellulose membrane, as described (Sambrook et al., 1989). Flanking lanes containing 0.2–9.5-kb RNA markers (Gibco-BRL) were cut from the gel prior to transfer and visualized by staining with ethidium bromide. Digoxigenin-labeled cRNA was synthesized from the PCR products listed in Fig. 1. The membrane was hybridized overnight at 70°C with a 20 ng/ml probe. Post-hybridization stringency washes at 70°C were as follows: twice each of 0.5×SSC, 0.1% SDS, and 0.1×SSC, 0.1% SDS (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7). Hybridized probe was detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and chemiluminescent substrate (CSPD, Boehringer Mannheim), as recommended by the manufacturer. Following exposure to the K^+-channel specific probes, the membrane was stripped by boiling in 0.1% SDS and re-hybridized with a chick β-actin probe (described above).

2.6. In situ hybridization

Whole cochlear ducts were removed from P7–P10 chicks and stripped of both the tegmentum vasculosum and tectorial membrane. Whole-mount in situ hybridization was performed with digoxigenin-labeled cRNA probes as described (Riddle et al., 1993), with the following exceptions. RIPA buffer (150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 8) was used for permeabilization. Hybridization was performed at 68°C, and at neutral pH. In all cases, final probe concentration was 1 μg/ml.

In order to determine cellular localization, some samples were equilibrated in 20% sucrose, embedded in OCT compound, cryosectioned (14 μm), mounted on superfrost plus slides (Fisher, Pittsburgh, PA, USA) with Vectashield (Vector Labs, Burlingame, CA, USA) or Advantage (Innovex, Richmond, CA, USA).

3. Results

3.1. PCR on chick cochlear tissue

The inward-rectifying and voltage-gated K^+ channel gene families are large, with many subfamily designations. Even so, we expected that as yet undescribed genes may exist in the highly specialized cochlear tissue. For this reason, we used multiplex display PCR, a single-sided PCR technique capable of detecting novel genes (Peale et al., 1998). Single-stranded chick cochlear cDNA was restriction-digested and an arbitrary oligonucleotide was ligated to one end of each fragment to serve as a sense PCR primer site. The antisense PCR primer was a degenerate gene-family-specific primer (one for voltage-gated and one for inward-rectifying K^+ channels).

Using this PCR method on cochlear tissue, we have identified fragments of two apparently novel voltage-gated K^+ channels. These have been designated cKv6.2 and cKv6.3. Fig. 1A shows an alignment of these channels with several related K^+ channel sequences. As seen in Fig. 1B, they share the highest degree of sequence identity among themselves (87%). They also share a high degree of sequence identity with rKv6.1 (78%), compared to 53% sequence identity with rKv2.1 (*Shab*). The sequence identity is only 39% for rKv3.1 (*Shaw*). Approximately 30% (20/67) of residues are conserved between all sequences, while ∼30% (19/67) are Kv6 subfamily-specific. In order to assess whether cKv6.2 and cKv6.3 are the chicken orthologs of rat Kv6.1, we determined the percent identity between known orthologs of Kv1.1–Kv1.6, Kv2.1, Kv2.2, Kv3.1, and Kv4.1 in a highly conserved region

A.

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          -- S4 --                    ------------ S5 ------------                    --- H5 --
          341                                                                          407
rKv6.1    RLARHSLGLQ TLGLTARRCT REFGLLLLFL CVAIALFAPL LYVIENEMAD SPE....... .FTSIPACYW WAVIT
cKv6.2    RLARHSLGLQ TLGLTVRRCT REFGLLLLFL CVAMALFSPL VYLAESELGA KQE....... .FTSIPTSYW WAVIS
cKv6.3    RLARHSLGLQ TLGLTVRRCT REFGLLLLFL CVAVTLFSPL VYLAENESGK VLE....... .FTSIPASYW WAIIS
rKv2.1    KLARHSTGLQ SLGFTLRRSY NELGLLILFL AMGIMIFSSL VFFAEKDEDD T.K....... .FKSIPASFW WATIT
rKv3.1    KLTRHFVGLR VLGHTLRAST NEFLLLIIFL ALGVLIFATM IYYAERIGAQ PNDPSASEHT HFKNIPIGFW WAVVT
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B.

	rKv6.1	cKv6.2	cKv6.3	rKv2.1	rKv3.1
rKv6.1	100				
cKv6.2	78	100			
cKv6.3	78	87	100		
rKv2.1	53	53	55	100	
rKv3.1	42	39	39	48	100

C.

