Membrane Properties of Chick Semicircular Canal Hair Cells In Situ During Embryonic Development

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Masetto, S., P. Perin, A. Malusà, G. Zucca, and P. Valli. Membrane properties of chick semicircular canal hair cells in situ during embryonic development. J. Neurophysiol. 83: 2740–2756, 2000: The electrophysiological properties of developing vestibular hair cells have been investigated in a chick crista slice preparation, from embryonic day 10 (E10) to E21 (when hatching would occur). Patch-clamp whole-cell experiments showed that different types of ion channels are sequentially expressed during development. An inward Ca2+ current and a slow outward rectifying K+ current (Ih) are acquired first, at or before E10, followed by a rapid transient K+ current (IK,trans) at E12, and by a small Ca-dependent K+ current (IKCa) at E14. Hair cell maturation then proceeds with the expression of hyperpolarization-activated currents: a slow Ih appears first, around E16, followed by the fast inward rectifier IKg around E19. From the time of its first appearance, IKCa is preferentially expressed in peripheral (zone 1) hair cells, whereas inward rectifying currents are preferentially expressed in intermediate (zone 2) and central (zone 3) hair cells. Each conductance conferred distinctive properties on hair cell voltage response. Starting from E15, some hair cells, preferentially located at the intermediate region, showed the amphotera shape typical of type I hair cells. From E17 (a time when the afferent calyx is completed) these cells expressed IK,1, the signature current of mature type I hair cells. Close to hatching, hair cell complements and regional organization of ion currents appeared similar to those reported for the mature avian crista. By the progressive acquisition of different types of inward and outward rectifying currents, hair cell repolarization after both positive- and negative-current injections is greatly strengthened and speeded up.

INTRODUCTION

Hair cells are the mechanoreceptor cells of the inner ear. Mechanical stimuli change the amount of current flowing through mechanotransduction channels located on hair cells’ stereocilia (Hudspeth 1989), thus generating a receptor potential whose final shape also depends on the interplay of different types of basolateral ion channels, the latter ones of which vary among hair cells from different inner ear organs, as well as from different regions of the same sensory epithelium (Art and Fettiplace 1987; Fuchs 1992; Masetto et al. 1994; Murrow 1994; Weng and Correia 1999).

Although the electrophysiological properties of fully developed hair cells have received considerable attention in the last two decades, far less is known about their ontogeny. The available data suggest that different types of ion channels are progressively acquired by developing hair cells. In the chick cochlea, as early as E10 (embryonic day 10), hair cells located at the basal or at the apical extremes express a slow or a fast outward K+ current, respectively (Griguier and Fuchs 1996). The later acquisition (E19) of a Ca-activated K+ current occurs at a time when auditory function begins to mature rapidly (Fuchs and Sokolowski 1990). In the mammalian cochlea, inner hair cells acquire only postnatally a fast K+ current, which turns them from spiking pacemakers into high-frequency signal transducers (Kros et al. 1998).

As far as vestibular organs are concerned, mouse utricular hair cells acquire IK,1 (the signature current of type I hair cells) (Correia and Lang 1990; Eaton et al. 1994; Rennie and Correia 1994), and the slow inward rectifying Ih a few days after birth (Rüscher et al. 1998). By contrast, no data are available about the electrophysiological properties of vestibular hair cells during in vivo prenatal development, either in avians or in mammals. We aimed at filling this gap, at least in part, by investigating the electrophysiological properties of avian vestibular hair cells as a function of development between E10 and E21. The chick has been chosen, because a large literature database is available regarding other aspects of inner ear development for this animal.

The crista slice preparation used here is a very suitable preparation for the proposed goal because it allows one to correlate electrophysiological data with cell location and morphology.

METHODS

Embryo dissection

Fertilized chicken eggs of the Cobb variety were obtained by a local supplier and incubated at 38.3°C. Although hatching occurred at embryonic day 21 (E21), when embryos were staged according to the criteria of Hamburger and Hamilton (1951), we observed that development consistently lagged behind this standard atlas up to E19; this is presumably the result of the higher incubation temperature (39.4°C) used by Hamburger and Hamilton in the first 9 days of incubation. Table 1 shows the relationship that we observed between stages of development and days of incubation.

