An M-Like Potassium Current in the Guinea Pig Cochlea

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Key Words

KCNQ · M current · Outer hair cells · Patch-clamp technique · Voltage-gated potassium channels

Abstract

Potassium M currents play a role in stabilizing the resting membrane potential. These currents have previously been identified in several cell types, including sensory receptors. Given that maintaining membrane excitability is important for mechano-electrical transduction in the inner ear, the presence of M currents was investigated in outer hair cells isolated from the guinea pig hearing organ. Using a pulse protocol designed to emphasize M currents with the whole-cell patch-clamp technique, voltage- and time-dependent, non-inactivating, low-threshold currents (the hallmarks of M currents) were recorded. These currents were significantly reduced by cadmium chloride. Results from RT-PCR analysis indicated that genes encoding M channel subunits KCNQ2 and KCNQ3 are expressed in the guinea pig cochlea. Our data suggest that guinea pig outer hair cells express an M-like potassium current that, following sound stimulation, may play an important role in returning the membrane potential to resting level and thus regulating outer hair cell synaptic mechanisms.

Introduction

The hearing organ acts as a mechano-electrical transducer, converting external acoustic stimuli to auditory nerve electrical activity. In the inner ear, the mechano-sensory hair cells are the elements ultimately responsible for auditory function. The inner hair cells are the primary sensory cells, releasing excitatory neurotransmitter(s) onto auditory afferents in response to sound stimulation. The outer hair cells (OHCs), on the other hand, provide modulation of cochlear sensitivity and frequency selectivity.

The endocochlear potential, which originates from maintenance of a high $[K^+]$ in the endolymph relative to the adjacent perilymphatic compartments, provides the driving force for potassium ions through the mechano-electrical transducer (MET) channels [1]. In addition to the MET channels, several K^+ channels are expressed by cochlear hair cells and provide the molecular correlates for hair cell ionic currents. The main potassium current in guinea pig OHCs is the $I_{K,n}$ [2]. It is half activated at -90 mV and fully deactivated at very negative potentials, thereby repolarizing the cell and maintaining the resting membrane potential. In addition, the cell may also be hyperpolarized by a calcium-activated potassium current, $I_{K(Ca)}$.

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Another ionic current that stabilizes membrane potential and thus excitability was first described in sympathetic neurons [3–5]; this ‘M current’ (I_M) is a voltage- and time-dependent, sub-threshold, non-inactivating potassium current. It shows little inward or outward rectification, and the half-activation potential is in the range of –30 to –50 mV. Muscarinic agonists (and other transmitters acting at G protein-calcium signaling pathways) suppress the I_M , which leads to a small cell depolarization. An I_M has been identified in neurons of both the central and peripheral nervous systems [4, 6, 7], including the visual and olfactory systems [8, 9]. However, I_M have not previously been identified in sensory cells from the hearing organ.

The molecular basis for the I_M is provided by KCNQ subunits belonging to the KQT-like subfamily of potassium channels [http://www.gene.ucl.ac.uk/nomenclature/genefamily/KCN.shtml]. KCNQ2 and KCNQ3 channel subunits were recently found to assemble as hetero-tetramers to make up a K^+ conductance nearly identical to M channels in terms of biophysical properties and pharmacology, and their expression pattern overlaps with the I_M distribution [10].

Several genes that are members of the KCNQ family are expressed in the rat, mouse and human cochlea. *KCNQ1* is expressed in marginal cells of stria vascularis, where it participates in forming small conductance (I_{SK}) potassium channels involved in potassium recycling in the endolymphatic compartment. Cochlear expression of *Kcnq1–3* has been shown by RT-PCR in the mouse [11], but the cellular location is not known. *Kcnq4* has been demonstrated by RT-PCR in the mouse and rat cochlea [11, 12] and has also been found to be expressed in both inner and outer hair cells [13]. KCNQ4 probably underlies the OHC main potassium current, $I_{K,n}$ [14]. However, KCNQ3/4 may also form functional ion channels with M-type properties [11]. It is possible that differential co-expression, heteromerization, and alternative splicing of the KCNQ subunits mRNAs underlie variants of the I_M depending on their localization. However, expression of M channel subunits has not yet been described in cochlear tissues from the guinea pig.