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          311                                                  351
hKir2.3   LYGMGKEELE SEDFEIVVIL EGMVEATAMT TQARSSYLAS E
cKir2.3   LYGIGKEELE TENFEIVVIL EGMVEATAMT TQARSSYLAS E
cKir2.1   LYDLSKQDMD NADFEIVVIL EGMVEATAMT TQCRSSYLAN E
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Fig. 1. Amino acid sequence comparison of potassium channels. PCR-derived fragments of putative K^+ channels were aligned with K^+ channels from an amino acid homology search of GenBank. Numbers above the sequence correspond to amino acid residues of the uppermost sequence. A: Voltage-gated K^+ channels. B: Percent amino acid identity between sequences shown in A. C: Inward-rectifying K^+ channels. Non-identity is indicated in boldface type. GenBank accession numbers are as follows: cKv6.2, U62139; cKv6.3, U62140; cKir2.3, U62138.

of the S5 transmembrane domain. For each channel, the percent sequence identity between orthologs in species as diverse as humans and *Drosophila* ranges from 92% to 100%. In contrast, rat Kv6.1 is only 76% identical to cKv6.2 or cKv6.3 in the same region. This suggests that neither cKv6.2 nor cKv6.3 is the chick ortholog of rKv6.1.

Voltage-gated K^+ channels share a characteristic membrane topology consisting of 6 membrane-spanning regions, designated S1–S6, separated by alternating extracellular and intracellular loops, and a hydrophobic membrane loop, 'H', located between S5 and S6 (Tempel et al., 1988). Both the N and C termini are on the cytoplasmic side of the membrane. Several reviews describe this topology and the functional significance of specific regions, including the regions shown in Fig. 1 (Salkoff et al., 1992; Jan and Jan, 1997). The S4 transmembrane segment is the putative voltage sensor. The S5, H, and S6 segments together form the pore region. The PCR fragments shown in Fig. 1 begin in the S4 region (indicated above the sequence) and extend through the beginning of the 'H', or pore region. The intracellular loop between the S4 and S5 transmembrane domains exhibits a high degree of conservation within subfamilies (85%, 100%, 100%, 100%, 92%, and 85% for Kv1, 2, 3, 4, 6, and 9, respectively). However, only 15% of the residues in this region are conserved across all subfamilies. Functional roles for this region were delineated in the Shaker H4 channel by Yellen and coworkers (Holmgren et al., 1996). This cytoplasmic loop interacts with the amino terminus during N-type inactivation and also may act as part of the 'mouth' of the pore.

PCR also identified an inward-rectifying K^+ channel, cKir2.3, in the chick cochlea. This has not been previously described in inner ear tissue. Fig. 1C shows an alignment of cKir2.3 with its human ortholog, hKir2.3 (Périer et al., 1994), and another subfamily member, cKir2.1. Kir2.1 has been cloned from the chick cochlea (Navaratnam et al., 1995). Human and chick Kir2.3 share 93% identity at the amino acid level. In contrast, cKir2.3 shares only 78% identity with cKv2.1.

3.2. Cloning and sequencing of cKv6.2

Our K^+ channel clones were isolated in the course of a study seeking genes involved in hair cell regeneration following gentamicin-induced hair cell death. We were interested in determining whether hair cell or support cell channel expression might change with drug damage. We hypothesized that some of these channels might be involved in the proliferation and regeneration events after drug damage. However, in situ hybridization for cKv6.2 and cKv6.3 in 3-day post-gentamicin-treated tissue showed no gross change in expression compared to non-treated tissue (data not shown). Thus, we were able to use a cDNA library constructed from chick cochlear tissue 3 days post-gentamicin treatment for isolation of full-length clones. 5/160 000 cKv6.2 transcripts were present in this library. Two of 5 clones isolated with the cKv6.2 probe were full-length (4400 bp). The remaining 3 clones were 3400–2900 bp in

length. We were unable to isolate a clone of cKv6.3 after screening 320 000 plaques. This is not unexpected since a Northern blot (see Fig. 4, below) shows this K^+ channel is much less abundant in the cochlear duct than the cKv6.2 channel.

Fig. 2 shows the full-length sequence of cKv6.2, including 27 bp of 5′ untranslated and 118 bp of 3′ untranslated sequence. An in-frame stop codon is located 24 bp upstream of the assigned initiator methionine. This is followed by an open reading frame of 1554 bp, encoding a protein of 518 amino acids with a theoretical MW of 58.8 kDa. The Kozak sequence for

