Chapter 4

Kinetics of potassium current $I_{K,neo}$ in neonatal mouse outer hair cells
ABSTRACT

In the first week after birth mechanosensory hair cells from the inner ear express different channels compared to mature cochlear hair cells. The major current during this period is carried by potassium via a delayed rectifier-like current termed $I_{K,neo}$. We investigated the kinetics of activation and inactivation of this current. For this purpose we used the whole-cell voltage-clamp technique in acutely isolated outer hair cells of newly born mice to measure $I_{K,neo}$ currents in response to membrane potential steps and describe the current activation using a Hodgkin-Huxley model. The current activation contains indications of two underlying mechanisms. Also the partial inactivation is shown to consist of two voltage-dependent as well as two time-dependent components. We therefore conclude that $I_{K,neo}$ is brought about by more than one channel. The detailed kinetic description can help to clarify the function of $I_{K,neo}$ in early postnatal development.
INTRODUCTION
The mammalian cochlea contains four rows of sensory hair cells consisting of two populations: one row of inner hair cells (IHC) and 3 rows of outer hair cells (OHC). As evidenced by the existence of transducer currents, the hair bundles already contain mechanotransducer channels at birth (unpublished observations T.D.; Géléoc and Holt, 2003) even though the hair cells are still under development and hearing does not start until postnatal day 12 (Ehret, 1983). Close to the onset of hearing, the ion channel expression in the basolateral membrane changes, turning the hair cells into high frequency mechanoreceptors with graded receptor potentials in response to mechanical disturbances to the hair bundle.

Not much is known about the exact types of potassium channels expressed in IHCs and OHCs during the period just after birth. Both populations of sensory cells possess a current with delayed rectifier characteristics, termed $I_{K,neo}$ (Kros et al., 1998), which is the dominating conductance in the basolateral membrane (Marcotti and Kros, 1999; Marcotti et al., 2003). Despite their similar outward potassium currents, differences exist in the excitability between the two populations of hair cells. Under experimental conditions, IHC for instance show spontaneous spiking behaviour (Kros et al., 1998), whereas OHCs need a current injection to give rise to an action potential (Marcotti and Kros, 1999).

The kinetic characteristics of the currents and related channels possibly underlying the differences in excitatory behaviour have not yet been described in great detail. In this study we obtained data about the activation and inactivation kinetics of $I_{K,neo}$ in OHC. Based on this information we conclude that $I_{K,neo}$ is brought about by at least two channel types.

METHODS
Preparation
OHC recordings were made from acutely isolated organs of Corti from SE mice (Harlan, The Netherlands) ageing between one to six days after birth (P0-P6 where P0 is the day of birth). Mouse pups were decapitated, after which the head was sagitally cut in halves. Both sides were placed in extracellular solution on ice, containing (in mM): 135 NaCl, 0.7 NaH$_2$PO$_4$, 5.8 KCl, 1.3 CaCl$_2$, 0.9 MgCl$_2$, 2 NaPyruvate, 5.6 D-Glucose, 10 HEPES-NaOH, pH 7.4, vitamins and amino acids.
were added from concentrates. The cochlea was subsequently isolated and transferred in fresh extracellular solution. Using fine forceps the bone was gently removed. Scala tympani, -vestibuli and the stria vascularis were carefully removed to isolate the organ of Corti, which was then transferred to an experimental recording chamber and placed under a nylon grid stretched over a stainless steel ring to mechanically stabilise the tissue. The remnants of the Reisner membrane were taken off or bent underneath the nylon grid. The tectorial membrane was partially removed using a suction pipette.

The tissue was continuously perfused (~30 ml/h) with extracellular solution at room temperature so that the preparation could be used for several hours. The hair cells were visualised using an upright transmitted-light microscope (Axiotron, Zeiss, Germany), which was modified into a fixed stage microscope, using a Zeiss ACM focussing unit for the translation of the objective (40x water immersion, NA 0.8) along the optical axis. The visualisation of the preparation was enhanced by Nomarski differential interference contrast optics. The hair bundles on the apical side of the cells could be clearly visualised while individual stereocilia of the inner hair cell bundles could be distinguished in many cases. The compact appearance of the bundle and the absence of blister-like spots in the cell body were used as criteria to judge the quality of the preparation.

