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K^+ CHANNELS IN THE INNER EAR – ELECTROPHYSIOLOGICAL AND MOLECULAR STUDIES

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Stockholm 2005

Abstract

Potassium channels are involved in several fundamental cellular processes. Heavy metals, such as mercury and lead, are widespread toxic pollutants and have been shown to act on various ion channels in the central nervous system. In the present thesis the effects of mercury and lead compounds on potassium currents in auditory sensory hair cells were investigated. During the electrophysiological recordings a new type of ion current, an M-like potassium current, was observed. M currents, involved in e.g. stabilizing membrane excitability, have been described not only in the central nervous system but also in the peripheral visual and olfactory systems. The corresponding M channel is formed by KCNQ protein subunits. To elucidate the molecular basis of the cochlear M currents, *Kcnq* expression and localization of KCNQ channel proteins were pursued in the inner ear.

Using whole-cell patch clamp recordings it was shown that $HgCl_2$ blocked onset peak outward and inward K^+ currents of guinea pig sensory outer hair cells in a voltage- and dose-dependent manner, probably by modifying channel gating mechanisms. Pb^{2+} on the other hand did not produce any significant effects on hair cell potassium currents.

Patch-clamp experiments using a particular voltage protocol revealed an M-like potassium current in guinea pig outer hair cells. The low-threshold, voltage- and time-dependent, non-inactivating current displayed a -75 mV reversal potential and a -35 mV half-activation. This M-like current was significantly reduced by cadmium chloride.

Results from one-step RT-PCR indicated that genes encoding M channel subunits KCNQ2 – 5 were expressed in the mammalian cochlea. Two *Kcnq2* splice variants were expressed in the modiolus (containing the auditory spiral ganglion neurons) and the organ of Corti. Sequence data showed that exon 15a, which is important for M current kinetics, was spliced out from both *Kcnq2* transcripts. To verify the molecular substrate of the recorded M current, the presence of KCNQ channel proteins within the cochlea was investigated using immunohistochemistry. Antibodies against KCNQ2 and KCNQ3 stained both spiral ganglion cells types (type I and II), and their afferents projecting to the sensory inner and outer hair cells. In the organ of Corti, hair cell bodies, nerves, nerve endings of efferent, afferent fibers stained for KCNQ2. For the sensory cells, a base-to-apex gradient was seen, while for nerve fibers and nerve endings, apex-to-base and radial gradients of KCNQ2 subunit expression were observed. KCNQ3 exhibited a similar pattern of immunostaining but without involving nerve fibers.

In conclusion, although both mercury and lead are harmful to the auditory system, only Hg^{2+} seems to be acting at the level of the outer hair cell K^+ channels. The presence of an M-like potassium current was shown in guinea pig outer hair cells. Its molecular substrate was further demonstrated using RT-PCR and immunohistochemistry. The functional role of M currents in the cochlea remains unclear but it is suggested that an M current may be involved in controlling hearing sensitivity by modulating outer hair cell excitability. However, more work is needed to clarify this hypothesis.

Keywords: Cochlea, gene expression, hair cell, immunohistochemistry, KCNQ, lead, M current, mercury, patch clamp, RT-PCR, voltage-gated potassium channels

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Published and printed by Karolinska University Press

Box 200, SE-171 77 Stockholm, Sweden

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ISBN 91-7349-971-4

It is teamwork that makes great things possible
(Samir Chaudhuri)

To all of you who made this thesis possible!

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Liang GH, Järlebark L, Ulfendahl M, Moore EJ (2003) Mercury (Hg^{2+}) suppression of potassium currents of outer hair cells. *Neurotoxicology and Teratology 25; 349-359.*

II. Liang GH, Järlebark L, Ulfendahl M, Bian JT, Moore EJ (2004) Lead (Pb^{2+}) modulation of potassium currents of guinea pig outer hair cells. *Neurotoxicology and Teratology 26; 253-260.*

III. Liang G, Ulfendahl M, Moore EJ, Järlebark L (2005) M-like potassium current in guinea pig cochlea. *ORL - Journal for Oto-Rhino-Laryngology and Its Related Specialties. In press.*

IV. Liang G, Jin Z, Ulfendahl M, Järlebark L (2005) Molecular analyses of KCNQ1-5 potassium channel mRNAs in rat and guinea pig inner ears: expression, cloning, and alternative splicing. *Submitted for publication to Acta Oto-Laryngologica.*

V. Liang G, Jin Z, Ulfendahl M, Järlebark L (2005) Expression and localization of K^{+} channel subtypes KCNQ2 and 3 in the mammalian cochlea. *Manuscript.*

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
BK ($I_{K(dr)}$)	Big conductance voltage-dependent and Ca^{2+} -activated K^+ channel
Ca	Calcium
$CdCl_2$	Cadmium chloride
E_K	Equilibrium potential for potassium
Hg	Mercury
$HgCl_2$	Mercury chloride
IHC	Inner hair cell
$I_{K,n}$	K^+ channel activated at negative potentials
I_M	M current
K	Potassium
K_A	Potassium transient outward currents
K_v	Voltage-sensitive potassium channel
MeHg	Methyl mercury
MEM	Minimum essential medium
OHC	Outer hair cell
PBS	Phosphate buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
SK	Small conductance voltage-dependent and Ca^{2+} -activated K^+ channel
SM	Scala media
ST	Scala tympani
SV	Scala vestibuli
TEA-Cl	Tetraethylammonium chloride

1 INTRODUCTION

1.1 THE EAR – STRUCTURE AND FUNCTION

1.1.1 Sound and hearing

Sound can provide happiness and excitement, with a favorite song or the voice of someone dear, but can also give rise to unpleasant feelings and even lead to irreversible damage. Sound, whether pleasant or damaging, will reach the external ear and auditory canal as air conducted pressure alterations. At the tympanic membrane, the pressure wave elicits a vibratory motion that, amplified by the middle ear, is transmitted via the ossicular chain (malleus, incus and stapes) to the oval window of the cochlea, the auditory part of the inner ear. Vibrations if the oval window produces a pressure gradient across the hearing organ (organ of Corti), which evokes a traveling wave along the basilar membrane, from the base of the cochlea to the apex. Vibration of the basilar membrane and hearing organ results in mechanical stimulation of the auditory sensory cells, the inner and outer hair cells, and thus nerve impulses in the auditory nerve, the 8th cranial nerve.

1.1.2 Inner ear structures

Well protected within the hard temporal bone of the skull, the inner ear houses the sensory organs of hearing and equilibrium (Fig. 1). The hearing organ is located within the snail-shaped cochlea, a helical system of three fluid filled tubes. The central tube, scala media (SM), is filled with endolymph, a fluid having a high K^+ concentration (Sterkers et al. 1984). Above and below the SM are the scala vestibuli (SV) and scala tympani (ST), respectively, both containing perilymph, a normal extracellular fluid having a low K^+ concentration. In a cross-section, the SM has a triangular shape formed by Reissner's membrane, stria vascularis and the basilar membrane on which the organ of Corti is resting like a longitudinal ridge. The organ of Corti is composed of three rows of outer hair cells (OHCs), one row of inner hair cells (IHCs), and several types of supporting cells (e.g., pillar, Deiters', Hensen's, Böttcher's and Claudius' cells). The hair cells are mechanoreceptors having hair-like protrusions projecting from their apical poles. The sensory hairs, stereocilia, are in close contact with the tectorial membrane, a gelatinous structures protruding from the spiral limbus and overlying the hearing organ. The IHCs, goblet-shaped cells with centrally placed nuclei are thought to be the true receptor cells of the cochlea while the main function of the OHCs is to modulate the input to the IHCs. The base of an individual hair cell makes a synapse with a small number of both afferent and efferent nerve fibers.

Crossing the base of the tunnel of Corti, unmyelinated afferent fibers originate from bipolar type I spiral ganglion cells located in the spiral ganglion (Fechner et al. 2001). Six to 100 OHCs within the same row are connected to one spiral afferent axon from the type II spiral ganglion cells (Smith 1975; Berglund and Ryugo 1987; Dannhof and Bruns 1993). Most efferent axons arise in the superior olivary complex of the brainstem and innervate OHCs after crossing the tunnel half-way up the tunnel of Corti (Pickles 1988; Slepecky 1996). Efferent nerves control cochlear afferent reactivity by inhibiting the fast voltage-dependent amplification mechanism provided by OHCs (Guinan 1996). It was suggested that, contrary to typical inhibitory synapses, synaptic inhibition in OHCs is achieved via activation of neuronal α9-acetylcholine receptors (nACHR).