Once removed from the eggs, embryos were decapitated and semicircular canals dissected out. The bone surrounding the ampullae was removed in embryos older than E14. The ampullae were incubated in Dulbecco’s modified Eagle’s medium (DMEM; Catalog number 31600-026, Gibco BRL, Life Technologies) to which was added 1.5% newborn calf serum (Catalog number N-4637, Sigma, St. Louis, MO), 24 mM NaHCO3, 15 mM piperazine- N,N′-bis(2-ethanesulfonic acid) (PIPES; Sigma), buffered at pH 7.4 with NaOH, and carbonated (95% O2, 5% CO2) in a humidity-saturated chamber at 37°C. After an incubation period of 2–6 h, the organ was removed from the culture medium and embedded in 4% agar wt/vol (Sigma) in a slicing solution containing (in mM): 145 NaCl, 3 KCl, 15 N-2-hydroxyeth-
vibratome (Campden, UK). Slice thickness varied between 150 and immersed in the slicing solution (partially frozen) and cut with a R
voltage protocols in figures. All calculated data in this study and figure (TEA)Cl, 2 4-aminopyridine, 5 BaCl2, 3 KCl, 15 HEPES, 5 CsCl, 0.6 once the whole-cell configuration was achieved, were perfused with recordings, slices were kept in the standard extracellular solution and,
through Ca2+ channels. Averaging of three to five sweeps was used to improve the signal-to-noise ratio for currents through Ca2+ channels.

Current traces were not corrected for residual capacitive artifacts. Voltages were not corrected for the liquid junction potential with the K-based intracellular solution (3 mV negative inside the pipette), but were corrected for the liquid junction potential (5 mV) with the NMDG-based intracellular solution. For current-clamp traces the true stimulus pipette current is shown as current protocol, because patch-clamp amplifiers in current-clamp mode can introduce artifacts evident as distorted stimulus currents (Magistretti et al. 1996; Masetto et al. 1999).

Test solutions were applied locally through a gravity-fed multibarrelled micropipette. Recordings were made at room temperature (22–24°C).

Morphological criteria
Recordings were made from hair cells in selected regions or zones of the neuroepithelium of all three (posterior, horizontal, and anterior) semicircular canals. Figure 1A shows microphotographs of an E12 vertical and an E20 horizontal canal crista slice from chick embryos. To maintain consistent nomenclature, we named the zones in accordance with the pigeon crista (Masetto and Correia 1997) and bullfrog crista (Myers and Lewis 1990) regional subdivision on the basis of differences in components of ionic currents, hair bundle morphology, hair cell density, and primary afferent arborizations. Given that the crista increased in size during embryo development, it was not possible to give a fixed size for each zone. The distinct zones were therefore defined as follows: in the dumbbell-shaped vertical canal crista (Fig. 1B, left), zone 1 is the most peripheral region of the sensory epithelium, contacting the planum semilunatum (PS), and extending for one third of segment S1; zone 3 is the most central region, extending for one third of segment S2 on either slope of the crista apex; zone 2 is an intermediate region, extending for one third of both segments S1 and S2 (see Landolt et al. 1975 or Weisleder and Rubel 1993 for scanning electron microscopic pictures of the avian crista). Because the horizontal crista (Fig. 1B, right) resembles one of the symmetrical sides of the vertical crista, it was similarly divided into zones 1, 2, and 3 starting from the PS.

Analysis
Analysis of traces and results were performed with Clampfit (pClamp version 6.0.3, Axon Instruments), Microcal Origin (version

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>10</th>
<th>11</th>
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<th>15</th>
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<td>36+</td>
<td>38</td>
<td>39̅−</td>
<td>39+</td>
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Data from Hamburger and Hamilton (1951).