Here we present the first observation of an M-like potassium current in auditory OHCs and show that M channel subunit genes *KCNQ2* and *3* are expressed in the guinea pig cochlea.

Materials and Methods

All animal procedures were performed in accordance with national regulations for care and use of animals (ethical approval no. N7c/98).

Isolation of OHCs

OHCs were dissected from the sensory epithelium of pigmented adult guinea pigs. The epithelium was incubated in collagenase (0.125–0.25 mg/ml, type I, Sigma Chemical Co.) in Minimum Essential Medium (MEM, Life Technologies/GibcoBRL) for 5–10 min at room temperature (22–24°C) followed by rinsing three times in MEM. The cells were gently dissociated from the sensory epithelium using a constricted glass pipette. The cell suspension was added to 1–2 ml of medium in the experimental chamber, and the cells were allowed 5 min to settle to the bottom of the chamber. Most cells obtained were from the middle and apical turns of the cochlea, with lengths ranging from 60 to 80 μ m. Cells were perfused with medium at a rate of 0.3 ml/min.

Electrophysiological Recording

Voltage-gated potassium currents [confirmed using 4-aminopyridine, apamin and tetraethylammonium (TEA); not shown] were recorded from isolated OHCs using the whole-cell configuration of the patch-clamp technique. Voltage-clamp command pulses were applied through the pipette (borosilicate glass; 8–15 Mohm), and an AgCl-pellet/3.0 M KCl-agar bridge was used as reference electrode. The pipette potentials were corrected for a liquid junction potential of approximately 5 mV using the Henderson equation of the pClamp software [15]. A patch-clamp amplifier (EPC 7, List-Medical) was used to record membrane currents passing through the pipette. Data were acquired using pClamp 7 software (Axon Instruments) with a 12-bit A/D converter (Digidata 1200A/B, Axon Instruments). Capacitative currents were canceled manually. No series resistance compensation was used. Currents were low-pass filtered at 5 kHz with a 3-pole Bessel filter and sampled at 10 kHz. Outward currents are plotted in the positive (up) direction, inward currents in the negative (down) direction. The internal solution consisted of (in mM): 100 KCl, 2 MgCl₂, 5 HEPES, 1 ATP disodium salt, 0.1 GTP sodium salt, 10 BAPTA [1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetra-acetic acid, tetra-potassium salt]; pH 7.3 (adjusted with 1 M KOH); 296 mOsm. The external solution (MEM) consisted of (in mM): 1.26 CaCl₂, 5.37 KCl, 0.44 KH₂PO₄, 0.49 MgCl₂, 0.40 MgSO₄, 128 NaCl, 4.2 NaHCO₃, 0.31 Na₂HPO₄; buffered with HEPES to pH 7.4; 305 mOsm. The equilibrium potential for potassium (E_K) was –81 mV. Cadmium chloride (CdCl₂, 1.0 mM; Sigma) and linopirdine (100 μ M; Research Biochemicals International) were added to the external solution in order to suppress K^+ currents of interest. All experiments were conducted at room temperature.

Total RNA Extraction

Pigmented guinea pigs were decapitated and the temporal bones were rapidly transferred to cold RNase-free physiological saline where the bony capsule of the cochlea and the stria vascularis were removed. The remaining cochlear tissue was excised, pooled (6 cochleas from 3 guinea pigs) and homogenized. Total RNA was isolated from fresh tissue homogenates using TRIzol. Total RNA pellets were washed with 75% ethanol, dried and suspended in Tris-EDTA buffer, pH 7.6. RNA concentration and

Table 1. Oligonucleotide primers for RT-PCR amplification are given with primer name, location (forward/reverse), nucleotide sequence, calculated size of PCR product, and literature reference

Primer	Nucleotide sequence	Size, bp	Reference
<i>KCNQ2</i> fwd	5'-CAAGTACCTCAGACCTGGAAC	551	29
<i>KCNQ2</i> rev	5'-CAGCTCTTGGGCACCTTGCT		
<i>KCNQ3</i> fwd	5'-CCAAGGAATGAACCATATGTAGCC	461	11
<i>KCNQ3</i> rev	5'-CAGAAGAGTCAAGATGGGCAGGAC		
β -actin fwd	5'-ACTGGGACGACATGGAGAAG	852	this paper
β -actin rev	5'-GTGGATCAGCAAGCAGGAGT		
<i>GAPDH</i> fwd	5'-TCCCTAAGATTGTCAGCAA	309	30
<i>GAPDH</i> rev	5'-AGATCCACAACGGATACATT		

purity was quantified by spectrophotometry (absorbance ratio A_{260}/A_{280}), and the isolated mRNA was stored at -80°C until further analysis.