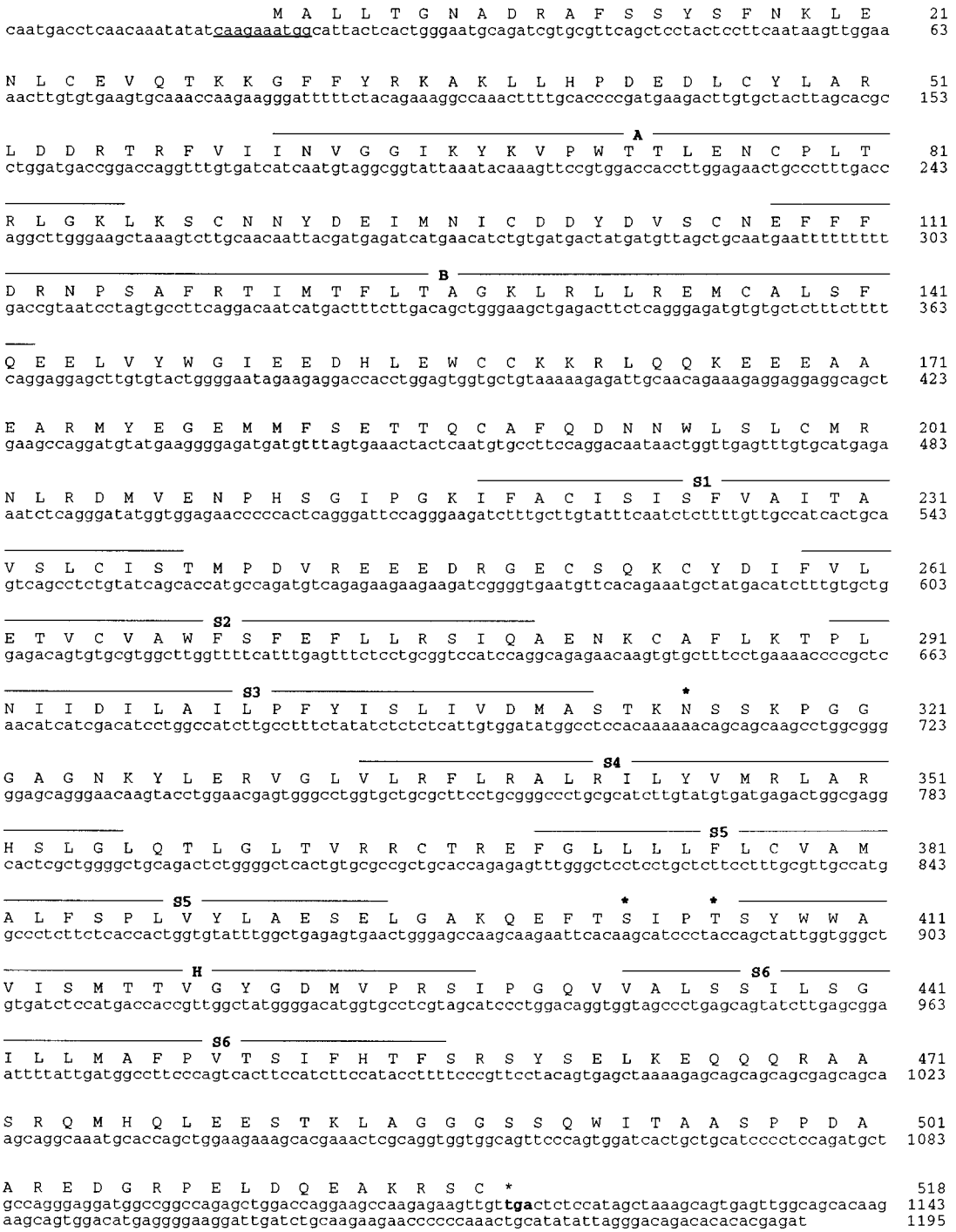

Fig. 2. Sequence of cKv6.2 and inferred amino acid sequence. Full-length sequence of cKv6.2; all sequence shown was verified by sequencing in both directions. The PCR fragment shown in Fig. 1 is identical to the corresponding region of the full-length clone. The Kozak sequence for initiation of translation is underlined. 'A' and 'B' indicate the highly conserved 'subunit assembly regions', as described in the text. S1–S6 are the highly conserved transmembrane domains characteristic of voltage-gated potassium channels, and 'H' is the pore region. Asterisks above amino acids indicate potential glycosylation sites.