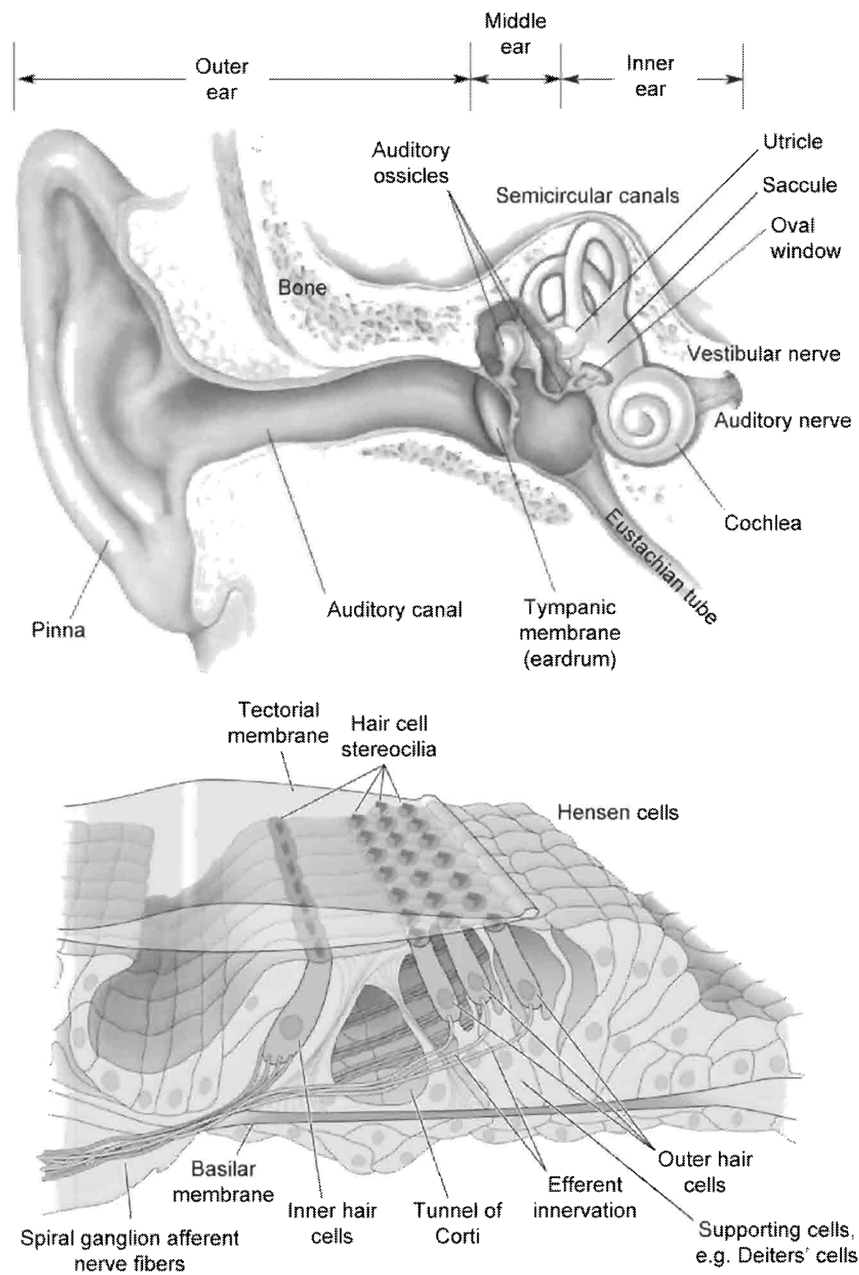

Figure 1. Anatomy of the human peripheral auditory system consisting of the outer, middle, and inner ear. The labyrinthine inner ear comprises the organs for hearing (cochlea) and balance (semicircular canals, utricle and saccule) (*top*). Cross-section through the hearing organ, organ of Corti, showing an intricate structural organization of sensory hair cells, innervation pattern, and supporting cells.
Adapted from http://www.neurophys.wisc.edu/yin/neuro524-2001/audition.htm.

The spiral ganglion is composed of two different cell types: type I and type II. It is widely accepted that the type I large bipolar spiral neurons (95% of the total) (size: 15-30 μm), with a large, regular round nucleus (Spoendlin 1969, 1972; Morrison et al. 1975), are connected to the IHCs (Spoendlin 1971, 1972). The type II spiral ganglion neurons (5% of the total) are small (10-15 μm) (Roberts et al. 1982), with an excentric, irregular and even lobulated nucleus. However, SGCs of type I and II are quite similar in size in guinea pig (Spoendlin 1971). The major neurotransmitters in the cochlea are believed to be glutamate (afferent transmission) and acetylcholine (efferent fibers), but other possibilities exist as well (Sewell 1996).

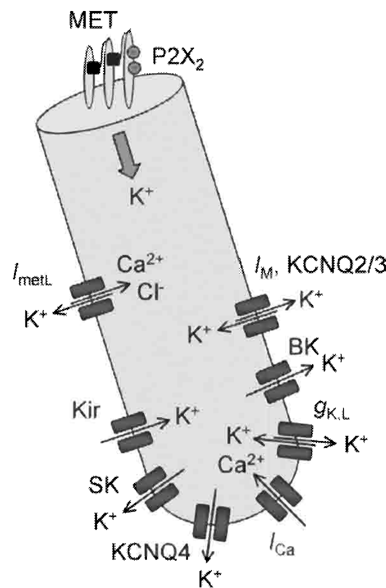

Figure 2. Ion channels and currents of outer hair cells: mechano-electrical transducer channels (MET, TRPA1); ATP-gated receptor ion channels ($P2X_2$);mechano-electrical transducer of the lateral membrane (I_{metL}); inward rectifier potassium channel (Kir); small-conductance calcium-activated K^+ channel (SK); K^+ channel activated at negative potentials ($I_{K,n}$, KCNQ4); voltage-gated Ca^{2-} current (I_{Ca}); low-conductance K^+ channel ($g_{K,L}$); large-conductance Ca^{2+}-activated K^+ channel (BK, I_{kdr}); M current (I_M, KCNQ2, KCNQ3, KCNQ5?).

1.1.3 K^+ recycling in the cochlea

Sound sensory transduction in the cochlea depends on the cycling of K^+ between the endolymphatic high concentration reservoir and the perilymph low concentration reservoir. The high K^+ endolymph is created by the K^+ channels in stria vascularis (Sakagami et al. 1991; Marcus et al. 1997). The huge voltage gradient across the OHCs membrane (+80 mV at the endolymph and -70 mV intracellularly) drives K^+ flow efficiently, but must be maintained by quick recycling of K^+ back into the endolymph. K^+ flows into the hair cells via the apical mechanoelectrical transducer (MET) channels (Dallos 1996; Hudspeth 2001) and is mainly removed from the hair cells by the I_{Kn} channel (Housley and Ashmore 1992; Kubisch et al. 1999; Marcotti and Kros 1999; Kharkovets et al. 2000) (Fig. 2). K^+ may either go into the perilymph and absorbed by fibrocytes in the spiral ligament (Spicer and Schulte 1996; Crouch, Sakaguchi et al. 1997) or it may pass gap junctions from cell to cell into strial intermediate cells (Kikuchi et al. 1995; Xia et al. 1999, 2000, 2001). Strial marginal cells take up K^+ across their basolateral membrane (Neyroud et al. 1997; Marcus et al. 1998; Delpire et al. 1999) and secrete K^- from the apical membrane via KCNQ1/KCNE1 channels into the endolymph, thus regenerating the high K^+ concentration and the endocochlear potential (Vetter et al. 1996; Neyroud et al. 1997). Any protein mutation involving this K^+ recycling will generate hearing loss.

1.2 POTASSIUM CHANNELS

1.2.1 Channel diversity and classification

Intracellular and extracellular fluids contain a wide range of ions at different concentrations. The electrically charged ions are subjected to electrochemical forces, which tend to move the ions across the cell membranes. The resulting ion currents, or rather their onset and termination, play a fundamental role in cellular function. The ions primarily pass through protein channels embedded in the cell membrane. A channel complex not only forms a pore, often selective, for the ions but also controls the ion conductivity. In the inner ear, with its unique K^+-rich extracellular fluid surrounding the transducer channels in the sensory hair bundles, potassium ion channels are of special functional importance. Recent progress in the understanding of the molecular diversity, structure, and function of potassium channels has greatly contributed to our knowledge of many cellular processes. Important functions of K^+ channels include neuronal excitability, neurotransmitter release, cell proliferation and cell degeneration. K^+ channels are membrane-spanning proteins that selectively allow K^+ ions to quickly pass through water-filled permeation pathways (Doyle et al. 1998) across the cell membrane. A single K^+ channel contains four α-subunits, each consisting of six transmembrane domains and a highly conserved pore region (Fig. 3). Since the first K^+ gene from Drosophila shaker mutant (Papazian et al. 1987) was cloned, more than eight types of K^- channels of spanning 70 different subunits have been identified in humans (Jentsch 2000; Shieh et al. 2000; Robbins 2001). Four different protein subunits from separate genes are assembled together in the membrane to form one functional channel (MacKinnon 1991; Papazian 1999).

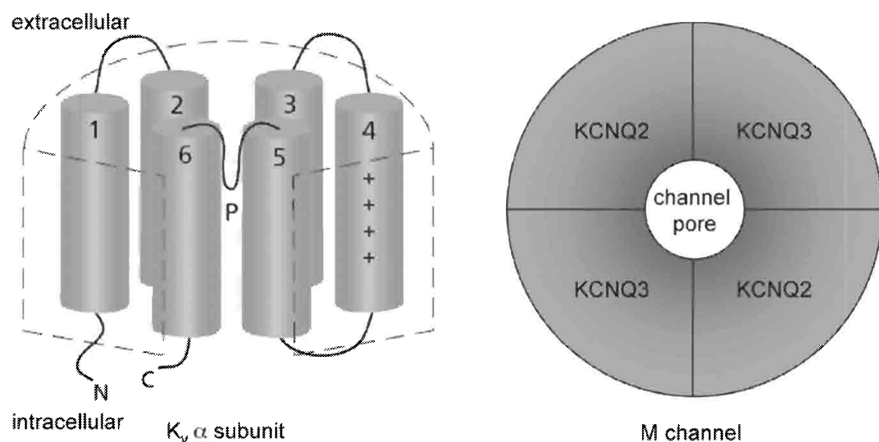

Figure 3. Tertiary and quatenary structures of voltage-gated potassium ion channels (K_v). An α subunit of K_v channels has six trans-membrane (TM) domains. The loop between TM5 and TM6 contains the P-segment, which lines the central pore of the ion channel. The voltage sensor is located in membrane-spanning segment 4. Four α subunits assemble to form a functional ion channel, e.g. a KCNQ2/KCNQ3 hetero-tetrameric M channel.