**Electrophysiology**

The basolateral membrane of an OHC was exposed and cleaned using a suction pipette (tip diameter 4-6 µm) carefully removing neighbouring cells. Suction forces were kept minimal in order not to compromise the integrity of the hair cell and its bundle. Recording electrodes were pulled on a Sutter P97 (Sutter Instruments, CA, USA) electrode puller from soda glass capillaries (Harvard Apparatus Ltd, Edenbridge, UK), resulting in tip resistances of about 2.5-3.5 MΩ in extracellular solution. The shank of the electrodes was coated with a layer of surf wax (Mr Zoggs SexWax, CA, USA) to reduce the electrode capacitance. The pipette solution contained (in mM): 145 KCl, 3 MgCl₂, 5 Na₂ATP, 1 K₂EGTA, 5 HEPES-KOH, pH 7.3. Total membrane currents were recorded under voltage clamp at room temperature in the whole-cell tight-seal configuration using an Axopatch 200B amplifier (Axon Instruments Inc., CA, USA). Only OHCs from the basal and basal-middle coil were used in this study. The holding potential was -84 mV, including a correction for the junction potential of -4 mV. Step changes in membrane potential were induced by the D/A output of a CED Power 1401 (CED, Cambridge, UK) under computer control via the external command input (1:50) of
the amplifier. Protocols were designed using the Signal software package (CED, Cambridge, UK). The amplifier output was low-pass filtered at 5 kHz (8-pole Bessel, Krohn-Hite, MA, USA) and digitised at 20 kHz using the 16 bit A/D converter of the CED Power 1401 and stored on hard disc.

Off-line analysis was performed using the Signal software package. Recordings were corrected for linear leak, which was estimated from the change in current elicited by a 10 mV step in the holding potential. Membrane potentials and currents were corrected for the residual series resistance \( R_s \) after compensation \( (R_s = 2.2 \pm 1.0 \text{ M}\Omega, \text{ compensation ranging from 40-80%}) \). Holding current is always displayed as zero current. Fitting procedures and final plots were performed using Microcal Origin 6.0.

RESULTS

Fig. 1A shows representative OHC currents in response to incrementing steps in membrane potential measured in the whole-cell patch-clamp configuration. Activation is slow, with an initial delay, and is characterised by an increased rate of activation upon increasing depolarisation. At the more depolarised membrane potentials a slow and incomplete inactivation can be observed, which is most
significant at the largest depolarisations (> 0 mV). The reversal potential of this current (ranging between -54 and -68 mV) indicates that this current is carried mainly by potassium, which has a theoretical equilibrium potential ($E_K$) of -83 mV at 23 °C. The I/V relationship (Fig. 1B) shows that activation starts close to -60 mV. This delayed rectifier-type K$^+$ current has been previously named $I_{K,neo}$ (Kros, 1998), and has been shown to be the dominating current in apical OHCs during the first week after birth (Marcotti and Kros, 1999).

**Kinetics of $I_{K,neo}$**

**Activation**

To minimise the distorting effects of inactivation, the voltage dependence of activation is expressed as the conductance at the maximal current response. Maximal currents were determined in each trace (black dots in Fig. 2A) and normalised peak conductance is then calculated by dividing the maximal current by the driving force, where the driving force is defined as the applied membrane potential subtracted by the theoretical value for $E_K$, -83 mV obtained using the Nernst equation. The normalised peak conductance was plotted as a function of the applied membrane potential, $V_m$ (Fig. 2B), and fitted by a first-order Boltzmann function:
\[
\frac{G}{G_{\text{max}}} = \left(1 + \exp\left(\frac{V_h - V_m}{S}\right)\right)^{-1},
\]

where \(V_h\) is the potential of half-maximal activation and \(S\) is the slope factor.

Fitting Eq. 1 to the normalised peak conductance yields an average value of \(V_h = -16 \pm 5\) mV and \(S = 10 \pm 2\) mV \((n = 9)\).