	A	B
cKv6.2	INVGGIKYKVPWTTLENCPLTRLGK	EFFFDRNPSAFRTIMTFLTAGKLRLLREMCALSFQ
Kv1	IN*SGLRFETQ**TL**FP*TLLG*	EYFFDRNRPSFD*ILY*YQSGGR**RPVNV*D*F*
Kv2	*NVGGLAHEVLWRTLDRLPRTRLGK	EYFFDRHPGAFTSILNFYRTG*LHMMEEMCALSFS
Kv3	*NVGG*RH*TYRSTL*TLPGTRLA*	EFFFDRHPGVFA**LNYYRTGKLHCPADVCGPL*E
Kv4	*NVSG*RF*TW**TL*RYPDTLLGS	*YFFDRDPD*FRH*LNFYRTG*LH*PR*ECI*A*D
Kv5	VNVGGVRQVLYGDLLSQYPETRLAE	EFYFDRDPDAFKCVIEVYYFGEVHMKKGICPICFK
Kv6	INVGGIKYSLPWTTLDEFPLTRLGQ	EFFFDRNPGAFGTILTFLRAGKLRLLREMCALSFQ
Kv7	LNVGGVRHETHVSTLRTVPNSRLSR	EYFFDRHPSVFNSIIDFYRTRELHVPLEVCGAVVK
Kv8	VNVGGSRFVLSQQALSCFPHTRLGK	EYFFDRSSQAFRYVLHYYRTGRLHVMEQLCALSFL
Kv9	*NVGG**R*L****L*RFP*TRLGR	EFYFDR*P**F***LHFY*TG*LHV**ELCVF*F*

Fig. 3. Amino acid sequence comparison of A and B subunit assembly domains. Subfamily designations are listed on the left. The amino acids shown at each position are conserved between all subfamily members except *Drosophila*, which show generally greater divergence. An asterisk indicates non-identity at the indicated position. Identity with cKv6.2 is indicated with boldface type. The Kv5, 6, 7, and 8 subfamilies consist of single sequences. The position of the first amino acid shown on each line is as follows: 61, cKv6.1; 42, mKv1.1; 31, rKv2.1; 94, rKv3.3; 45, rKv4.1; 39, rKv5.1; 67, rKv6.1; 68, aKv7.1; 47, hamKv8.1; 23, rKv9.1.

initiation of translation is underlined. A Kyte and Doolittle plot indicates the presence of 7 hydrophobic stretches of amino acids, corresponding to the S1–S6 transmembrane domains and the pore region characteristic of voltage-gated K^+ channels (data not shown). There seems to be no N-terminal signal peptide. Possible *O*-glycosylation sites located at S403 and T406 occur in the extracellular loop between S5 and the pore region. There is one possible site for *N*-linked glycosylation at N315, in the extracellular loop between S3 and S4.

In order to confirm the Kv6 subfamily designation of our cKv6.2 clone, we aligned representative members of each of the nine α subunits of the voltage-gated K^+ channel subfamilies with MultAlin (Corpet, 1988). Since these sequences are highly divergent at the extreme N and C termini, we started the alignment 9 residues upstream of the highly conserved 'A subunit assembly region' (amino acid 22 in rKv2.1), and continued through 15 residues downstream of the S6 transmembrane domain (amino acid 427 in rKv2.1). We used this alignment to calculate the percent of identical amino acid residues between different pairs of K^+ channels. Percent identity within each subfamily ranges from 94% to 60%, compared to less than 50% between subfamilies. Since rKv6.1 and cKv6.2 share 70% identity overall, they probably belong to the same subfamily.

The N-terminal region of voltage-gated K^+ channels contains two highly conserved segments designated 'A' and 'B'. These have been shown to be critical for the subfamily-specificity of channel assembly and as such are a determinant of subfamily type (Li et al., 1992). Fig. 3 shows an amino acid sequence comparison of the A and B subunit assembly domains. Note the high percentage of identical amino acids between subfamily members in Kv1–4 and Kv9 (until now, only one family member has been identified for each of Kv5–8). rKv6.1 bears the greatest identity with cKv6.2 in these regions (83%). The next most similar subfamily is Kv2, at 55% identity.

3.3. Tissue distribution of all three PCR products

In Fig. 4, a Northern blot using RNA from various tissues of P8 chicks shows that cKir2.3 and cKv6.3 each exist as a single transcript of sizes 3.4 and 3 kb, respectively. The blot for cKv6.2 shows two bands of ~4.4 and ~8 kb. The 4.4-kb band is consistent with the size of the full-length transcript which was sequenced; in our probing of the library we did not observe any transcripts larger than ~4.4 kb; the longer 8-kb form may therefore represent unspliced mRNA.

Expression of these K^+ channels is not limited to the cochlea. cKir2.3 is highly expressed in the brain. The signal for cKir2.3 in the cochlea is visible in the original gel, but did not reproduce well. Kir2.3 has been cloned from human, rat and mouse (Bond et al., 1994; Lesage et al., 1994; Morishige et al., 1994; Périer et al., 1994). While it is highly expressed in brain in all these species, it is expressed in heart only in human and rat. cKv6.2 is strongly expressed in the cochlea, with intermediate levels of expression in the brain and weak expression in the heart. (This band is visible on the gel but did not reproduce well.) cKv6.3 is strongly expressed in the cochlea, and weakly expressed in the brain. In contrast, rat Kv6.1 is very abundant in the adult liver, lung, and brain, and in the neonatal heart (Drewe et al., 1992).