RT-PCR

Primers based on published sequences were purchased from Invitrogen and used to identify gene expression for the different KCNQ subunits. Primers for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin were used as controls. Primers were designed and/or confirmed based on sequence data in GenBank and using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primer sequences and the predicted sizes of the amplified PCR products are listed in table 1.

The one-step RT-PCR system combining superscript reverse transcriptase with platinum *Taq* polymerase (Invitrogen) was used. A 50- μl reaction contained 25 μl of $2 \times$ reaction mix provided with the kit (containing 2.4 mM MgSO_4 and 0.4 mM of each dNTP), and template RNA (100 ng/reaction), 1 μl of each primer in a pair (final concentration 0.2 μM), and 5 units of RT/*Taq* mix. Reverse transcription was performed in a thermocycler for 30 min at 47°C or 50°C , followed by 35 cycles of PCR each consisting of: 15 s denaturation at 94°C , 30 s annealing at 50°C and 60 s primer extension at 72°C ; with a final extension step of 10 min at 72°C . Positive control reactions for amplification of housekeeping genes GAPDH and β -actin were performed for each cDNA sample. A negative control reaction in which the target cDNA was omitted (no template) was run with every reaction. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

DNA Sequencing

In order to verify PCR products not only by size but also by their DNA sequence, fluorescent bands corresponding to PCR products were excised from agarose gels and purified using the QIAquick MinElute gel extraction kit (QIAGEN). Sequence reactions were performed in the thermocycler using the DYEnamic ET terminator cycle sequencing kit (cat. US81050, Amersham Pharmacia Biotech), and analyzed on an ABI model 377.

Results

Potassium Currents

When recording ion currents initiated from a holding potential of around -70 mV, most of the potassium currents in the OHC will be included, thus masking the relatively smaller I_M . However, the I_M does not inactivate above -70 mV. Therefore a standard protocol was applied where the currents were pre-activated by depolarizing the cell to a holding potential of -20 or -30 mV (to allow full activation of I_M and minimizing other potassium components) and then stepped to more hyperpolarized potentials [5, 16]. This made it possible to bring out a current with characteristic M-like kinetics, i.e. the slow closing of M channels (fig. 1b, inset). Figure 1a shows potassium currents in response to 10-mV voltage steps between -90 and 0 mV from a holding potential of -20 mV. From the holding potential -20 mV, a depolarizing step to -10 mV induced a slowly increasing outward current, while hyperpolarizing steps to between -30 mV and -70 mV caused a slow and sustained increase of the inward I_M relaxation currents (i.e., a negative slope of the current trace), whereas further negative steps activated an additional outward relaxation current. The reversal potential for the M-like current (i.e., the transition from an inward relaxation current to an outward relaxation current) is shown in figure 1b as the intersection between the instantaneous [$I(V)_H$] and steady-state (I_{ss}) current-voltage (I/V) curves. In 5 cells it was -75 mV $\pm$ 10 mV (mean $\pm$ SD). This is close to the calculated potassium equilibrium potential ($E_K = -81$ mV). Tail currents consisting of a fast and a slow component were observed after the pulse returned from the conditioning voltage steps to the holding potential (cf. fig. 1a). The fast component is considered to be related to the delayed rectifier (I_K) potassium current. The amplitude of the slow component of the tail current (indicated by dotted line in fig. 1a) when