The time dependence of activation was analysed by describing the onset of the current traces with the Hodgkin-Huxley model (Hodgkin and Huxley, 1952; Hille, 1992). The activating current traces up to the peak current are then described by the equation:

\[
I(t) = I_\infty \left(1 - \exp\left(-t/\tau_{\text{act}}\right)\right)^N,
\]

where \(I_\infty\) is the steady state current, \(\tau_{\text{act}}\) is the time constant of activation, which is voltage dependent, and \(N\) is the number of gates needed to open the channel, which is chosen to be an integer number. Fig. 3A shows the first 25 ms of the activated currents in response to different membrane potentials. Eq. 2 was used to describe the activation of the currents. In cases where the peak was reached within these 25 ms, the range of the fit was limited up to the peak of the current. \(I_{K,\text{neo}}\) currents can be described using a model with three gates \((N = 3, \text{solid lines})\) with voltage dependent values for \(\tau_{\text{act}}\). The model quite well describes the current kinetics except

**Figure 3: Activation kinetics of \(I_{K,\text{neo}}\)** (A) Current recordings in response to membrane potential steps ranging from -34 to 61 mV (grey) following a holding potential of -84 mV. Solid lines show fits of Eq. 2 with \(N = 3\). (B) The resulting activation time constants, \(\tau_{\text{act}}\), as a function of membrane potential. Error bars indicate the standard deviation \((n = 7)\) in \(\tau_{\text{act}}\) and \(V_m\) (due to correction for \(R_s\)). The voltage dependence of \(\tau_{\text{act}}\) is described by: \(t_1 + t_2 \exp(-V_m/V_t)\), with \(t_1 = 2.2\) ms, \(t_2 = 2.9\) ms, \(V_t = 14.3\) mV.
for a small deviation during the beginning of the rising phase where the model lags behind the actual current while tending to underestimate the peak current. The 3-gate model, however, best describes the delayed onset. The voltage-dependence activation time constant, $\tau_{\text{act}}$, is shown in Fig. 3B as a function of membrane potential. Information on $\tau_{\text{act}}$ was only obtained at the membrane potentials leading to an activation of the $I_{K,\text{neo}}$. No measurements of the rate of deactivation have been done. The voltage dependence of $\tau_{\text{act}}$ is therefore fitted by an exponential function (see legend Fig. 3).

**Inactivation**

When applying depolarising membrane potentials during 150 ms, inactivation of the evoked responses, such as displayed in Fig. 1A, is only visible for the largest depolarisations. The 150 ms duration does not allow for the estimation of the extent of inactivation or its kinetic properties. To do so a double pulse protocol is used consisting of a 9 s conditioning step to induce (steady state) inactivation at membrane potentials ranging from -120 to 40 mV with 10 mV increments (Fig. 4A). To probe which part of the current has not been inactivated during the preceding conditioning pulse, it is followed by a 200 ms test pulse to +100 mV, forcing open all non-inactivated channels. Since the driving force is equal during each test pulse, the peak response is proportional to the number of non-inactivated channels. To allow the channels to return to the steady state at -84 mV, each recorded trace was followed by an 11 s period at this holding potential. The current traces during the conditioning pulses clearly show partial inactivation (70 ± 7% at 40 mV, $n = 10$), which nearly reaches a steady state at 9 s after the onset of the conditioning pulse. The response to the test pulse is more clearly visible in Fig. 4B (right panel). The most hyperpolarised conditioning pulse gives rise to a maximal response in the test pulse, since all channels have recovered from inactivation during the condition pulse at these negative potentials. In contrast, the test responses after a highly depolarised conditioning pulse show only an increased current due to the increased driving force during the test pulse. Peak currents during the test pulse are normalised to the maximal response and plotted against the membrane potential of the conditional pulse (Fig. 4C). The voltage-dependence of inactivation clearly shows two voltage dependent components and a non-inactivating voltage-independent component, and was therefore fitted by a summation of two Boltzmann curves and a constant:

$$
\frac{I}{I_{\text{max}}} = \gamma_1 \left( \frac{1}{1 + \exp \left( \frac{V_m - V_1}{S_1} \right)} \right) + \gamma_2 \left( \frac{1}{1 + \exp \left( \frac{V_m - V_2}{S_2} \right)} \right) + \gamma_3, \tag{3}
$$
where $I/I_{\text{max}}$ is the current during the test pulse normalised to the maximal current, $V_m$ is the membrane potential during the conditioning step, $V_1$ and $V_2$ are the voltages at which half of the channels are inactivated for each of the two inactivation components, $S_1$ and $S_2$ are the respective slope factors, $\gamma_1$ and $\gamma_2$ represent the contribution to the inactivation of both components and $\gamma_3$, which is equal to $1 - (\gamma_1 + \gamma_2)$, is the component that does not inactivate. At the holding potential ($V_h = -84 \text{ mV}$) nearly all channels are in the activatable state. However, at the resting membrane potential, ($V_r$, -53 mV), approximately 30% of the current is