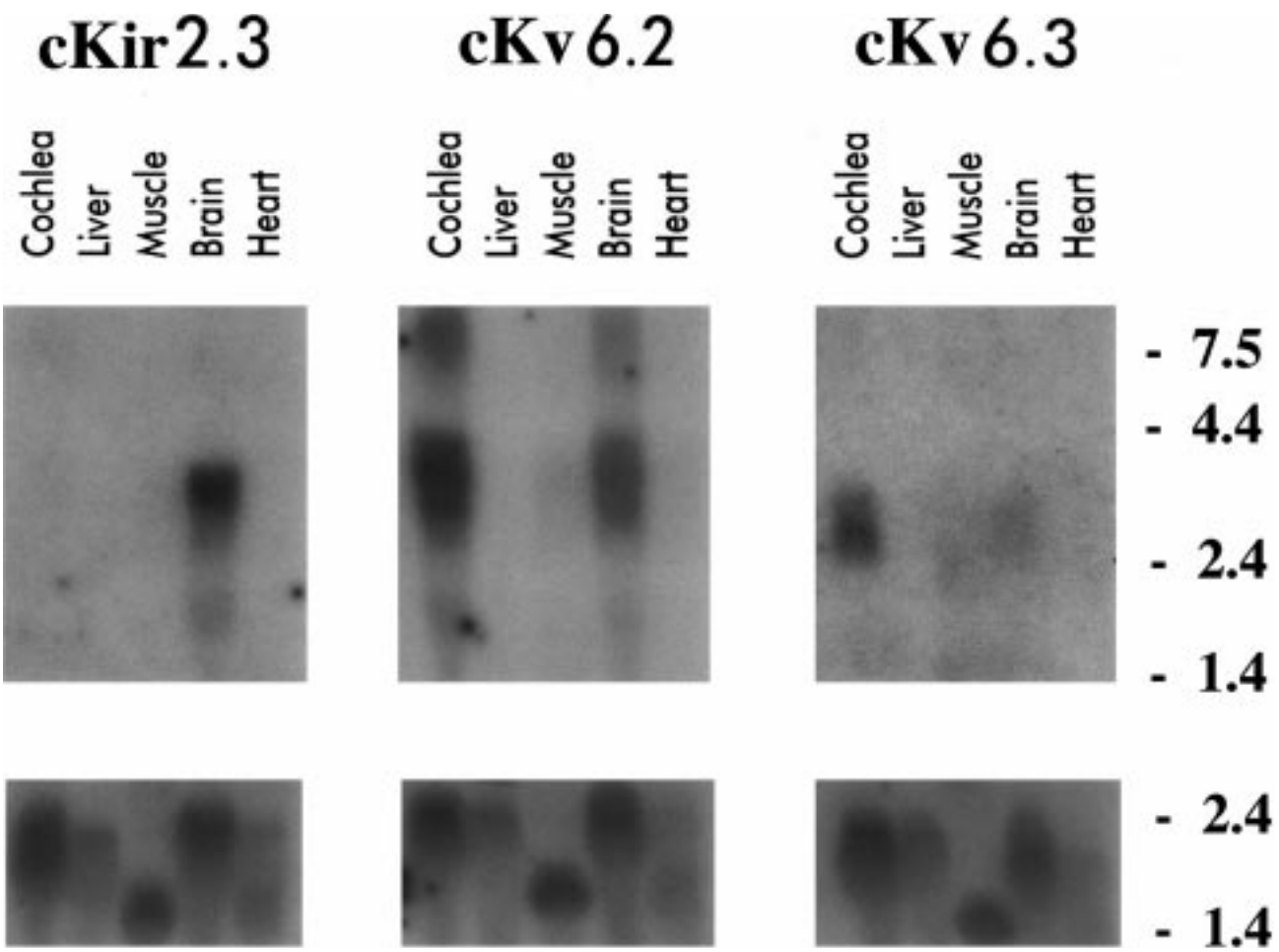

Fig. 4. Tissue distribution of K^+ channels. Tissue type is indicated above the lanes. Upper panel: The digoxigenin-labeled K^+ channel probes are indicated above each blot. Lower panel: The membrane from the upper panel was stripped and reprobed with digoxigenin-labeled actin probe. Sizes are indicated to the right. Chick cytoplasmic β-actin is 2.4 kb, chick muscle actin is 1.8 kb.