The classification of K^+ channels are mainly based on the number of transmembrane domains (TMD) (pore-forming α-subunit) and pore loop of the channels (Papazian 1999; Shieh et al. 2000; Robbins 2001). To date, the system of six groups with eight, seven, six, four, or two putative transmembrane domain segments is used (Robbins 2001). K^+ channels usually have the following important structural parts (based on a 6TMD-1P confirmation):

- *Pore and selectivity filter*: There is a conserved sequence G(Y/F)G between S5-S6 for all K^+ channels. This G(Y/F)G is considered as a K^+ selectivity signature motif (Heginbotham et al. 1994). Four of these pore sequences construct a functional K^+-conducting pore (MacKinnon 1991). Tetramers from the same or different subunits link together to build up the K^+ channel protein (MacKinnon 1995; Jan and Jan 1997).

- *Voltage sensor and channel activation*: In the voltage-gated ion channel, S4 contains the positive charged amino acid residues (lysine or arginine), or voltage sensor sequences, which can change the channel protein shape, allowing K^+ ions to flow through the pore, after the membrane had been depolarized (Armstrong and Bezanilla 1974).

- *Inactivation*: Voltage-dependent K^+ channels must be activated and inactivated by a positive membrane potential. Inactivation is a non-conducting state during a maintained depolarization.

1.3 M-TYPE POTASSIUM CHANNELS

1.3.1 Function, properties, distribution

The M channel is a relatively recently described potassium channel (Brown and Adams 1980). The M-type K^+ channel is quite different from the potassium channels that repolarize excitable cells after an action potential. M channels are always open in a neuronal resting status and are further activated by small membrane depolarizations. These channels need about 100-fold more time to close and open than channels involved in the action potential. Because of its unique modulation by neurotransmitters, voltage sensitivity and multiple regulatory pathways, M channels play a crucial role in stabilizing membrane excitability, maintaining the resting potential, and controlling the frequency of action potentials (Brown and Adams 1980; Constanti and Brown 1981; Adams et al. 1982; Brown 1988; Bosma et al. 1990; Smith et al. 1992; Devaux et al. 2004). The K^+ current passing through the this type of ion channel was termed M current, I_M, because it was first blocked by muscarine. An I_M has been identified in neurons of not only the central nervous systems (Constanti and Brown 1981; Halliwell and Adams 1982; Jones 1987) but also in the visual system and olfactory systems (Constanti and Galvan 1983; Beech and Barnes 1989).

1.3.2 Molecular correlates of the M channel

Since the first report of an M current (I_M), several candidate genes have been suggested to constitute the M channels (Stansfeld et al. 1997). The M channel gene was only expressed in the nervous system. Molecularly similar to the *KCNQ* gene (Wang et al. 1996), which is often coupled to the accessory *KCNE* gene (Takumi et al. 1988; Abbott et al. 1999; Piccini et al. 1999; Angelo et al. 2002), and that is mainly expressed in heart and mutated in the long-QT syndrome, the brain channel was named KCNQ2. Three more neuronal *KCNQ* genes were subsequently found. Mutation of either *KCNQ2/3* was associated with benign familial neonatal seizures (BFNS) (Charlier et al. 1998). *KCNQ4* was identified in the same way. Its mutation was related to a form of dominantly inherited deafness (Kubisch et al. 1999). *KCNQ5* was cloned from retinal degeneration disease (Lerche et al. 2000; Schroeder et al. 2000). The different *KCNQ* subtype genes contain various numbers of exons. In particular, the *KCNQ2* gene contains over 35 alternative exons. Exon 15a is well conserved among all KCNQ channels and located in the long C-terminus. Exon 15a has been suggested to play many important roles in the determination of deactivation, activation of channels, assembly and recognition of different subunits (Schmitt et al. 2000; Pan et al. 2001). A change in the deactivation rate and a shift of the steady-state activation of the KCNQ1 channel were observed in a clinical exon 15a mutation (Chouabe et al. 1997). KCNQ2 splice variants missing an intact C-terminal tail also fail to give rise to measurable current when expressed in heterologous systems (Nakamura 1998; Smith et al. 2001).

1.3.3 *Kcnq* splice variants

A number of KCNQ splice variants are transcribed, while some of them have a tissue-specific distribution (Demolombe et al. 1998; Murray et al. 1999; Schroeder et al. 2000). Over 11 alternatively spliced KCNQ2 mRNA isoforms have been isolated from the brain and sympathetic ganglia (Tinel et al. 1998; Biervert and Steinlein 1999; Pan et al. 2001; Smith et al. 2001) because of its complex genomic structure (Biervert and Steinlein 1999). The main functional splice variants underlying I_M are located in the long C terminus (Biervert et al. 1998; Nakamura 1998; Tinel et al. 1998; Pan et al. 2001) and two of them were deleted from key exon 15a (Pan et al. 2001). The long Q2 variant is preferentially expressed in adult neurons while the short Q2 message is dominantly in immature proliferating preneurons, such as those in fetal, tumor or embryonic stages (Nakamura 1998; Smith et al. 2001). The long Q2 sequence is crucial for modulation of I_M function. Cochlear expression of *Kcnq2/3* has only been shown by RT-PCR in mouse (Kubisch et al. 1999), and their cellular location is therefore not known. *Kcnq4* is mainly expressed in the OHCs (Kubisch et al. 1999; Beisel et al. 2000; Kharkovets et al. 2000), although there seems to be interspecies variation. *Kcnq4* probably underlies $I_{K,N}$ (Marcotti and Kros 1999), both of which have a gradient of expression, being more expressed in basal than apical turns of the cochlea (Mammano and Ashmore 1996; Kharkovets et al. 2000). Q4 is also expressed in hippocampal CA1 or CA3 by RT-PCR but was not related to function (Shah et al. 2002). Four neuronal KCNQ subunits probably form functional M channels homologously or heterologously in vitro and the latter seems to be limited to certain compositions (Schroeder et al. 1998, 2000; Kubisch et al. 1999; Lerche et al. 2000). All of the heteromultimeric currents are similarly activated at a membrane potential of positive to -60 mV, with

little or no inactivation. Co-expression of *Kcnq2* and *Kcnq3* produces current amplitudes much higher than expected from those obtained from either *Kcnq2* or *Kcnq3* alone (Schroede et al. 1998; Wang et al. 1998; Yang et al. 1998; Main et al. 2000). Moreover, the kinetics and sensitivity to inhibitors are also slightly different (Wang et al. 1998; Hadley et al. 2000). Unlike KCNQ2, KCNQ3 can co-assemble with all KCNQ subunits in heterologous cells (Kubisch et al. 1999; Lerche et al. 2000; Schroeder et al. 2000) to form functional ion channels with M-type properties. It is possible that differential co-expression of alternative splicing of the KCNQ subunits underlie variants of the I_M depending on their localization. However, expression of *Kcnq5* has not yet been described in cochlear tissues.

1.3.4 M channel proteins in the CNS

Although labeling is widespread, the expression of M channel proteins varies in different tissues. KCNQ2 and KCNQ3 were co-localized in a somatodendritic pattern in several neuronal populations, especially the cortex and hippocampus for control of synchronization and rhythmic neuronal activity (Wang et al. 1998; Cooper et al. 2000; Cooper et al. 2001). Immunostaining of KCNQ2, but not KCNQ3, was also fundamental in some terminal fields, such as in axon initial segments and nodes of Ranvier (Devaux et al. 2004), suggesting a presynaptic role for a distinct subgroup of M channels in the regulation of action potential propagation and neurotransmitter release. The distribution of KCNQ2 and KCNQ3 subunits in the inner ear has not been described previously.

1.4 VOLTAGE-GATED CHANNELS FOUND IN OHCs

Several different voltage-gated ion channels and their related currents have been identified in OHCs. For example:

- I_{Kn}, a potassium current that is activated at negative potentials, the main current exists in apical region of the cochlea, is fully activated at resting potentials, and is a Ca-activated K^+ current. It is fully activated at -60 mV and is turned off at -130 mV, with half-activation kinetics at -92 mV and is blocked by cesium (Housley and Ashmore 1992), TEA (Hadley, Noda et al. 2000), linopirdine (Marcotti and Kros 1999), and XE991 (Schroder et al. 2003). $I_{K,n}$ can maintain the cell at the normal Nernst equilibrium potential, E_K, and create maximum driving force for K^+ entry through the apical MET channels (Housley and Ashmore 1992; Mammano and Ashmore 1996). I_{Kn} is important for maintaining OHC viability as well as to reduce the membrane time constant of OHCs to maximize the frequency response of electromotility (Housley and Ashmore 1992; Mammano and Ashmore 1996; Nenov, Norris et al. 1997; Marcotti and Kros 1999).

- BK channels ($I_{K(dr)}$): BK channels have been discovered in guinea pig OHCs and suggested to carry the majority of the outwardly rectifying currents in these cell (Ashmore and Meech 1986; Santos-Sacchi and Dilger 1988; Gitter et al. 1992; Housley and Ashmore 1992; Nenov et al. 1997). It is activated at -35 mV, half-activated at -17 mV and sensitive to TEA (Hille 1967; Stanfield 1983; Housley and

Ashmore 1992), 4-AP (Baker et al. 1993; Nenov et al. 1997), $CdCl_2$ (Nenov et al. 1997), linopirdine (Marcotti et al. 2003) and ChTx (Galvez et al. 1990; Nenov et al. 1997). BK channels are colocalized with Ca^{2+} channels at sites of transmitter release, the hair cell's presynaptic active zones (Roberts et al. 1990; Issa and Hudspeth 1994). Many physiological processes, such as neurosecretion, smooth muscle tone, and hearing are controlled by Ca-activated K^+ channels of large conductance. The channel provides a calcium- and voltage-activated pathway through OHCs lateral membranes for passive K^+ transport (Gitter et al. 1992). BK channels can regulate neurotransmitter release coupled to the Ca^{2+} current of hair cells.