Fig. 1. Whole-cell patch-clamp recordings from an OHC isolated from the guinea pig cochlea. **a** Whole-cell currents from an OHC at a holding potential (V_H) of -20 mV during 10-mV voltage steps ranging from -90 to 0 mV. Pulse duration was 300 ms. Arrows indicate the current responses at conditioning membrane potentials (V) from -70 mV to -10 mV, corresponding to the range previously reported for I_M [5]. Tail currents for I_M at voltage steps from -70 to -10 mV are seen as the slow component current relaxations. Steps more negative to -80 mV gave rise to slowly relaxing outward tail currents of increasing amplitudes. The horizontal dashed line represents the zero I_M level corresponding to -75 mV (cf. **b**). The horizontal dotted line represents the zero current level corresponding to the resting membrane potential. **b** Current-voltage (I/V) rela-

tions obtained from current and voltage of the instantaneous current [the amplitude of the fast transient; $I(V_H)$], and the steady-state current [total current measured after the slow relaxation is complete; $I_{ss}(V)$] in figure 1a. The instantaneous (circles) and steady-state (triangles) currents are plotted against the conditioning potential. The intersection of the two curves gives the reversal potential, approximately -75 mV (inset, after [3]). **c** Activation curve obtained from tail currents (measured at the time indicated by the vertical dotted line in figure 1a) corresponding to conditioning potentials from -70 mV to -10 mV. Current values were normalized against peak current and fitted with the Boltzmann relation, $I_{norm} = 1/1 + \exp[-(V - V_{1/2})/k]$ where V is voltage, $V_{1/2}$ is half-activation voltage and k is a constant.

stepped back to a holding potential of -20 mV from conditioning pulses between -90 and -10 mV was used to derive the activation curve for I_M [3, 5, 18]. As seen in figure 1c, the activation curve was sigmoid in shape and

occurred predominantly at conditioning voltages between -50 and -20 mV. Half-maximal activation occurred at -35.5 mV for this cell.

Fig. 2. Effects of CdCl_2 and linopirdine on whole-cell current recordings in an OHC. **a** Membrane currents were superimposed from the cell before (Control), in the presence of 1 mM CdCl_2 , or 1 mM CdCl_2 and 100 μM linopirdine. The cell was held at -20 mV and stepped in 10 mV increments to values between -130 mV and $+50$ mV for 300 ms. (Note: only -130 mV and $+50$ mV traces are shown). CdCl_2 mainly reduced the outward current while the addition of linopirdine significantly reduced the inward current. **b** Activation curve obtained from tail currents for control current and subtracted CdCl_2 -sensitive and linopirdine-sensitive currents. Current values were normalized against peak current and fitted with the Boltzmann relation. The half-maximal activation voltage was -58.4 mV for the control, -80.0 mV for the linopirdine curve and -43.7 mV for the CdCl_2 curve.

Effects of CdCl_2 and Linopirdine on Potassium Currents

Cadmium chloride (CdCl_2) and linopirdine were used to modulate the potassium currents in the OHCs (fig. 2). Cadmium chloride (1 mM) caused a significant decrease ($\sim 50\%$) of the outward peak and steady-state currents (fig. 2a), but only a slight decrease of the steady-state inward current was seen. When 100 μM linopirdine was added (in the presence of CdCl_2) there was a minor further reduction of the outward currents, whereas inward currents were substantially decreased (fig. 2a).

Characteristics of CdCl_2 -Sensitive Currents

To highlight individual current components, the current remaining in the presence of CdCl_2 was subtracted

from the control current (cf. fig. 2a). The resulting CdCl_2 -sensitive current exhibited a clear M-like time- and voltage-dependency (similar to the one in fig. 1a) in the range of -70 to -10 mV. In addition, there were substantial CdCl_2 -sensitive outward currents, presumably $I_{\text{K}(\text{Ca})}$ (BK). To further illustrate the different current components, the activation curves for control, CdCl_2 -sensitive and linopirdine-sensitive currents are plotted in figure 2b. Activation of the CdCl_2 -sensitive current was seen between -80 and -10 mV. Half-maximal activation occurred at -43.7 mV. The activation curve for the linopirdine-sensitive currents in figure 2b exhibited a sigmoidal increase above -130 mV, maximizing at about -30 mV (half-maximal activation at -80.0 mV).