Borosilicate glass pipettes (Drummond Scientific, Broomall, PA) were pulled to tip diameters between 0.5 and 1.0 μm, fire-polished, and partially coated with Sylgard (Dow Corning 184, Midland, MI). The micropipettes were filled with intracellular solutions of different compositions: for total current (in mM): 134 KCl, 2 MgCl2, 10 HEPES, 1 CaCl2, and 11 EGTA; pH 7.4 with KOH; for currents through Ca2+ channels (in mM): 140 N-methyl-D-glucamine (NMDG), 2 MgCl2, 10 HEPES, 1 CaCl2, and 11 EGTA; pH 7.4 with HCl. Adenosine 5‘-triphosphate (ATP; 1 mM) was freshly added each day of the experiment to both intracellular solutions. Micropipettes had a resistance in the bath of 2–3 MΩ when filled with the K-based intracellular solution. The patch-clamp amplifier was a List L/M-EPIC-7 (Germany). Series resistance (Rs) and cell-membrane capacitance were read in voltage-clamp mode directly from the amplifier’s compensation dials. After electronic compensation by the amplifier, residual series resistance was between 1 and 11 MΩ [mean value = 4.2 ± 2.3 (SE) MΩ; n = 152]. Nominal voltages are shown for voltage protocols in figures. All calculated data in this study and figure diagrams have been corrected for voltage drop across residual Rs, unless otherwise stated. The amplifier’s filter bandwidth was set at 3 kHz. Digital sampling frequency of voltage- and current-clamp protocols was at least 3 times the analogue bandwidth of the signal recorded. Current and voltage were measured and controlled through a DigiData 1200 interface (AD/DA converter; Axon Instruments, Foster City, CA) connected to a personal computer (Pentium PC) running pClamp software (version 6.0.3, Axon Instruments). Resting membrane potential with the K-based intracellular solution was measured as the zero-current voltage in current-clamp mode (Vh), by averaging seven measurements taken at 10-s intervals. Leakage current was measured as the current in response to 250-ms, 10-mV hyperpolarization from a holding potential of −60 mV (average of 7 measurements). In hair cells without inward rectifying currents (see RESULTS), or where they were blocked pharmacologically (Ba2+ extracellular solution), current measurements in the above-mentioned voltage range reflected only passive properties of the hair cell membrane and seal leakage, because other currents activated above −60 mV. This limited the use of leakage corrections to type II hair cells as noted in figure legends. No leakage correction was performed when Ileak was present (presumptive type I hair cells). For minimizing contamination by residual unblocked outward current and leakage current, Ba2+ currents through Ca2+ channels were sometimes isolated by subtracting currents recorded in the presence of 200 μM CdCl2, which blocked all Ca2+ channels. Averaging of three to five sweeps was used to improve the signal-to-noise ratio for currents through Ca2+ channels.

Electrical recordings
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FIG. 1. Slices of chick embryo semicircular canals. A: differential interference contrast light photomicrographs of a longitudinal slice of the posterior ampullary crista from an E12 embryo (left) and of the horizontal ampullary crista from an E20 embryo (right). The nylon mesh used to immobilize the slices is partially visible. Calibration bar = 100 μm. B: schematic representations of a vertical (left) and a horizontal (right) crista slice, showing zones 1, 2, and 3 as used for patch-clamp experiments. Top: slices of a vertical and a horizontal canal crista of an E15 chick embryo. The regions of the crista indicated in solid white were not employed for patching to avoid hair cell misclassification. S1 and S2 are indicated as described in METHODS. Bottom: superior view of the cristae depicting ideal slice orientation and zone limits. PS, planum semilunatum; EC, eminentia cruciata.
describes the inward current relaxation and reflects the transition from after the residual capacitive artifact.

\[
I = e^{t - \tau} + C
\]

where \( \tau \) is the inactivation/activation time constant; \( k \) (here and in Eqs. 2 and 3 below) is a nonzero delay for starting of fit. For \( I_{C(V)} \) (isolated with appropriate protocols, see RESULTS) and \( I_{K(V)} \) (when \( I_{K(A)} \) was absent) time-dependent inactivation, fitting was started when current amplitude had decayed to 90% of the peak value to avoid contribution from current activation. In those hair cells in which \( I_{K(A)} \) was present together with \( I_{K(V)} \), \( I_{K(A)} \) contributed to the peak value, and therefore fitting for the \( I_{K(V)} \) time-dependent inactivation was started at a similar point as for the \( I_{K(V)} \) present in isolation. In this way contribution from \( I_{K(V)} \) activation was avoided, and \( I_{K(A)} \) inactivated almost completely before starting the fitting (cells with a small \( I_{K(A)} \) were chosen for this analysis). For \( I_{K(A)} \) activation time course, fitting was started immediately after the residual capacitive artifact.

Time-dependent sigmoidal activations of \( I_{K(V)} \) and \( I_{K(A)} \) were fitted by a second-order power equation of the form:

\[
A \cdot e^{1 - \tau} + C
\]

where \( \tau \) is the activation time constant. Fitting was started immediately after the residual capacitive artifact.