**Figure 4: Steady state inactivation**

(A) Representative example of OHC membrane currents in response to a two pulse voltage protocol consisting of incrementing conditioning steps to membrane potentials ranging from -113 to 24 mV followed by a test pulse of 200 ms to 100 mV. (B) Details of the current responses given in A showing the activation during the first 200 ms of the conditioning step and the current response during the 200 ms test pulse starting at 9 s. The membrane potentials applied during the conditioning pulse are plotted next to the traces. (C) Inactivation curve consisting of the peak current to the test pulse normalised to the maximal test response ($I/I_{\text{max}}$, filled circles). The solid line is a fit by Eq. 3, $V_1 = -61.2 \text{ mV}$, $S_1 = 8.7 \text{ mV}$, $V_2 = -13.4 \text{ mV}$, $S_2 = 5.6 \text{ mV}$, $\gamma_1 = 0.40$, $\gamma_2 = 0.44$. For comparison the activation curve, obtained using the normalised peak conductance, is given ($G/G_{\text{max}}$, open circles), fitted by Eq. 1, $V_h = -9.4 \text{ mV}$, $S = 11.4 \text{ mV}$. Dotted line, $V_r$, indicates the resting membrane potential.
inactivated (Fig. 4C). 21.4 ± 9.5 % (n = 4) of the current does not inactivate ($\gamma_3$). The inactivating part of the current can be subdivided in a component of 35.5 ± 4.7 % ($\gamma_1$) with a half inactivation point at -60.5 ± 3.2 mV ($V_1$) and slope factor of 6.8 ± 2.8 ($S_1$) and a component of 44.8 ± 12.8 % ($\gamma_2$) with a half inactivation point at -17.8 ± 4.8 mV ($V_2$) and slope factor 7.1 ± 4.7 ($S_2$) (all $n = 4$).

The rate of inactivation was measured by exponential fits to the decay phase of the current during the conditioning pulse (Fig. 5A). Similar to the two voltage dependent components describing the inactivation (Eq. 3), the time dependence has to be fit using two summed exponential decay functions, each with a decay time constant and a residual component that does not inactivate. The two time constants of the current decay (Fig. 5C) cluster around 450 ms (filled circles) and 4 s (open circles) and do not show a strong voltage dependence between -30 and 40 mV. The fast decaying component contributes 27.3 ± 3.6 %, the slow decaying component 49.0 ± 3.8 % and the non-inactivating component 23.9 ± 3.9 % (n = 4).

The rate of recovery from inactivation was measured at different hyperpolarised membrane potentials. The membrane was initially depolarised to +40 mV for 6 seconds to induce channel inactivation. The membrane was subsequently hyperpolarised for a duration that increased with each sweep to allow recovery of inactivation before a test pulse +40 mV was applied. Fig. 5B presents the superimposed traces showing the recovery in the subsequent test pulses. Peak responses to the test pulse as a function of the duration of recovery were used to acquire the rate of recovery. This time-dependence of recovery was described using a single exponential function and was found to be strongly dependent on membrane potential, becoming faster with increasing hyperpolarisation (Fig. 5C, triangles). Extrapolation of the exponential fit to the peak responses obtained after a maximal recovery period of 500 ms, did not result in a full recovery (data not shown). However, the 10 second interval between consecutive sweeps during which the membrane was held at the holding potential did allow for full recovery since peak responses during the conditioning pulses were equal in size. Although it was not experimentally tested this suggests that a second slower component is needed to completely describe the recovery.