3.4. Transcripts for all three channels are present in the apical end of the basilar papilla

In order to further localize the expression of these K^+ channels within the basilar papilla, we performed whole-mount in situ hybridization. Fig. 5(A, B, D, E, G, H) shows that transcripts of all 3 channels are present in the apical end of the normal basilar papilla. This general pattern of expression was always observed in the many in situ experiments that were performed ($n > 6$ experiments, on average, using 6 cochlear ducts in each experiment). Tegmentum vasculosum has been removed from the basilar papillae prior to in situ hybridization. In initial experiments, it did not show specific signal above background for any of the probes. To assess whether this apex-specific expression might represent an artifact related to variable probe penetration, we used a cRNA probe made from chick cytoplasmic β-actin (residues 21–261). In situ hybridization with this probe showed uniform expression over the entire length of the basilar papilla; sectioning revealed actin transcripts in both hair cells and support cells of the sensory epithelium (data not shown). This confirms previous findings of β-actin expression in chick basilar papilla (Pickles, 1993; Lee and Cotanche, 1995).

The 3 panels to the right (C, F, I) in Fig. 5 show cryosections of whole-mount in situ hybridization experiments. The hair cell stereocilia are oriented towards the top, and support cell nuclei are located below the row of hair cell nuclei. Similar levels of expression of cKir2.3 and cKv6.2 were seen in hair cells and support cells. In contrast, cKv6.3 was clearly expressed at much higher levels in hair cells, while very little (if any) expression was detected in support cells. There was no apparent gradient in expression between the superior and inferior edges of the cochlea.

4. Discussion

Ionic currents are critical for the functioning of the inner ear auditory sensory epithelium (Fuchs, 1992). Several different types of K^+ channel currents have been mapped to distinct positions in hair cells along