Fig. 3. Expression of *KCNQ2* and *KCNQ3* K⁺ channel mRNAs in adult guinea pig cochlea. Ethidium bromide-stained PCR products analyzed on a 2% agarose gel (100 V, 90 min) after 35 cycles of PCR. Template was omitted for the negative control; *β-actin* and *GAPDH* primers were used for positive control reactions; DNA size marker with 100-bp interval (600-bp band has double intensity; Invitrogen). The cDNAs were obtained by reverse transcription of total RNA extracted from guinea pig cochleas. The results shown are representative of at least 5 experiments.

Identification of *Kcnq2/3* Transcripts in Guinea Pig Cochlea

Our molecular data from guinea pig cochlea lend support to I_M recordings in guinea pig outer hair cells. Expression of the molecular genetic substrates for potassium I_M , *Kcnq2* and *Kcnq3*, was detected in total RNA isolated from guinea pig cochlea using reverse transcription-polymerase chain reaction (RT-PCR). Parallel negative control reactions lacking target cDNA were always performed and yielded negative results. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide (fig. 3). Identity of PCR-amplified DNA fragments was indicated by their sizes corresponding to the calculated amplicon lengths (table 1; fig. 3), and further confirmed by direct sequencing of excised gel bands (data not shown). PCR product sizes were as follows: *β-actin*: 852 bp; *GAPDH*: 309 bp; *KCNQ3*: 461 bp, while the wild-type *KCNQ2* product of estimated size 551 bp was not identified. However, two bands of smaller size (520 and 486 bp) were amplified by the *KCNQ2* primer set and identified by sequencing as splice variants of *KCNQ2*. Fluorescence intensities may provide an estimate of expression levels, as indicated by the bright *β-actin* band and moderately fluorescent product for *GAPDH*, which correspond, respectively, to known high and intermediate levels of expression. Similarly, *KCNQ3* may be expressed at a higher level than *KCNQ2* in the guinea pig cochlea.

Discussion

The I_M is a relatively small potassium current that may be modulated by a number of neuroactive substances, such as muscarine (hence the name M channel [3]). It plays an important role in stabilizing the resting membrane potential in a variety of cell types. This mechanism would obviously be of value also in cochlear OHCs, but I_M has not previously been described in guinea pig OHCs. Patch-clamp recordings from OHCs are usually performed at holding potentials close to the resting potential of around -70 mV. However, it has been shown (in other cells, e.g. sympathetic neurons), that the I_M is more readily identified when cells are studied at a somewhat depolarized state, frequently at holding potentials of -30 or -20 mV [5, 16]. Thus in the present study this protocol was applied in order to investigate whether I_M are present also in guinea pig OHCs.

From both the original and subtracted current recordings it is evident that there is a non-inactivating current with a time- and voltage-dependency similar to what has been described in other cell types [3, 4, 6, 7, 17]. The reversal potential and the activation curve provide further evidence for the presence of an M-like current in guinea pig OHC. The reversal potential of -75 mV (cf. fig. 1b) is very similar to that originally shown by Brown and Adams [3]. The activation of the current occurred at potentials more negative than -20 mV and with half-maximum activation around -40 mV (cf. fig. 1, 2b). This is in agreement with published observations [4]. The current was significantly reduced by external application of CdCl_2 as previously demonstrated [18]. Taken together, the present observations clearly suggest that there is an M-like current in guinea pig OHCs.

Similar to the I_M in olfactory cortex neurons [9], the M-like current shown in the present study is also relatively small, which, in addition to the specific protocol used here, probably explains why it has not been reported previously. There are, however, a number of factors that could contribute to reducing the size of the recorded currents. First, when using preparations of isolated OHCs there is usually a bias towards long cells, i.e., cells originating from the apical regions of the cochlea. It cannot be excluded that the expression of the I_M , like that of some other potassium currents [2], differs with location along the length of the cochlea/basilar membrane and that cells from the basal turn would show larger M-like currents. Second, in the present experiments the Ca^{2+} chelator BAPTA was used in the internal solution in order to reduce calcium-activated potassium currents. However, as

BAPTA has been shown to gradually suppress the I_M [19], this may adversely influence our results, and may well be the reason M channel kinetics in OHC are less apparent than in, for instance, superior cervical ganglion neurons [10].