- Inwardly rectifying K^+ channels (K_{ir}): Kir is a channel that allows a net influx of K^- ions through the membrane at membrane voltages negative to the K^+ equilibrium potential. These channels regulate membrane excitability, heart rate, vascular tone, insulin release and salt flow across epithelia.

- Mechanoeletrical transducer (MET) channels: The MET channel (Lukashkin and Russell 1997; Hudspeth 2001) is located in the tip of the stereocilia of the OHCs (Corey and Hudspeth 1983; Ohmori 1985; Crawford et al. 1989; Dallos 1996). Sound-induced vibrations of the hearing organ are detected by deflections of the hair cell's hair bundle, a structure containing the voltage-dependent (Lukashkin and Russell 1997) mechanosensitive channels and mainly consisting of a cluster of actin-filled stereocilia (Corey and Hudspeth 1983; Ohmori 1985; Crawford et al. 1989). Opening of the MET will depolarize the OHCs and thus lead to the opening of voltage-gated Ca^{2+} channels (*Ica*) (Fuchs et al. 1990; Roberts et al. 1990; Chen et al. 1995), and an influx of Ca^{2+} and K^+ ions.

1.5 HEAVY METALS AND THEIR EFFECTS ON ION CHANNELS

1.5.1 Mercury

Mercurial compounds are ubiquitous environmental contaminants that harm especially the developing and immature CNS (Bakir et al. 1973; Choi 1989; Clarkson 1992). Generally, a few weeks or months are needed to manifest numbness, ataxia, dysarthria, constriction of the visual fields, loss of hearing, and even death from acute or subacute Hg^{2+} poisoning (Bakir et al. 1973; Falk et al. 1974; Miller 1998). The duration of sickness in fatal cases was from about four weeks to four years. Up to 80% of patients with Minamata disease developed hearing loss (Kurland et al. 1960) and only 7% of them showed improvement with pure-tone audiometry test 10-20 years later (Mizukoshi et al. 1989). In the initial stage, some of the damages appeared to be reversible.

Sulfhydryl groups of cysteinyl residues of proteins are the most reactive of all amino acid side chains (Kenyon and Bruice 1977). In order to stabilize protein structure, it is important to form a disulfide bridge between cysteine residues by oxidation that occur quite frequently in extracellular or transmembrane segments of proteins (Branden and Tooze 1991; Thorn et al. 1992).

Hg^{2+} has a high affinity to sulfhydryl and disulfide (S-S) groups (Hughes 1957) and the formation of disulfide bonds changes channel conformation, producing changes in the permeability and gating kinetics of ion channels (Zaidi et al. 1989; Ruppersberg et al. 1991; Salama et al. 1992; Hare et al. 1993; Islam et al. 1993; Sarafian 1993; Chiamvimonvat et al. 1995; Benitah et al. 1997), hence, reduced currents of voltage-gated channels (Quandt et al. 1982; Bohme et al. 1992; Pekel et al. 1993; Rossi et al. 1993; Busselberg et al. 1994; Gallagher et al. 1995; Dyatlov et al. 1996; Leonhardt et al. 1996; Hurley et al. 2001), altered the I/V curve of channels, and depolarized the membrane voltage (Shrivastav et al. 1976; Quandt et al. 1982; Kauppinen et al. 1989; Hare and Atchison 1992; Hille 1992) in various biological preparations. On the other hand, some of the blocking effects by both organic and inorganic mercurials could be readily reversed by reducing agent, e.g., 1,4-dithiothreitol (Islam et al. 1993; Koivisto et al. 1993; Chiamvimonvat et al. 1995; Kurata et al. 1998).

1.5.2 Lead

Lead (Pb^{2+}), both in inorganic and organic forms, cause medical health effects, including central auditory processing impairment (Dietrich et al. 1992; Mendola et al. 2002), cognitive dysfunctioning (Bellinger et al. 1987), psychologic and classroom deficits (Needleman et al. 1979; Cullen et al. 1983), changes in auditory evoked potentials (Otto et al. 1985; Holdstein et al. 1986; Lille et al. 1988), and purported decreased hearing sensitivity (Schwartz and Otto 1987, 1991; Otto and Fox 1993; Staudinger and Roth 1998), particularly of the young. A blood lead level (BLL) higher than 0.5 μM (10.34 μg/dl) is the criteria for protecting children from lead intoxication (Counter et al. 1997; Staudinger and Roth 1998).

Many experiments have been carried out on ion channels to understand the basic mechanisms of Pb effects. K^+ channels have been the focus of different studies in neurons of *lymnaea stagnalis L* (Szucs et al. 1994), rat dorsal root ganglion (Dai, Ruan et al. 2001), of cloned neurons (Madeja et al. 1995) and hippocampal neurons (Talukder and Harrison 1995; Madeja et al. 1997; Yu et al. 2003). Pb^{2+} reversibly inhibited the amplitudes of I_h, $I_{K(dr)}$ in a dose-dependent manner; shifting the I/V curves to positive potentials (Madeja et al. 1995, 1997; Dai et al. 2003). Contrary to these findings are reports of essentially normal hearing sensitivity in a group of children from the Andes exposed to BLLs of 5.32 μM (110 μg/dl). Thus, it is not clear whether or not Pb^{2+} causes significant peripheral hearing loss from acute or chronic exposure, and if so, the underlying mechanisms at the membrane level responsible for the supposed sensory deficit remain unknown.

2 AIMS OF THE STUDY

The overall aim of this study was to characterize the potassium currents in the inner ear, especially the M current, and to explore both the expression of *Kcnq* genes and the localization of gene products. The specific aims have been:

- To investigate effects of heavy metals on voltage-sensitive K^+ channels in guinea pig outer hair cells

- To explore the presence and characteristics of an M type K^+ current in guinea pig outer hair cells

- To investigate the expression and localization of novel voltage-gated K^+ channel genes for KCNQ subtypes

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

In Papers 1-3, adult pigmented guinea pigs (body weight 250-350g) were used for electrophysiological and molecular studies. In Papers 4-5 adult rats (Sprague-Dawley; body weight 200-300g), and adult mice (C57; body weight 20-27g) were used. The animals were deeply anaesthetized and decapitated prior to any further dissection. All experiments were approved by local and national regulations for the care and use of animals (approvals N7c/98, N146/99 and 10/01).

For experiments involving patch-clamp whole-cell recordings (Papers I-III), the cochlear bullae were excised and transferred to ice-cold minimum essential medium (MEM), where the coils of the sensory auditory epithelium were gently isolated from the modiolus. The tissue was then incubated in collagenase (0.125–0.25 mg/ml, type I, Sigma-Aldrich, St. Louis, MO) in MEM for 5–15 min at room temperature. After rinsing the cochlear coils three times in MEM, the outer hair cells (OHCs) were dissociated mechanically from the sensory epithelium by gently pipetting the media once using a constricted glass pipette. The resulting cell suspension (in MEM) was attached to the chamber bottom for 5 minutes before electrophysiological experiments commenced. Most cells obtained were from the middle and apical turns of the cochlea, with cells ranging from ~60 to 80 μm in length.

For molecular experiments (Papers III-V), the rats, mice and guinea pigs were sacrificed. The cochleas were either quickly dissected out (for whole cochlea preparations) or micro-dissected into the modiolus, organ of Corti, and lateral wall in cold RNase-free physiological saline under observation using a dissection microscope. The tissues were immediately put in TRIzol and/or RNAlater, homogenized, and used directly in molecular applications, e.g. RT-PCR, or frozen and stored at -80°C until further processing.

For immunohistochemistry, the cochleas were removed, put into cold saline or phosphate-buffered saline (PBS) for further dissection. This included opening the cochleas at the apex, round and oval windows to allow intra-cochlear perfusion with a solution containing 4% (w/v) paraformaldehyde (also commercial 4% formalin solution, containing 10-15% methanol, was used) in 0.1 M phosphate buffer, pH 7.4, and then post-fixed overnight with the same fixative.

3.2 PATCH-CLAMP TECHNIQUES

Voltage-sensitive K^+ currents were recorded from isolated guinea pig outer hair cells using the whole-cell patch-clamp technique with the dual purposes of: (1) determining whether voltage-gated K^+ channel in the cochlea may be involved in the hearing loss induced by heavy metals, and (2) exploring the presence of M-type K^+ channels in the cochlea. Voltage-clamp command pulses were applied through the pipette (borosilicate glass; 8-15 MOhm), and a AgCl-pellet/3.0 M KCl agar bridge was used as a reference electrode. The pipette potentials were maintained by initially correcting for the liquid

junction potential of ~5 mV using the Henderson equation of the pClamp software [Barry, 1994 #140;Barry, 1994 #16]. A patch-clamp amplifier (EPC 7, List-Medical, Darmstadt, Germany) recorded membrane currents passing through the pipette. Data were acquired using pClamp 7 (Axon Instruments, Union City, CA) with a 16-bit A/D converter (Axon Instruments). P/4 leak subtraction was used for some of the data and capacitive currents were subtracted manually. Currents were sampled at 10 kHz and filtered with a three-pole Bessell filter from DC -5 kHz.