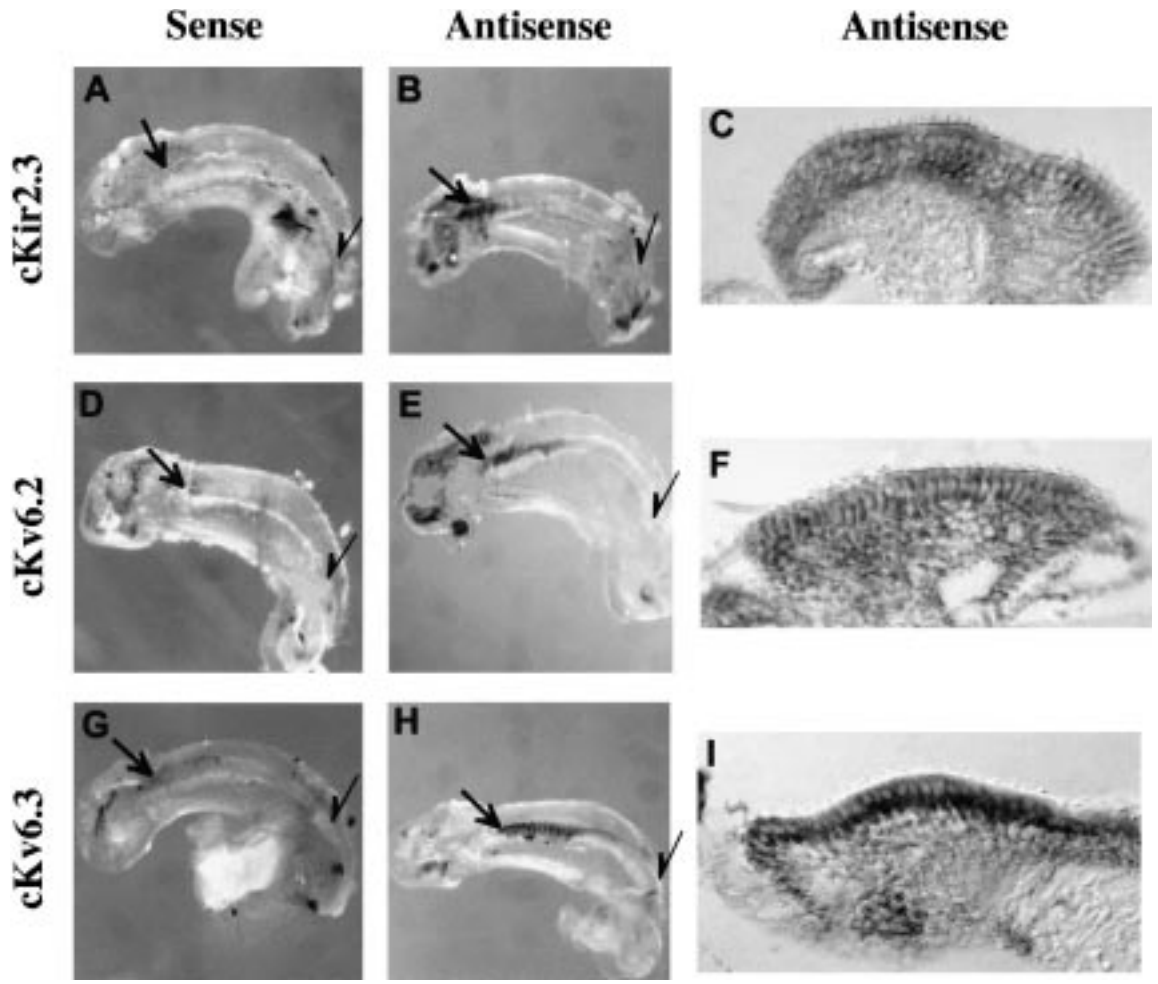

Fig. 5. Transcripts for all three channels are present in the apical end of the basilar papilla. In situ hybridization using digoxigenin-labeled K^+ channel cRNA probes on whole chick cochlear ducts; A, B, C: cKir2.3; D, E, F: cKv6.2; G, H, I: cKv6.3. A, D, G: sense probes; B, C, E, F, H, I: antisense probes; C, F, I: 14-μm cryosections of whole-mount in situ on chick cochlear ducts, using a 100× objective, showing just the sensory epithelium (antisense shown). Note that the large arrowheads indicate the apical end of the sensory epithelium, the half-arrowheads indicate the basal end. Surrounding tissue is lagena on the left, superior and inferior cartilaginous plates on the top and bottom, respectively. The overlying tegmentum vasculosum and tectorial membrane have been removed to allow probe penetration. Note that the signal over the lagena seen in B and E may be due to non-specific trapping of probe; we often saw non-specific signal here in sense controls with these probes.

the chick basilar papilla (Fuchs et al., 1988; Fuchs and Evans, 1990; Murrow and Fuchs, 1990; Murrow, 1994; Pantelias et al., 1998). These currents include a large outward current due to Ca^{2+}-activated K^+ channels in the basal 2/3 of the cochlea, a small outward current due to the rapidly inactivating A current in short hair cells in the basal end, a small inward current due to an inward-rectifying K^+ channel in the apical end, and a small outward current from a delayed-rectifying K^+ channel in the apical end. No doubt, each of these different K^+ currents serves a unique purpose to the hair cells of the basilar papilla. In this work, we find K^+ channels in support cells as well. To our knowledge, there has been no previous description of K^+ channels in support cells of the chick basilar papilla, nor any discussion of possible functions of K^+ channels in this cell type.

Here, we report the presence of an inward-rectifying K^+ channel, cKir2.3, in hair cells and support cells in the apical end of the basilar papilla. Another member of the same subfamily, cKir2.1 (cIRK1), has also been detected in the apical end of the basilar papilla, although its presence in hair cells has not been demonstrated (Navaratnam et al., 1995). Our failure to obtain PCR products representing cKir2.1 is not surprising since our PCR protocol would have yielded a 680-bp fragment, too large to resolve on a 6% acrylamide sequencing-style gel. hKir2.3 (the human ortholog of cKir2.3) and cKir2.1 are steeply inward-rectifying channels that exhibit similar magnitudes of conductance (13 and 17 pS, repectively) (Périer et al., 1994; Navaratnam et al., 1995) and are blocked by Cs^{2+} and Ba^{2+}. The apical end of the chick basilar papilla expresses a steeply inward-rectifying K^+ channel which exhibits sensitiv-

ity to both Cs^{2+} and Ba^{2+} (Fuchs and Evans, 1990). These functional data as well as the apical expression patterns suggest that either hetero- or homomultimers of Kir2.1 and Kir2.3 may account for the corresponding inward-rectifying current in the basilar papilla.

Several roles for inward-rectifying K^+ channels have been suggested. Currents through inward-rectifying K^+ channels can play a significant role in setting the resting membrane potential of a cell, buffering external K^+ and modulating action potential waveforms (Fuchs and Evans, 1990; Hille, 1992). A possible role for inward-rectifying K^+ channels in electrical tuning of hair cells was suggested by the work of Goodman and Art (1996a). These authors suggest that the inward-rectifier can enhance the sharpness of tuning due to a negative-slope region in the current-voltage relation in the vicinity of the resting membrane potential.