Time-dependent sigmoidal activation of \( I_b \) was fitted by a sum of two exponential functions of the form:

\[
A_1 \cdot e^{k - \tau} + A_2 \cdot e^{k - \tau} + C
\]

where the fast-time constant \( \tau_1 \) accounts for the very initial flat portion of the inward current and describes channel transition from closed-state \( C_1 \) to closed-state \( C_2 \), whereas \( \tau_2 \) (slow-time constant) describes the inward current relaxation and reflects the transition from \( C_2 \) to the open state (Hestrin 1987). Fitting was started immediately after the residual capacitive artifact.

Steady-state activation and inactivation (current–voltage) curves were fitted with Boltzmann functions:

\[
I = I_{max}/[1 + e^{(V_{1/2} - V_{m})/S}]
\]

where \( I \) is current, \( I_{max} \) is maximum current, \( V_m \) is membrane potential, \( V_{1/2} \) is the potential at which the current is half-maximally activated, and \( S \) is the exponential slope.

**RESULTS**

**General membrane properties of developing crista hair cells**

Before E15 all hair cell properties were homogeneous enough to be pooled together; primarily on the basis of morphological criteria, we observed that these immature hair cells resembled type II hair cells. Hair cells with a distinctive morphology, resembling type I hair cells, were recognizable starting from E15, and will be dealt with in a separate section (see below). The membrane properties of immature hair cells changed along with embryonic development from E10 to E21. Figure 2 shows cell membrane input capacitance \( C_m \), resting potential \( V_m \), and peak chord conductance for total outward current \( G_p \), measured in hair cells from all regions of the crista at various embryonic ages. Regression analysis shows that both \( C_m \) and \( G_p \) increased during development: \( C_m \) increased from 3.5 pF (±0.8; mean ± SE; \( n = 22 \)) at E10–E11...
to 5.9 pF (±1.6; n = 33) at E20–E21, whereas Gk for the same cells increased from 6.7 nS (±3.8; n = 22) to 14.5 nS (±5.2; n = 33). Vc varied largely among hair cells at the same embryonic day, but a trend toward more negative values along with development is evident.

**Ionic currents in crista hair cells before E12**

The pattern of ionic currents expressed by hair cells changed during development. At E10 and E11 (Fig. 3), all hair cells, independent of the crista region, almost exclusively displayed a slow-voltage–dependent outward current on depolarization (Fig. 3A), and no current on hyperpolarization (Fig. 3B). The slow outward current reversed close to the K⁺ equilibrium potential; from tail-current measurements in two hair cells at E10 and E11 the reversal potential resulted in −87 and −85 mV, respectively (estimated EK = −96 mV).

Consistent with voltage-clamp results, the cell-voltage response to depolarizing current steps revealed the presence of a slow repolarizing current, whereas it behaved almost passively in response to hyperpolarizing currents (Fig. 3C).

The outward K⁺ current activated in a sigmoidal manner, and could be well fitted by a second-order power function (Eq. 2; Fig. 4A). In a sample of cells from E10–E12 embryos, the average activation time constant decreased from 46.8 ms (±14.3; n = 4) at −40 mV to 10.1 ms (±1.4; n = 4) at −10 mV. The inactivation time course was not voltage dependent: single-exponential fits from E10–E12 hair cell outward currents (Eq. 1; Fig. 4B) gave decay time constants of 2.8 ± 0.3 s at −40 mV and 2.7 ± 0.6 s at −10 mV (n = 3). This current resembles the delayed rectifier K⁺ current observed in the pigeon crista type II hair cells, although it activates and inactivates significantly more slowly (e.g., at −40 mV, the average activation and inactivation time constants were 12.8 and 191 ms, respectively) (Masetto and Correia 1997); hence it will be named I_{K(V)}.

The I_{K(V)} inactivation time course did not change appreciably during development: in E19–E21 hair cells, the inactivation time constant was 2.4 ± 0.1 s at −40 mV and 2.7 ± 1.1 s at −10 mV (n = 5). If the I_{K(V)} inactivation time course could be well fitted also in hair cells expressing a small additional fast and transient current component (see METHODS), the latter precluded a good fitting of I_{K(V)} activation time course from its very beginning in some cells. In three cells among those investigated at E19–E21, in which I_{K(V)} was apparently the sole outward current, its activation time constant was 39.7 ± 9 ms at −40 mV and 8.7 ± 2.1 ms at −10 mV, that is, not significantly different from the values found at E10–E12.