3.2.1 External and internal solutions

The contents of external solution (MEM) and internal solutions for heavy metal and M current papers (Papers I-III) are listed in Table 1. The calculated equilibrium potential for K^+ (E_K) was -81 mV (M current, Paper III). In some of the experiments, tetraethylammonium chloride (TEA-Cl, 25 mM), cadmium chloride ($CdCl_2$, 100 µM or 1 mM), 4-aminopiridine (4-AP, 100 µM), or linopirdine (100 µM; Research Biochemicals International, Natick, MA, USA) were used to suppress certain K^+ currents sensitive to these compounds. Experiments were conducted at room temperature.

Table 1. Solutions used in patch clamp experiments.

	Heavy metal experiments		M current experiments	
	Internal solution (mM)	External solution (MEM)	Internal solution (mM)	External solution (MEM)
KCl	140	5.37	100	5.37
$MgCl_2 \cdot 6H_2O$	2.0	0.49	2	0.49
HEPES	5.0		5	
ATP (Na-salt)	1.0		1.0	
GTP (Na-salt)	0.1		0.1	
BAPTA			10	
$CaCl_2 \cdot 2H_2O$		1.26		1.26
KH_2PO_4		0.44		0.44
$MgSO_4 \cdot 7H_2O$		0.4		0.4
NaCl		128		128
$NaHCO_3$		4.2		4.2
Na_2HPO_4		0.31		0.31
EGTA	0.5			
pH (adjusted by)	7.3 (KOH)	7.4 (HEPES)	7.3 (KOH)	7.4 (HEPES)
Osmolality (mOsm/kg)	290	305	296	305

3.2.2 Voltage protocols

The voltage protocols used in the three electrophysiological studies differed slightly:
Paper I (Hg^{2+}) – Voltage protocol 1
Cells were held (holding potential, V_H) at a membrane potential of -70 mV, and a 300 ms pulse was stepped to potentials ranging from -130 to +50 mV in 10 mV steps. Each test potential was applied once every 20 ms and stepped back to V_H.
Paper II (Pb^{2+}) – Voltage protocol 2
A 300 ms pulse at a V_H of -70 mV was stepped to -130 mV five times and to +50 mV five times, with each pulse separated by a 1-minute interval.
Paper III (M current) – Voltage protocol 3
Holding the cell at resting potential, the recording of ion currents will include most of the potassium currents in the outer hair cell (OHC), thus masking the relatively smaller M current (I_M). However, the I_M only activates in a very narrow range within the sub-threshold potential, therefore a standard M current protocol was applied, where the I_M is pre-activated by depolarizing the cell to sub-threshold potentials (= holding potential) for a sufficiently long time (Adams et al. 1982; Lamas et al. 1997).

3.3 RT-PCR

In order to detect and analyze the mRNA expression of *Kcnq* genes in the cochlea in a convenient, sensitive, and reproducible way, one-step RT-PCR (Reverse Transcription Polymerase Chain Reaction) was performed in a single tube using gene-specific primers. Total RNA from whole or micro-dissected cochleas was isolated from fresh tissue homogenates according to the TRIzol protocol (GibcoBRL/Life Technologies). RNA concentration and purity was quantified by spectrophotometry (absorbance ratio A_{260}/A_{280}). Primers based on published sequences were purchased from Invitrogen and used to identify gene expression of the different KCNQ subunit mRNAs. As neither nucleotide nor protein sequences for guinea pig *Kcnq1–5* were reported in public gene sequence databases, primers were from other species: rat/mouse (often identical) or human. Primers for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kreisberg et al. 1997) and β-actin (Overbergh et al. 1999) were used as controls. Primers optimized to recognize *Kcnq4* were designed based on sequence data in GenBank with the online primer design tool at the Whitehead Institute, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCR primers (tentatively named "Q2L" and "Q2ALL") for additional work on characterization of KCNQ2 splice variants, i.e. with the aim to elucidate what KCNQ2 isoform may contribute to M channels in the cochlea, were designed on the basis of our own sequence data. The primer nucleotide sequences and the predicted sizes of the amplified PCR products are listed in Table 2.

Table 2. PCR primer sequences and expected sizes of their amplicons.

Primer	Nucleotide sequence	Size	Reference
KCNQ1 fwd	5'-GCTCTGGCCACCGGGACCCT	434	Wulfsen et al. 2000
KCNQ1 rev	5'-GATGCGGCCGGACTCATTCA		
KCNQ2 fwd	5'-CAAGTACCCTCAGACCTGGAAC	551	Jow and Wang, 2000
KCNQ2 rev	5'-CAGCTCTTGGGCACCTTGCT		
KCNQ2L fwd	5'-CGCCCAGTCAGAAGGTCA	625	Paper IV
KCNQ2L rev	5'-TTGGTGCGGTCCTTGTC		
KCNQ2All fwd	5'-ACTCATCCCACCTCTCAACC	618	Paper IV
KCNQ2All rev	5'-TTGGTGCGGTCCTTGTC		
KCNQ3 fwd	5'-CCAAGGAATGAACCATATGTAGCC	461	Kubisch et al. 1999
KCNQ3 rev	5'-CAGAAGAGTCAAGATGGGCAGGAC		
KCNQ4 fwd	5'-GGGCCTCTCTAAGACTCAAG	235	Paper IV
KCNQ4 rev	5'-AGGTGTCCTGCTGAATACTG		
KCNQ5 fwd	5'-CCATTGTTCTCATCGCTTCA	200	Kananura et al. 2000
KCNQ5 rev	5'-TCCAATGTACCAGGCTGTGA		
β-actin fwd	5'-ACTGGGACGACATGGAGAAG	852	Paper III
β-actin rev	5'-GTGGATCAGCAAGCAGGAGT		
GAPDH fwd	5'-TCCCTAAGATTGTCAGCAA	309	Kreisberg et al. 1997
GAPDH rev	5'-AGATCCACAACGGATACATT		

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 2x reaction mix provided with the kit (containing 2.4 mM $MgSO_4$ and 0.4 mM of each dNTP), 1 µl of each primer in a pair (final concentration 0.2 µM), template RNA (100 ng/reaction), and 5 units of RT/*Taq* mix. Reverse transcription was performed in a thermocycler for 30 minutes 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 RNA sample. A negative control reaction in which the target cDNA was omitted (no template) was run with every reaction. Alternatively, the RT step was omitted to serve as negative control. PCR products were separated by 2-2.5% agarose gel electrophoresis and stained with ethidium bromide. For semi-quantitative analysis of RT-PCR products, the numbers of cycles were optimized by stopping the RT-PCR reactions every two cycles between 14 and 40 cycles.

3.4 IMMUNOHISTOCHEMISTRY

Cochleas fixed overnight were decalcified under vacuum for <1 day in 0.1 M EDTA at room temperature. Decalcification was checked by gently pressing on the cochlea with a needle. On non-decalcified cochleas, the bony shell was trimmed away carefully by the use of a scalpel. The tissues were cryoprotected by processing in PBS containing 10% and 30% sucrose, 24 hours for each concentration, respectively. The tissues were prepared for cryosectioning by embedding in a solution containing 30% sucrose and O.C.T. compound (1:1) for 3 hours and then oriented on a cryomicrotome pedestal under a microscope. Tissues were frozen at -20°C and cut at 12 μm in a cryostat, and captured on gelatin/chrome-alum- or poly-D-lysin-coated objective glass slides, or SuperFrost slides. Selected sections mounted on slides were re-hydrated in PBS or TBS prior to incubation with primary antibodies at 4°C overnight. The anti-KCNQ2 and anti-KCNQ3 polyclonal antibodies were generous gifts from Dr EC Cooper, University of Pennsylvania. After incubation with the primary antiserum (initially at different titers to evaluate immuno-staining quality; see Paper V for actual titers used), the samples were washed several times in PBS and incubated with labeled secondary antibodies. Different methods of visualization were used: (1) fluorescence-conjugated secondary antibodies (e.g. fluorescein isothiocyanate, FITC, 1:200, Dako) for 2 hr at room temperature; (2) the Vector ABC Elite kit based on biotinylated secondary antibodies, avidin-biotin-peroxidase complex formation, and peroxidase substrate (e.g. diaminobenzidine, DAB) which is converted to a dark brown insoluble product. For immunofluorescence, the sections were thoroughly rinsed in TBS and mounted in Fluosave mounting media in order to prevent fading. For additional nuclear staining, sections were embedded with Vectashield mounting medium with 4',6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Slides developed by immunoperoxidase methods were preserved in permanent mounting medium.
In all immunostaining procedures it is crucial to determine what is true positive staining and what belongs to background signal. To test the specificity of the antibody-antigen reaction, every antibody should optimally be controlled by pre-absorption of the antibody with the appropriate antigen, which should abolish staining, or by omission of the primary (and secondary, if one wishes to test its specificity) antibody, which in our case resulted in no detectable labeling. The tissue sections were finally observed in a Zeiss Axioplan Universal Microscope equipped with appropriate excitation and emission filters (Zeiss, Germany).

4 RESULTS AND DISCUSSION

4.1 HEAVY METAL EXPOSURE AND K^+ CHANNELS OF THE AUDITORY SYSTEM

4.1.1 Effects of inorganic mercury

Blocked outward and inward K^+ currents

In paper I, the whole-cell patch-clamp technique was used to clarify the possible contribution of Hg^{2+} to K^+ channels of guinea pig OHCs. This cell was selected, because the OHCs from this animal are easier to prepare than other species and its current are well characterized. However, the cell were difficult to harvest from basal turns of the cochlea and hard to get them in great number and the cells usually survived for only 30 or 40 min after preparation.

With the whole cell patch clamp technique we recorded outward and inward K^+ currents on freshly isolated OHCs of the guinea pig cochlea. The suppression of the outward currents by compounds 4-AP (Baker et al. 1993; Nenov et al. 1997), $CdCl_2$ (Nenov et al. 1997) and TEA (Housley and Ashmore 1992) in our experiments demonstrated that these currents belonged to $I_{k(dr)}$. The inward current blocked by TEA (data not shown in Paper I) (Hadley et al. 2000), and linopirdine (Marcotti and Kros 1999) were inferred as $I_{K,n}$.