The magnitude of the conductance of the inward-rectifying K^+ channel in the turtle basilar papilla varies systematically with the cell's resonant frequency (Goodman and Art, 1996a). It is possible that this variation in conductance may also occur in the chick basilar papilla. Possible explanations for this phenomenon include the existence and differential expression of splice variants of these channels or differing levels of channel expression in different cells. Alternatively, there could be gradients of expression of various channels, with any given cell expressing a distinct complement of channels slightly different from its neighbors.

Twenty functional α subunits of voltage-gated K^+ channels within the families Kv1–4, 7, EAG, and KvLQT have been identified. These form functional homotetrameric channels. Heteromultimerization between these subfamilies is not observed (Covarrubias et al., 1991). In contrast, there is a growing number of genes with sequences that are very similar to Kv1–4, but which are unable to form functional homotetramers either in *Xenopus* oocytes or in cultured mammalian cells (Drewe et al., 1992; Hugnot et al., 1996; Jegla and Salkoff, 1997; Salinas et al., 1997; Carranza et al., 1998). These have been designated to new subfamilies based on their sequences, and the total number of these putative channels (including our two) is now nine. Functional roles for these α subunits have not been determined. However, Lazdunski and coworkers have suggested a possible regulatory effect on other functional K^+ channels based on their colocalization with various members of the Kv1–4 subfamilies and their ability to inhibit functional α subunits when coexpressed in *Xenopus* oocytes (Hugnot et al., 1996; Salinas et al., 1997). In this work, PCR on chick cochlear tissue with primers specific for voltage-gated K^+ channels yielded two fragments of new members of the Kv6 subfamily, related to rat Kv6.1 (Drewe et al., 1992). By itself, rat Kv6.1 does not assemble into a functional K^+ channel. However, it can alter the kinetic properties of rKv2.1 and rKv2.2 when coexpressed in *Xenopus* oocytes (Salinas et al., 1997).

To date, cKv3.1 is the only delayed-rectifying type of voltage-gated K^+ channel described in the apical end of the chick cochlea (Navaratnam et al., 1997). Cellular localization has not been determined for this channel. If cKv6.2 and cKv6.3 behave as rKv6.1, they may regulate cKv3.1 or other as yet undescribed channels in the cochlea. Alternatively, within this and other 'non-functional' subfamilies, heteromultimerization and/or association with a β subunit may be required for proper channel assembly and function. There are several possible roles for voltage-gated K^+ channels in the basilar papilla. In the turtle, Goodman and Art suggest that the interplay of voltage-gated K^+ channels with Ca^{2+}-activated K^+ channels helps to shape electrical tuning (Goodman and Art, 1996b). A similar mechanism may also exist in the chick basilar papilla. Additionally, Fuchs notes the similarity between this channel and one which repolarizes squid axons during an action potential (Fuchs, 1992).

Our present results do not accurately define gradients of expression of functional K^+ channels. The signal derived from in situ hybridization is not necessarily linear with respect to mRNA expression levels, nor is protein expression necessarily a linear function of mRNA levels. Nevertheless, the preferential expression of mRNA for Kir2.3, Kv6.2 and Kv6.3 in the apical end of the cochlea suggests that these channels might contribute to the tonotopicity of the cochlea. We detected no gradients in expression of any of these 3 channels across the width of the cochlea.

The PCR method used in this work does not necessarily detect all genes of a given family. There is no reason to expect that cKir2.1 and cKir2.3 are the only inward-rectifying channels in the basilar papilla, nor that cKv6.2, cKv6.3 and cKv3.1 are the only voltage-gated K^+ channels. In support of this notion, recent work shows that multiple inward-rectifying K^+ channels are found in cDNA libraries constructed from rat cochlear hair cells (Shao et al., 1998). Additionally, another putative K^+ channel related to Kv9.1 and Kv9.2 is expressed in a subset of rat cochlear hair cells (Carranza et al., 1998). In order to begin to understand the complex functions of K^+ currents in the basilar papilla, all of the component channels must first be identified and their cell surface expression mapped with respect to cell type and the tonotopic map.

Some of the data have been presented and/or published in preliminary form (Mason et al., 1998; Peale et al., 1998).

Acknowledgments

We thank Lorraine Gibbs for sectioning tissue, An-

ish Sudra for help with in situ hybridization experiments, Dr. Jialin Shang and Laura Sugden for assistance with dissections, and Laura and Art Sugden for assistance with the figures. We thank Anastasia Pantelias and Pablo Monsivais for comments on the manuscript. We are especially indebted to Drs. Tom Reh, Olivia Bermingham-McDonough, William Zagotta, Galen Eaholtz, and Bruce Tempel for helpful contributions to the work in progress and for critical reading of the manuscript. This work was funded by NIDCD Grants DC02863 and DC00181 and from Oberkotter and NOHR funds.

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