Linopirdine has been widely used as a blocker of I_M in a variety of cells. However, in the present study we had a substantial reduction of the current using CdCl_2 , and the subtracted CdCl_2 -sensitive current had the same properties as I_M . Subsequent addition of linopirdine caused only a small further decrease (cf. fig. 2a). It should be noted that linopirdine does not exclusively affect the I_M but blocks also other currents, e.g., $I_{\text{K(Ca)}}$, $I_{\text{K,n}}$ [14, 20], and that the effect of CdCl_2 may involve a calcium component overlying the I_M .

Our findings provide the first evidence for I_M in auditory OHCs and expression of the underlying *KCNQ2* and *KCNQ3* subunit genes in guinea pig cochlea, which support the presence of inner ear M channels. Expression of *KCNQ2* and 3 in the guinea pig cochlea is in agreement with the previous finding, also by RT-PCR, of *KCNQ2* (although weakly expressed) and *KCNQ3* in mouse cochlea [11]. Alternative splicing of *KCNQ2* suggests heterogeneity of M channels assembled from *KCNQ2* isoforms and *KCNQ3*, and may contribute to functional diversity of cochlear I_M , i.e. M-like but not 'classic' properties of potassium currents recorded in guinea pig OHCs, in analogy with heterologously expressed *KCNQ2* splice variants [21–23]. Other *KCNQ* genes can produce M-type currents, and *KCNQ4* is expressed in the cochlea. However, it is believed to underlie $I_{\text{K,n}}$ in OHCs, together with an unknown subunit [12, 14]. In inner hair cells, *KCNQ4* was recently shown to be involved in setting the resting membrane potential and submembrane calcium concentration, which is important for activation of large conductance calcium- and voltage-dependent (BK) K^+ channels [24]. OHC M channels composed of *KCNQ2/3* subunits (or other combinations, including so far unidentified accessory β -subunits) may have similar functional roles.

Channelopathies, diseases involving mutations in ion channels, are known for two *KCNQ* genes involved in hearing [25]. *KCNQ1* and *KCNQ4* are associated with Jervell and Lange-Nielsen syndrome (cardiac arrhythmia with congenital deafness) and DFNA2 [12] (non-syndromic, autosomal dominant progressive hearing loss), respectively. Mutations in *KCNQ2* or *KCNQ3* genes result in benign familial neonatal seizures (a type of epilepsy in the newborn), but pathological manifestations in

the inner ear are not yet known, although defective M channels may underlie inner ear disorders related to dysfunction of the cochlear amplifier or hyperexcitability of the auditory system, such as tinnitus.

In general, I_M helps to determine cell excitability, to maintain resting potential as well as set the frequency and pattern of spike discharges of action potentials [5]. However, the functional significance of I_M depends on the cell type. For example, I_M exerts a subtle control over the passive membrane properties and repetitive firing behavior of olfactory cortex pyramidal cells. In rod photoreceptors, I_M can set the dark resting potential and accelerate the voltage response to small photocurrents [8, 9]. The I_M is critical for subthreshold excitability and responsiveness to synaptic input; in *Aplysia* sensory neurons, serotonin closes the M channel, thus inducing a slow depolarization of the resting potential and an increase in duration of the action potential. This facilitates the presynaptic terminal and leads to increased neurotransmitter release [26].

Rapid repolarization of the OHCs is a key element in cochlear transduction, allowing the cell to respond to events at auditory frequencies. However, in addition to mechano-electrical transduction, OHCs express electromechanical properties, i.e. the cells respond mechanically to alterations in trans-membrane potential [27]. The latter is the basis for the cochlear amplifier [28], which is of fundamental importance to cochlear sensitivity and frequency selectivity.

In summary, we show here that OHCs express potassium currents with characteristics closely resembling I_M recorded in many other cell types. This M-like current will, in addition to the other voltage- or calcium-activated potassium channels present, act to re-polarize the OHC following acoustic stimulation. Thus it will stabilize not only membrane potential but also OHC function manifested in the mechano-electrical and electromechanical properties underlying the cochlear amplifier. Furthermore, OHC M channels may provide a neuromodulatory (muscarinic) mechanism for efferent regulation of peripheral auditory function.

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