$HgCl_2$ at 1.0–100 μM blocked $I_{k(dr)}$ and $I_{K,n}$ in a voltage- and dose-dependent manner and were more pronounced on fast onset current than on steady-state current. Although the effect of $HgCl_2$ at 1.0 μM was partially washed out over several minutes, the effects at 10 and 100 μM were irreversible to washout. The resting membrane potential of OHCs were also shifted to a positive depolarized level by $HgCl_2$.

Mechanism 1: Permanent oxidization of the free sulfhydryl group of the voltage-gated K^+ channels at high concentrations

$HgCl_2$ quickly reduced the $I_{K,n}$ currents in Paper I which accorded well with previous gating studies. Like most metals, Hg^{2+} can form a sulfur-Hg-sulfur complex (Ballatori 1991; Kerper et al. 1992; Mokrzan et al. 1995; Kajiwara et al. 1996) by reversibly oxidizing the free sulfhydryl to disulfide (S-S) groups of proteins (Koivisto et al. 1993; Gyori et al. 1994; Chiamvimonvat et al. 1995; Kurata et al. 1998). Sulfhydryl oxidation of the rat brain K_A channels expressed in oocytes deleted the function of fast inactivation of the channel (Ruppersberg et al. 1991). This loss of inactivation was shown to be due to the oxidation of a critical cysteine residue located near the ball domain. Sulfhydryl oxidation also produced a rapid and reversible closure of the ATP-regulated K^+ channel in pancreatic β cells (Islam et al. 1993). Further more, in a whole cell recording of voltage-gated Ca^{2+} currents from rat DRG neurons, 2 μM $HgCl_2$ irreversibly reduced the fast onset voltage-gated Ca^{2+} currents (Pekel et al. 1993) to a steady state in a rapid concentration-dependent manner. Its I-V relation was shifted to more positive values, suggesting a binding of Hg^{2+} to the channel protein and/or

modifying its gating properties. Similar results also were found in the Ca^{2+} release channel (Zaidi et al. 1989; Salama et al. 1992; Hare et al. 1993; Sarafian 1993; Chiamvimonvat, et al. 1995; Benitah et al. 1997). The transient $I_{K,n}$ current was quickly, completely and irreversibly abolished by $HgCl_2$ at concentrations of 10 - 100 μM, with the steady-state current (probably Kir) unchanged. As suggested by the above binding and gating experiments, $HgCl_2$ seemed to have a relatively high affinity to the binding site at the mouth of or in the $I_{K,n}$ channels by irreversibly oxidizing sulfhydryl groups. Similarly, the blocking effects of the gating mechanism can also be applied to $I_{k(dr)}$.

Mechanism 2: Mercurial oxidization of the free sulfhydryl group of voltage-gated K^+ channels at low concentrations can be temporary

Another clue for such a specific oxidizing action of Hg^{2+} is the reversal blocking effects of 1.0 μM $HgCl_2$ on OHCs K^+ channels. Unlike the 10 and 100 μM concentration where the effect was irreversible, superperfusion with normal solution substantially reversed the effects of the lower concentration of 1.0 μM $HgCl_2$, this may be an indication that the action of Hg^{2+} on OHCs was not due to an irreversible mechanism, instead, also by sulfhydryl oxidation. Similarly, many published data showed that the decreasing effects of hydrophilic sulfhydryl-oxidizing agent thimerosal could be readily reversed by 1,4-dithiothreitol (an agent that reduces disulfide bonds) on many different currents, such as Ca^{2-} channel, calcium-activated non-selective cation channel, ATP-regulated K^+ channel as well as Na^+ channel (Islam et al. 1993; Koivisto et al. 1993; Chiamvimonvat et al. 1995; Kurata et al. 1998). The reducing effect of external 1,4-dithiothreitol (Kurata et al. 1998; Hisatome et al. 2000) was an action directly on the external face of the channel (Branden and Tooze 1991; Thorn et al. 1992), which leads us to arrive at a similar explanation for low concentration of $HgCl_2$ in Paper I.

Mechanism 3: Cell membrane was depolarized by high concentrations

Unspecific binding of Hg^{2+} to the outside membrane surface might shift the resting membrane potential (Vr). The Vr values were range from –35 to –66 mV in ten cells before Hg^{2+} treatments. We observed that high concentration at 100 μM Hg^{2+} shifted 84% of the Vr positively, and this is apparently clear when comparing to the 40% shifting at 1 μM. This results are well in line with previous investigations (Shrivastav et al. 1976; Quandt et al. 1982; Kauppinen et al. 1989; Hare and Atchison 1992; Hille 1992). The changed positive charged electric field by the depolarized Vr probably resulted in an inappropriate status for $I_{K,n}$ gating. Unlike the normal Vr (-70 to – 100 mV) that is always opened at cell resting potentials, -10 to -30 mV or up will completely block the $I_{K,n}$'s activation. Depolarized Vr will not give enough driving force for $I_{K,n}$ to keep the Nernst equilibrium potential, E_k to the normal value, consequently the driving force for K^- entry through the apical MET (Housley and Ashmore 1992; Mammano and Ashmore 1996) will be lost.

Clinically observed long latency after exposure to MeHg could be explained by long time washout

High concentration of 10 μM Hg^{2+} used in paper I elicited a fundamental long effect on both outward and inward K^+ current. It is worthy to note that long time washout only partially restored the K^+ currents to their pretreatment values. This would suggest that blocking the deterioration of OHCs due to $HgCl_2$ poisoning does not occur immediately. This partly addresses why some patients from the poisoning cases in Iraqi (Bakir et al. 1973) and Minamata Bay (Tokuomi et al. 1982) needed a long latency to manifest phenotypic changes in electrophysiologic measures after exposure to MeHg.

$HgCl_2$-induced hearing loss

Mercurials in variant forms are harmful to all life now and in the past. In our studies, K^+ currents were recorded and confirmed in guinea pig OHCs by K^+ channel blockers of 4-AP, $CdCl_2$ and TEA. Bath-application of inorganic $HgCl_2$ remarkably blocked both outward and inward K^+ currents in a concentration-, voltage- and time-dependent manner, depolarized the membrane potential, hence, strongly indicated that Hg^{2+} can damage K^+ channels of the inner ear. The Ca^{2+} dependence of $I_{K,n}$ may permit many control mechanisms to alter the resting potential of OHCs in vivo. The functions of such current were suggested to hold the cell near the Nernst equilibrium potential, E_k, and therefore, maximize the driving force for K^+ entry through the apical MET (Housley and Ashmore 1992; Mammano and Ashmore 1996). As Hg^{2+} can damage not only outward $I_{k(dr)}$ but also inward $I_{K,n}$ as well as shift the resting membrane potential, OHCs will not be able to return the resting potential to normal, therefore, it cannot create the best driving force for K^+ recycling. OHCs will accumulate high K^+ concentration since the extrusion of the K^+ from $I_{K,n}$ is disrupted. Thus, Hg^{2+} will definitely result in acute or chronic deafness.

4.1.2 Lead acetate and K^+ channels

Albeit clinically it has been demonstrated that lead affects auditory function, our experiments using inorganic lead could not demonstrate any significant effects on the K^+ channels of OHCs.

4.2 M-LIKE POTASSIUM CURRENTS

4.2.1 M-like K^+ currents in guinea pig outer hair cells

M currents (I_M) were first recorded in 1980 from sympathetic cells (Brown and Adams, 1980). Later, I_M was found in the hippocampus as well as rod photoreceptors and olfactory cortex neurons (Constanti and Galvan, 1983; Beech and Barnes, 1989), i.e., in other sensory systems. Following that evidence and the notion that sensory cells also in the inner ear may exhibit M-type current, we set off on a search for M currents in the sensory epithelium of the hearing organ, initially focusing on the outer hair cells (OHCs).

The typical protocol for recording M currents involves holding the cell at -20 mV to -50 mV, which will inactivate most of the voltage-dependent potassium currents and pre-open the I_M (Adams et al., 1982; Lamas et al., 1997). Using a holding potential for the outer hair cells of – 20 mV made it possible to emphasize a current with characteristic M-like kinetics. Frequently, the I_M has a reversal potential of -60 mV to -80 mV, and a sigmoidal activation curve between -70 mV and -10 mV, with a half-maximal activation at -35 mV (e.g. Adams et al., 1982). A reversal potential of -75 mV and half-maximal activation around -40 mV were well in accordance with previous findings (Constanti and Brown, 1981; Adams et al. 1982; Constanti and Galvan, 1983; Beech and Barnes, 1989).

Linopirdine and its analog XE991, a "cognition enhancer" (Wang et al., 1998; Selyanko et al., 1999) and releaser of acetylcholine and other neurotransmitters (Kristufek et al., 1999), are the most characterized M channel blockers to date. $CdCl_2$ was used with the intent to remove the calcium-dependent components from the original recordings. After removing of this "calcium component" by $CdCl_2$, the remaining I_M should be further blocked by additional application of linopirdine.

Our observations of I_M in guinea pig OHCs, both original and, rather surprisingly, subtracted $CdCl_2$-sensitive currents, showed characteristics similar to what has been described for in other cell types (Brown and Adams, 1980; Constanti and Brown, 1981; Halliwell and Adams, 1982; Jones, 1987; Womble and Moises, 1992) under conditions of cell voltage-clamp at -20 mV, with the remaining inward currents unchanged and suggested to be the $I_{K,n}$ (Paper III). The activation curves and -75 mV reversal potential from both the native recording and subtracted $CdCl_2$-sensitive currents did indeed indicate the existence of an M-like, albeit somewhat atypical, potassium current in guinea pig outer hair cells.