DISCUSSION

In this study we investigated the outward potassium current of the baso-lateral membrane of the OHCs in neonatal mice, which has been previously termed $I_{K,neo}$ (Kros, 1998). It is the main conductance in the basolateral membrane during the
first week after birth and is characterised by a delayed onset and a voltage-dependent activation starting around -60 mV. It possesses the characteristics of a delayed rectifier type of channel. This outward conductance is also present in postnatal IHCs (Kros et al., 1998) up to about day 12 around the onset of hearing.
In OHCs $I_{\text{K,neo}}$ increases in current density during the first 5 days after birth (Marcotti and Kros, 1999). Around P8 $I_{\text{K,neo}}$ is down-regulated and the current becomes dominated by the linopirdine-sensitive $I_{\text{K,n}}$ (Marcotti and Kros, 1999).

In this study we attempt to further characterise the properties of $I_{\text{K,neo}}$ in postnatal OHC of mice by describing its activation and inactivation kinetics based on the Hodgkin-Huxley model with a fixed number of gates. In first approximation the activation kinetics of the current can be described by a model assuming three gates, which switch to the open conformational state before a conductance change occurs. The assumption of three gates most adequately describes the initial delay, but fails to accurately describe the kinetics during the rising phase. Several assumptions have to be made when using the HH model, which might cause the model to deviate from the measured current response. The HH model assumes fully independent gating particles, each being subjected to first-order kinetics, neglecting possibilities for co-operativity between the subunits. It also assumes one conductance level for an open channel, which need not necessarily be the case. Alternatively the channels underlying the $I_{\text{K,neo}}$ current could consist of different sub-units, which might give rise to different kinetics depending on their individual stoichiometry.

$I_{\text{K,neo}}$ is generated by more than one channel

Our data contains several indications suggesting that $I_{\text{K,neo}}$ consists of more than one channel type. The inactivation curve of $I_{\text{K,neo}}$ (Fig. 4C) is characterised by two membrane potential ranges causing partial inactivation. Moreover the rate of inactivation is characterised by a double exponential time course. If these properties were to be combined in one channel, this channel should have more than one conductance level, both of which should independently inactivate over different membrane potential regions. In IHC it has been shown that 4-AP blocks one part of the biphasic inactivation curve, removing the current that inactivates at the more hyperpolarised potentials (Marcotti et al., 2003).

In one of the OHCs the rate of inactivation could best be fit using a single-exponential function with a time constant of about 4 seconds. Likewise, the voltage dependence of inactivation also contained one component and was fit by a single Boltzmann function (Eq. 3, $\gamma_2 = 0$), which yielded the parameter values $V_1 = -66 \text{ mV}$ and $S_1 = 7.3 \text{ mV}$. These values match the ones obtained for the component inactivating at the more hyperpolarised potential in the other OHCs. It also had a similar extent of inactivation (78%), suggesting that the slow
inactivating component shows partial inactivation and the faster component is fully inactivating.

Also the measurements of the activation following the conditioning pulse in the inactivation protocol (Fig. 4B) clearly show two different activation kinetics depending on the extent of the preceding inactivation. After a depolarising conditioning pulse, which maximally inactivates the more hyperpolarised-inactivating component, the activation is very rapid. However, depolarising conditioning pulses in the range of the more hyperpolarised-inactivating component show a more gradual activation during the test pulse.

The fact that multiple aspects of the inactivating kinetics of $I_{K,neo}$ require two parameters suggests that at least two channels underlie this current, both of which have a similar activation curve (Fig. 2), but different activation kinetics. To test the possibility of two channels, the rate of activation was re-fitted using a model consisting of two channels one with two and one with four gates:

$$I(t) = I_\infty \left[ \alpha \left(1 - e^{-\frac{t}{\tau_2}} \right)^2 + (1 - \alpha) \left(1 - e^{-\frac{t}{\tau_4}} \right)^4 \right],$$

where $t$ is time, $I_\infty$ is the steady-state current, $\tau_2$ and $\tau_4$ are the activation time constant of the channels with 2 and 4 gates, respectively, and $\alpha$ is the relative contribution of the 2-gate channel to the current. Re-fitting the data results in an improved fit of the current activation, which more accurately describes the delay and the rising phase and the peak response (Fig. 6). The fit results in an almost equal contribution of both channels. The fit with an increased number of parameters suggests that rather two channels instead of one underlie the $I_{K,neo}$ current. An assumption has however to be made on the number of gating elements in each channel. It can therefore not rule out alternative combinations of gating elements per channel.