The steady-state activation and inactivation curves for I_{K(V)} are shown in Fig. 4C. I_{K(V)} tail currents were measured at −60 mV after 100-ms voltage steps at different voltages (see Fig. 3A legend). I_{K(V)} started activating less negative than −60 mV, and was fully activated for voltages more positive than 0 mV. When fitted with a Boltzmann function (Eq. 4), the I_{K(V)} activation curve between E10 and E12 gave a V_{1/2} value of −24 mV (±0.1; mean ± SE; n = 6) and a slope factor (S) of 6.1 mV (±0.1; n = 6). Analogous measurements between E19 and E21 gave V_{1/2} = −24 mV (±1.8; n = 9) and S = 12.3 mV (±5.2; n = 9). The change in slope factor paralleled a significant change in the percentage of channels open at most negative voltages: on average, at E10–E12, only 7% of K_V channels were open at −40 mV. At E19–E21, 21% were open at the same membrane voltage (P < 0.005; Student’s t-test). The difference observed could reflect a true change of I_{K(V)} voltage dependence, or a contribution from other K⁺ currents appearing at later stages of development (a fast transient K⁺ current and a Ca-dependent K⁺ current; see below). However, a similar shift was observed at E19–E21 in cells without the fast K⁺ current (n = 3), and in cells in which the Ca-dependent K⁺ current was blocked.
current was blocked by the substitution of \( \text{Mg}^{2+} \) for \( \text{Ca}^{2+} \) \( (n = 3) \); therefore, the \( I_{K(V)} \) activation curve appears to change along with development.

Steady-state \( I_{K(V)} \) inactivation (Fig. 4C) did not appear complete until positive membrane voltages, and was fully removed at \(-110 \text{ mV}\); at \( E10-E12 \), \( V_{1/2} \) was \(-62 \text{ mV} \) \((\pm 1.6; n = 4) \) and \( S \) was \( 14.9 \text{ mV} \). These parameters were not significantly different at later stages of embryonic development \((E19-E21; n = 3) \). The area under the crossing curves describing \( I_{K(V)} \) steady-state activation and inactivation (window current) indicates that a substantial fraction of \( K_v \) channels can contribute steadily to the membrane resting potential, and is permanently available for hair cell repolarization in response to depolarization in the physiological range (see DISCUSSION).

**Acquisition of the transient \( K^+ \) current**

The main change observed after \( E12 \) in hair cells’ electrophysiological properties was the functional expression of a transient outward current (Fig. 5A).

The voltage- and time-dependent properties of the fast transient current differed enough from those of \( I_{K(V)} \) to allow us to isolate it without pharmacological blockers. From tail-current measurements in two hair cells, the transient outward current reversed at \(-83 \text{ and } -85 \text{ mV}\), that is, close to the estimated \( K^+ \) equilibrium potential \((-96 \text{ mV}\). Tail currents (not shown) were measured at different test potentials after conditioning the cell for \( 5 \text{ ms} \) at \(-20 \text{ mV}\); in this way, <6% of the slower \( I_{K(V)} \) was activated, whereas the transient current had reached the peak.

Time-dependent inactivation could be well fitted by a single exponential \((\text{Eq. 1; Fig. 6B}\)); the decay time constant decreased from \( 30.4 \pm 6.9 \text{ ms (mean } \pm SE) \) at \(-40 \text{ mV} \) to \( 7.2 \pm 1.8 \text{ ms} \) at \(-10 \text{ mV} \) \( (n = 4) \). The transient current therefore inactivated 100 times faster than \( I_{K(V)} \). Time-dependent activation could be well fitted by a second-order power function similarly to \( I_{K(V)} \) \((\text{Eq. 2}\). Activation time constants decreased from \( 1.6 \pm 0.3 \text{ ms} \) at \(-40 \text{ mV} \) \( (n = 4) \) to \( 0.8 \pm 0.2 \text{ ms} \) at \(-10 \text{ mV} \); therefore, this current activated 10 times faster than \( I_{K(V)} \). Given the above-mentioned properties and similarities to the transient \( K^+ \) current described in semicircular canal hair cells of mature pigeons \((\text{Masetto and Correia 1997} \), the transient outward current will be called \( I_{K(A)} \). Instantaneous tail-current measurements \((\text{Fig. 6C}\) show that \( I_{K(A)} \) starts activating at \(-60 \text{ mV} \) and is completely activated at \( 0 \text{ mV}\). Boltzmann fit \((\text{Fig. 6D}\) yielded \( V_{1/2} = -36.5 \text{ mV} \) \((\pm 0.9; n = 5) \) and \( S = 8.2 \text{ mV} \) \((\pm 0.9; n = 5) \). Steady-state inactivation curve \((\text{Fig. 6D}\) ob-