4.2.2 Molecular evidence supporting I_M recordings: *Kcnq* gene expression in the cochlea

Gene expression profiles for neuronal Kcnq2–5 *subunits in the mammalian peripheral auditory system*

In Paper III, we showed a novel voltage-gated K^+ current in guinea pig OHCs with sub-threshold, time- and voltage-dependent characteristics matching that of M currents, I_M. The *Kcnq* gene super-family has five members, of which four may encode subunits, which underlie M channel assemblies. Both mono- and hetero-multimers of subunits *Kcnq2–5* are known to make up M channels in arrangements of four subunits per functional channel. The "neuronal" M channel subunit genes *Kcnq2–5* are all expressed in brain, except *Kcnq4*, which mainly appears in OHCs (Mammano and Ashmore 1996; Kubisch et al. 1999; Beisel et al. 2000; Kharkovets et al. 2000). In order to verify the I_M recorded in guinea pig OHCs, mRNA expression of *Kcnq1–5* was investigated by RT-PCR and DNA sequencing in the cochlea, and latter also in CNS regions, including the cochlear nuclei.

It was first shown, by RT-PCR, that there is cochlear expression of *Kcnq2* and *Kcnq3* genes, encoding the "classical" M channel subunits, in guinea pig cochlea (Paper III). We then expanded our investigation plan to include the entire *Kcnq* gene family, *Kcnq1–5*, in Paper IV. Here, we also included a brief look at *Kcnq* expression in rat cochlear nuclei (within the lateral wall of the brain stem). Cochlear *Kcnq* gene expression was confirmed; RT-PCR products were for detected *Kcnq1–5* in guinea pig and in rat. This was the first evidence for *Kcnq5* in guinea pig; also *Kcnq5* expression had not previously been described in rat cochlea.

Furthermore, in conjunction with immuno-localization of KCNQ2 and KCNQ3 protein in Paper V, the relative expression levels of *Kcnq2* and *Kcnq3* in micro-dissected cochlear subregions were assessed by expressed by semi-quantitative RT-PCR, indicating that *Kcnq2* and *Kcnq3* were more strongly expressed in modiolus (containing the spiral ganglion) than in the organ of Corti, and that *Kcnq2* was not expressed in the lateral wall (Paper V).

Our molecular investigation into the expression of *Kcnq* genes in the guinea pig cochlea not only confirmed published data of KCNQ mRNA expression in mouse and rat cochleas (Kubisch et al. 1999; Kharkovets et al. 2000), but we also showed that *Kcnq5* is expressed in guinea pig and rat cochlea. The expression of particularly *Kcnq2* and *Kcnq3* in the organ of Corti and spiral ganglion, but also *Kcnq5* and to some extent *Kcnq4* in the cochlea, as well as the presence of these subunit mRNAs in the cochlear nuclei, clearly indicates that I_M in the mammalian auditory periphery and central nervous system auditory pathways may be composed of KCNQ2-5 subunits.

Alternatively spliced Kcnq2 *isoforms contribute to* I_M *in the mammalian cochlea.*

In our expression profiling of *Kcnq1–5* in the mammalian cochlea, PCR products for *Kcnq2* did not consist of a single band, but instead of two bands, and these did not correspond to the expected amplicon size. However, the two PCR products for *Kcnq2* were identified by DNA sequencing to be shorter splice variants of *Kcnq2*, i.e. the cochlea does not express wild-type *Kcnq2*.

The *Kcnq2* gene may have up to 34 alternative exons, and has a long complex C-terminus that has at least 11 different forms of spliced variants found (Nakamura 1998; Tinel et al. 1998; Biervert and Steinlein 1999; Pan et al. 2001; Smith et al. 2001). The two sequenced cochlear *Kcnq2* transcripts were aligned other *Kcnq2* sequences in the public gene database and turned out to completely match (94% identity) *mKQT2.4* and *mKQT2.3* (Nakamura 1998) found in mouse brain. Both cochlear splice variants lack exon 8, while the shorter of the two transcripts also misses exon 12a. In addition, exon 15a was spliced out from both guinea pig cochlea KCNQ2 transcripts (the deduced amino acid sequences were tentatively named KCNQ2L1 and KCNQ2L2).

Exon 15a appears to be important in the function of the C-terminals of KCNQ2 subunits. Transcripts of exon 15a have been suggested to be involved in subunit recognition and assembly (Schmitt et al. 2000), as well as determination of activation and deactivation properties of the M channels (Pan et al. 2001). Heteromeric channels containing KCNQ2 including exon 15a and KCNQ3 subunits showed a much slower activation and deactivation (Pan et al. 2001). Lack of exon 15a transformed the channel

properties to faster kinetics. Also, a naturally occurring mutation in the KCNQ1 channel in this region changed the deactivation rate and shifted the steady-state activation properties (Chouabe et al. 1997). Slow deactivation of I_M will, basically, depress the neuronal firing probability for a longer time period following activation of the I_M (Pan et al. 2001). Both our cochlear KCNQ2L1 and KCNQ2L2 putative subunits were without exon 15a, and should thus exhibit faster I_M activation and deactivation, which would then confer rapid response and recovery of e.g. OHC or spiral ganglion excitability.

4.2.3 Localization of M channel subunits KCNQ2 and KCNQ3 by immunohistochemistry

Gene expression does not automatically translate into protein expression due to factors such as stability and degradation of mRNA, or post-translational modification and turnover of proteins. In order to confirm the existence of M channels composed of KCNQ subunits in the cochlea, and also pinpoint their cellular or sub-cellular location, the next logical step in our endeavor was to immuno-label the KCNQ channel proteins underlying I_M in the cochlea. KCNQ2 and KCNQ3 antibodies were chosen mainly because KCNQ2 and 3 appears to be the most common subunit combination as indicated by studies of KCNQ tissue co-expression (Cooper et al. 2000); also, co-expression of these two genes resulting in heteromeric subunit channels yield much higher currents *in vitro* (Schroeder et al. 1998; Wang et al. 1998; Yang et al. 1998; Main et al. 2000). Immuno-labeling for KCNQ2 and 3 (Paper V) was found to be located at several areas likely to be important for peripheral auditory function.

In guinea pig spiral ganglion, strong immuno-labeling for KCNQ2 was seen in the soma of all spiral ganglion neurons (SGNs), which differed somewhat in size, although most of the cells had centered and regular nuclei. These neurons belonged to either SGN type I or type II, however, in guinea pig there is no clear size or shape difference between these two cell types under light microscope (Spoendlin 1971). Type I spiral ganglion neurons are round, larger-sized cells, and with a centered nucleus in mouse and rat. In contrast, type II SGNs are clear, “small-size” neurons with an eccentric cell nucleus. Taken together, this indicated that SGN type I and II were stained by anti-KCNQ2 antibodies in rat, mouse, as well as guinea pig. Similarly, anti-KCNQ3 antibodies labeled SGN type I and II of mouse and rat cochleas, however with much weaker signal. In addition, KCNQ3 immuno-staining was evident in satellite cells surrounding SGNs.

In the organ of Corti, anti-KCNQ2 antibodies densely labeled the basal regions of inner and outer hair cells. Afferent nerve endings synapsing onto the OHCs appeared to be strongly stained, while the afferent nerve fiber was moderately labeled, in a pattern of an inner-to-outer row gradient. Interestingly, these afferent nerve extensions seem to have abundant branching between mid- and basal turns of the cochlea. KCNQ2 antibodies also labeled the nerve fibers after entering through *habenula perforata* (where auditory afferents lose their myelin sheath). In addition, KCNQ2 antibodies labeled cell bodies of IHCs and OHCs differently, with more pronounced staining in apical, and less in basal, cochlear turns. KCNQ immunostaining in the efferent system

was different from that of afferents. Only weak immuno-labeling was seen in efferent endings on the OHCs. KCNQ2 immuno-staining was not observed in efferent fibers at all outside of the tunnel of Corti. Furthermore, KCNQ2 seemed also to be localized, although at lower expression levels in most supporting cells (Böttcher's cells, border cells, inner sulcus cells, inner phalangeal cells), and without a discernible pattern. Anti-KCNQ3 antibodies stained the entire cell bodies of OHCs and IHCs, as indicated by immuno-fluorescence labeling. Neither afferent nor efferent nerves were labeled by the KCNQ3 antiserum. Pillar cells were stained strongly by both KCNQ2 and KCNQ3 antibodies in immuno-peroxidase and immuno-fluorescence experiments.

In summary, strong immuno-labeling was seen for both KCNQ2 and KCNQ3 in both types of spiral ganglion neuron. Various distribution patterns of KCNQ2/3 were seen in the hearing organ. KCNQ2 was mainly observed in the endings of afferent and efferent nerves to OHCs, and also distributed more to the outer row of the basal turn of the cochlea than that of the inner row of apical cochlea. OHCs and IHCs were labeled more strongly in the apical than in the basal turn. KCNQ3 was localized in OHCs and IHCs. This staining pattern led to the conclusion that SGN type I and II, inner (and outer) hair cells, as well as supporting cells should be further evaluated regarding the presence of M currents, also taking into consideration the localization of cells within the cochlea (apical-basal, medial-lateral).