It is interesting to consider how these components could be resolved. In a whole cell configuration it might be possible to separate them based on their different inactivating properties. Voltage clamping the cells to -30 mV for a prolonged period should maximally inactivate the slow-inactivating component of $I_{K,neo}$. However, the partial desensitisation leaves about 20% of this component not inactivated. Applying voltage steps incrementing from -30 mV should then mainly evoke activation of the fast-inactivating component, which starts inactivating from about -30 mV and higher.

To obtain more decisive evidence on the contribution of different components
to $I_{K,neo}$ and their individual rates of activation, inactivation and recovery, a single channel approach may be needed. Alternatively it might be possible to separate the two components based on differential pharmacology. A capsaicin-sensitive current, $I_{K,c}$, described in hair cells of the frog *crista ampullaris* (Marcotti and Kros, 1999) shows remarkably similar characteristics with respect to its activation kinetics and voltage dependence of activation, as well as its inactivation, exhibiting a double exponential time course with identical time constants. In contrast, the voltage dependence of inactivation is described by a single Boltzmann curve and nearly matches the voltage of the slow-inactivating component in OHCs. To test this, some preliminary attempts were made to investigate whether the same sensitivity was found in OHCs. We found however, that capsaicin had no clear effect on $I_{K,neo}$ (data not shown).

**Functional significance of the potassium current**

The neonatal OHCs used for this study already show mechano-electrical transducer currents at the day of birth (unpublished observations) and possibly even prior to birth, showing that the mechanotransducer channel is present well before the actual onset of hearing at about P12. The basolateral potassium current on the other hand clearly shows development and maturation during the first two weeks of life (Kros *et al.*, 1998; Marcotti and Kros, 1999; Marcotti *et al.*, 2003), with the general characteristic that close to the onset of hearing potassium current activation is speeded up and shifted towards more hyperpolarised membrane potentials. This
results in an increased resting conductivity, which contributes to a decrease in the membrane time constant. It also changes the hair cell response into a receptor potential rather than a (spontaneous) action potential.

In OHCs the change in channel expression also correlates with the onset of electromotility (Marcotti and Kros, 1999; Dallos and Fakler, 2002). Electromotility is thought to be caused by voltage-activated conformational changes in a membrane associated protein called prestin (Liberman et al., 2002). There is no reason to presume that electromotility would not be operational in the presence of $I_{K_{neo}}$ and a correlation of electromotility with up-regulation of $I_{K_{n}}$ might be non-causal.

A second process correlating with the time of $I_{K_{neo}}$ down-regulation is the change in ionic content of the endolymph giving rise to the endocochlear potential (EP). In mice the development of the EP takes place during P6-14 (Fernandez and Schmidt, 1963; Steel and Barkway, 1989; Sadanaga and Morimitsu, 1995). The endolymphatic concentrations of monovalent ions have been described to reach adult concentrations at P7 before the full development of the EP (Yamasaki et al., 2000). As a result of these ionic changes an increased driving force for cations (especially $K^+$) develops across the transducer channel. $I_{K_{neo}}$ would not be a suitable channel to expel the increased $K^+$ entry for its relative high activation potential and its inactivating properties. The gradual changes in OHC potassium conductance as described by Marcotti et al. (1999) nicely match the temporal development of the EP.

It is also very likely that the excitability of the neonatal hair cell membrane is used to strengthen auditory pathways during early development, as is suggested for IHCs that show spontaneous spiking under experimental conditions. However, OHCs only show spiking behaviour after a small current injection, and also show far less afferent contacts.

The present study investigated elementary kinetic aspects of $I_{K_{neo}}$. To fully understand the function of $I_{K_{neo}}$ in a still maturing cochlear environment more information needs to be obtained. Besides a single channel/pharmacological approach to segregate the underlying channels, their relative contribution to $I_{K_{neo}}$ along the basilar membrane might give insight to a possible role in the frequency distribution. The kinetic information can further be used to model the interplay between $I_{K_{neo}}$ and other ligand- and voltage-gated ion channels present in the basolateral membrane.