![FIG. 4. Properties of \( I_{K(V)} \), A: ionic currents evoked by depolarizing voltage steps from a holding voltage of \(-60 \text{ mV} \) (nominal test voltages indicated next to each trace). Current traces have been corrected for leakage off-line (see METHODS). Activation time constants (\( \tau \), shown next to each trace) come from best fits of the traces with a second-order power function \((\text{Eq. 2}\). \( R_e = 4.1 \text{ M} \Omega, C_m = 2.5 \text{ pF}\). B: protocol used to study steady-state inactivation of \( I_{K(V)} \). The cell was conditioned at different voltages for \( 10 \text{ s} \) and then stepped at \(-10 \text{ mV} \) for \( 500 \text{ ms} \); between each conditioning cycle, the cell was maintained at the holding potential \((-60 \text{ mV}\) for \( 60 \text{ s} \) to allow a full recovery from the previous conditioning step. Horizontal bars are \( 2 \text{ s} \) before the arrow and \( 400 \text{ ms} \) after the arrow. Tags in the current traces show the start \((\text{see METHODS}\) and the end of the inactivation fits with monoeponential functions \((\text{Eq. 1}\), which are shown for traces at \(-10, -20, -30, \text{ and } -40 \text{ mV}\). Leakage current subtracted off-line. \( R_e = 3.9 \text{ M} \Omega, C_m = 3.9 \text{ pF}\). C: steady-state activation and inactivation curves for \( I_{K(V)} \). The two activation curves describe \( I_{K(V)} \) activation measured at \( E10-E12 \) (filled squares, fit = dotted line) and at \( E19-E21 \) (triangles, fit = dashed line), and have been obtained by fitting with Boltzmann functions \((\text{Eq. 4}\) the normalized means values for the instantaneous tail current amplitude measured at \(-60 \text{ mV}\) \((\text{see Fig. 3A, inset}\); tail currents were fitted with a single monoeponential function and extrapolated to the onset of the voltage step), after correction for voltage drop across residual series resistance. Vertical and horizontal bars are means \( \pm SE \) for current amplitude and membrane voltage, respectively. Note the change in the voltage-dependence of the activation curve at later stages of embryonic development. Points in the inactivation curve \((\text{circles}\) are normalized means \( \pm SE\), vertical bars; \( n = 4\) ) for peak current amplitudes measured at \(-10 \text{ mV}\) after conditioning the cell at different voltages \((\text{as in Fig. 4B}\), at \( E10-E12\). Steady-state inactivation curve at later developmental stages is not shown because it is not significantly different. Experiments were chosen in which the greatest voltage error resulting from residual \( R_c \) calculated at the test potential \((-10 \text{ mV}\) after most negative conditioning voltages, was <4 mV. This would introduce a maximal error in estimating the percentage of activated or inactivated \( K(V) \) channels <2% (well in the range of the standard deviation), as estimated from best activation and inactivation curves.)
obtained by measuring the peak current amplitude at −40 mV after conditioning the cell at different voltages (as in Fig. 6A), shows that $I_{K(A)}$ is almost completely inactivated (96%) at −30 mV, whereas inactivation is completely removed at −120 mV ($V_{1/2} = −75$ mV; Fig. 6D). There is thus a significant window current contributing to the receptor potential steadily between −60 and −30 mV. $I_{K(A)}$ properties did not change appreciably throughout embryonic development.