In Paper III, we showed a novel K^+ current, the I_M, in guinea pig OHCs with a characteristic sub-threshold, time- and voltage-dependence. Papers III-V gave us further evidence in the form of molecular data that KCNQ2-5 mRNAs were expressed in both whole cochlea, and micro-dissected cochlear sub-regions: modiolus and organ of Corti (*Kcnq2*, *Kcnq3*), as well as lateral wall (*Kcnq3*). Immuno-staining with specific antisera recognizing KCNQ2 and KCNQ3, respectively, in the peripheral auditory nervous system and the sensory hair cells (Paper V) verified the existence of I_M recorded from guinea pig OHCs at the protein level.

4.2.4 Functional implications of cochlear *Kcnq* expression

The functional significance of the I_M will ultimately depend on the cell type in which it is expressed. In the central nervous system, I_M is crucial for determining cell excitability, maintaining the resting potential, setting the frequency and pattern of spike discharges (Adams et al. 1982; Wang and McKinnon 1995). In the peripheral sensory system, I_M can accelerate the voltage response to small photocurrents via setting the dark resting potential in rod photoreceptors, and exert a mild control over the passive membrane properties and repetitive firing behavior of the cells (Constanti and Galvan 1983; Beech and Barnes 1989).

We have identified expression of all *Kcnq* subunit mRNAs in cochlea, including two alternatively spliced forms of *Kcnq2*, both of which lack exon15a. Our data suggests that these are the only two KCNQ2 subunit isoforms available to assemble with KCNQ3 in guinea pig cochlea. Furthermore, the KCNQ2 immunostaining patterns in spiral ganglion type I neurons-inner hair cells and SGN type II-outer hair cells also suggest that KCNQ2/3 clearly are involved in auditory sensorineural function.

Mutations in either *Kcnq2* or *Kcnq3* genes results in reduced M channel function, such that excitatory ligand-gated and voltage-gated channels that are activated would remain open longer and lead to hyperexcitability, i.e. benign familial neonatal seizures (BFNS, a type of childhood epilepsy).

Kcnq4 is expressed in the guinea pig cochlea, in complete agreement with published data. Taken together with our finding of the expression of *Kcnq5*, here described for the first time (Paper IV), in the cochlea, it will be reasonable to include several other possibilities for subunit composition of cochlear M channels, i.e. KCNQ3/4, KCNQ3/5 may also contribute to the formation of M channels. It should be noted that different M channel assemblies containing KCNQ subunit splice variants and/or a selection of KCNQ subunits 2-5 will underlie different I_M properties in the cochlea, and that these currents may differ from "classical" KCNQ2/3-based M currents.

The functional role and pathological manifestations of M channels in the inner ear require further investigation. Together with other voltage- or Ca^{2+}-activated K^+ channels present, I_M will probably stabilize OHC membrane potential, and also influence its function manifested in the mechano-electrical and electromechanical properties underlying the cochlear amplifier. Furthermore, M channel activity may be regulated by a number of neurotransmitters or modulators, e.g. via muscarinic acetylcholine receptors. Thus, OHC excitability could be under control from the efferent nervous system via M channel activity.

In analogy with epilepsy resulting from KCNQ2 or 3 dysfunction, hyper-excitability in the auditory system, whether resulting from defective M channels or channels inhibited via neuromodulators, may underlie "uncontrolled" sensorineural activity, potentially underlying hyperacusis and tinnitus. Perhaps, here one could dare to speculate that neural influence on auditory excitability may be part of the explanation to mood- or stress-induced tinnitus.

Given that mutations in *Kcnq1* and *Kcnq4* genes are responsible for hearing disorders, Jervell and Lange-Nielsen syndrome and DFNA2, respectively, gene defects in other *Kcnq* genes expressed in the inner ear are likely to underlie disease conditions in the auditory periphery as well.

Obviously, much more work is needed in order to clarify the many remaining questions and issues regarding neuromodulation of cochlear M channels, their subunit composition and functional consequences of subunit heterogeneity, as well as physiological and pathological roles of these M channels in inner ear sensorineural cells and in hearing.

5 CONCLUSIONS

Voltage-gated potassium channels play essential roles throughout the body; in the nervous system, for example, these channels are intimately associated with maintenance of neuronal signaling. Obviously, sensory systems, and perhaps particularly the inner ear with its K^+-enriched endolymphatic fluids, also depend heavily on a number of potassium ion channels and transport proteins for normal, physiological function. Although much is known about various K^+ channels in the ear, several important issues have remained unanswered. This thesis work has advanced our knowledge in part of that unknown territory of cochlear K^+ channels, specifically:

- Several ion channels in the central nervous system are known to be targets for, and thus suppressed by, environmental pollutants that penetrate lipid membranes, such as many mercury- or lead-containing substances, some of which affect hearing. This prompted the investigation of whether the hearing loss associated with heavy metal poisoning had its origin in ototoxic mechanisms directed towards voltage-sensitive K^+ channels in sensory hair cells. Current findings suggest that while such an effect can be excluded for lead (Pb^{2+}), mercuric choride ($HgCl_2$) can indeed block both the inward and outward gating of OHC K^+ channels, and possibly mediate the ototoxicity leading to hearing loss from Hg^{2+} exposure.
- M currents assembled from KCNQ2 and KCNQ3 subunits play crucial roles in maintenance of resting potential and regulation of neuronal excitability, as evidenced by the epileptic seizures resulting from mutations in the underlying genes. *Kcnq1* and *Kcnq4* had previously been associated with inherited deafness disorders defects, but M currents had not been identified in the cochlea (as in other sensory peripheral systems, i.e., visual and olfactory). This study has indicated the presence of time- and voltage-dependent K^+ currents with M-like characteristics in guinea pig OHCs.
- The recorded M currents have now been verified; expression of *Kcnq2-5* genes was detected in cochlear preparations, with the novel *Kcnq5* gene suggesting molecular heterogeneity of M channels in the inner ear. Furthermore, alternatively spliced forms of *Kcnq2*, lacking exon 15a, provide additional functional diversity and confer faster kinetics to cochlear M currents (perhaps adapting them to an environment of high frequency operation?). Finally, M channels subunits KCNQ2 and 3 have been immunolocalized in the cochlea, primarily labeling sensorineural elements that take part in the transduction of acoustic stimuli to electric signals conveying sound information to the brain, i.e. sensory hair cells and spiral ganglion neurons. Taken together, the electrophysiological, molecular and immunohistochemical findings provide strong support for M channels in the cochlea and suggest important roles in normal hearing and potential involvement in auditory pathologies related to hyper-excitability, e.g. hyperacusis and tinnitus.

6 ACKNOWLEDGEMENTS

The present study was performed at the Center for Hearing and Communication Research and the Department of Clinical Neuroscience, Karolinska Institutet and Karolinska University Hospital, Stockholm. I sincerely thank all the persons who helped and supported my studies in the past years, especially:

Professor Mats Ulfendahl, my supervisor, thank you very much for your earnest tuition I have got during all these years, for your kind enthusiasm to guide me through this work, for sharing your extensive knowledge in not only neuroscience but also medical research. Your encouragement, made my life so memorable and enjoyable in and out of the laboratory. Your friendship and honesty will stay with me in my future.

Dr. Leif Järlebark, my co-supervisor, grateful thanks to you for sharing your molecular biological knowledge and the general communications over different fields as well as thesis writing, paper work, and many other things.

Professor Bo Rydqvist, Dept. of Physiology and Pharmacology, my second co-supervisor, for invaluable input and comments on electrophysiology.

Professor Ernest J. Moore, Dept. of Molecular Pharmacology & Biological Chemistry, Northwestern University, Chicago, thank you very much for your grateful tuition in patch-clamp, molecular knowledge, for your kindness of taking care of my studies and life.

Dr. Anders Fridberger, thank you very much for your careful teaching in neuroscience course as well as for sharing your extensive scientific knowledge of electrophysiology.

Dr. Jacques Boutet de Monvel, for your computer support and friendship.

Zhe Jin, my good friend and nice cooperator, for your wonderful thinking of molecular biology and the general discussions over different fields.

Dr. Maoli Duan, for sharing your knowledge of many medical research fields.

Xilong Zhao, my friend at the Dept. of Molecular Pharmacology & Biological Chemistry, Northwestern University, Chicago, for sharing your broad knowledge in neuroscience, the medical research. I will cherish your encouragement, and your friendship in the future because your suggestions were such nice to my situation in the lab and in my daily life.

Professor Jay Yeh, Professor, Ph.D., my friend at the Dept. of Molecular Pharmacology & Biological Chemistry, Northwestern University, Chicago, grateful thanks for sharing your extensive communications in science and the medical lab work.

Anette Fransson and Paula Mannström, great thanks for your support, your friendship and honesty that made my staying in Stockholm funny and enjoyable.

Professor Åke Flock, thank you very much for your scientific communications and thinking.

Professor Malou Hultcrantz, for helpful discussions in scientific subjects.

Professor Allen Counter, Harvard University, for helpful discussions over broad fields.

Åsa Skjönsberg, for your kind help over science and life.

Dr. Ann-Christin Johnson, for sharing your scientific input and your encouragement in daily life

Louise von Essen, for administrative support.

Jian Li, my friend at the Dept. of Orthopedics, Karolinska University Hospital, Solna, for helpful discussion in general science.

Nailin Li, my friend at the Dept. of Clinical Pharmacology, Karolinska University Hospital, Solna, your friendship make my life colorful.

Zhiqing Xu, my friend at the Dept. of Neuroscience, Karolinska Institutet, for helpful discussion in electrophysiology.

This work was supported by grants from the Swedish Research Council, the Foundation Tysta Skolan, the Swedish Association for the Hearing Impaired (The Hearing Foundation), Wenner-Grenska Samfundet, the Petrus and Augusta Hedlund Foundation, the Swedish Council for Working Life and Social Research, the Magnus Bergvall Foundation, Åke Wiberg Foundation, and the Northwestern Drug Discovery Program.

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