To illustrate $I_{K(A)}$ contribution to hair cell currents in the different zones, we plotted the time-to-peak ($t_p$, Fig. 5C) and steady-to-peak ratio ($I_s/I_p$, Fig. 5D) of hair cell total currents versus embryonic day of development, measured at −20 mV. The parameters considered can reveal the dominant current component of the total outward current, given the considerably faster activation and inactivation kinetics of $I_{K(A)}$ versus $I_{K(V)}$. From these considerations, $t_p$ would be smaller in hair cells with a significant $I_{K(A)}$, and larger in hair cells dominated by $I_{K(V)}$. On average, from $E_{12}$, when $I_{K(A)}$ first appears, to $E_{21}$, $t_p$ was 14.1 ms (±2.6; $n = 54$) in zone 1 hair cells, 60.2 ms (±36.8; $n = 42$) in zone 2 hair cells, and 32.6 ms (±40.1; $n = 24$) in zone 3 hair cells. These values were statistically significantly different among zones ($P < 0.05$ between all pairs of zones; one-way ANOVA-Tukey). The time-to-peak was <6.6 ms (the median for all hair cells from $E_{12}$ up to $E_{21}$) in 44/54 (81.5%) zone 1 hair cells, in 6/42 (14.2%) zone 2 hair cells, and in 10/24 (41.6%) zone 3 hair cells, investigated from $E_{12}$ to $E_{21}$.

As far as $I_s/I_p$ is concerned, both $I_s$ and $I_p$ were measured at −20 mV after having conditioned the cell at −120 mV for 100 ms to remove most $I_{K(A)}$ inactivation; $I_s$ was measured at the end of the test potential, lasting 100 ms; that is, when $I_{K(A)}$ had almost completely inactivated, whereas $I_p$ was measured in the first 6 ms of the test potential, when $I_{K(A)}$ had already reached the peak. The ratio $I_s/I_p$, therefore, will be 1 if the maximal current amplitude attained in the first 6 ms of the test-voltage step equals the current amplitude attained at the end of the test-voltage step. Because $I_{K(A)}$ reaches the peak in the first 6 ms at −20 mV, whereas $I_{K(V)}$ is much slower (we estimated that at −20 mV on average <20% of $K_v$ are open in the first 6 ms of the voltage step), an $I_s/I_p$ value close to 1 would indicate a similar contribution to the total outward current from $I_{K(A)}$ and $I_{K(V)}$; this ratio will be <1 if the total outward current declines from the peak attained in the first 6 ms to a lower steady level (total outward current dominated by $I_{K(A)}$), and otherwise >1 if the steady outward current is greater than the current amplitude attained in the first 6 ms (total current dominated by the slow $I_{K(V)}$). From $E_{12}$ to $E_{21}$, $I_s/I_p$ was on average 0.6 (±0.3; $n = 48$) in zone 1 hair cells, 2.4 (±1.4; $n = 35$) in zone 2 hair cells, and 1.4 (±1.1; $n = 22$) in zone 3 hair cells. These values were statistically significantly different among zones ($P < 0.05$ between all pairs of zones; one-way ANOVA-Tukey). The preceding results indicate that $I_{K(A)}$ is not evenly expressed across the crista: indeed it was by far the predominant current in zone 1 hair cells from $E_{12}$ on, but less important in zone 3 and very small (or even absent) in zone 2 hair cells.

Another aspect worth considering is the relative importance of $I_{K(A)}$ along with development. From Fig. 5D it can be seen that for $I_{K(A)}$ hair cells the ratio $I_s/I_p$ does not change significantly once $I_{K(A)}$ is expressed. In fact, it was 0.7 ± 0.5...
This would introduce an error in estimating the percentage of activated or potential (2% (well in the range of the standard deviation), as estimated from best activation and inactivation curves. The inset shows the difference trace obtained by subtracting the current evoked after conditioning at −60 mV from the current evoked after conditioning at −110 mV (vertical bar = 0.2 nA; horizontal bar = 50 ms); the inactivating phase of this difference current could be well fitted by a monoeponential function (τ = 9.4 ms; Eq. 1). Rm = 6.8 MΩ; Cm = 3.8 pF. C: protocol used for IKA activation. The hair cell was held at −70 mV, depolarized every 5 s at different voltages, and repolarized at −60 mV. Step duration was adjusted (first step = 2.5 ms, 0.3-ms increase for each step) so that instantaneous tail current at −60 mV reflected peak IKA at each test potential, just before it began to inactivate. A returning voltage of −60 mV was chosen to avoid IKV activation overlap with IKA tail. Leakage current subtracted off-line. Rm = 4.5 MΩ; Cm = 4.8 pF. D: average steady-state activation and inactivation curve for IKA. Points were fitted with Boltzmann functions (Eq. 4). Points for the activation curve (filled squares) are normalized means for instantaneous tail current amplitudes (± SE, vertical bars; n = 5). Tail currents were fitted with a monoeponential function (Eq. 1) and extrapolated to the onset of the voltage step. Current is plotted versus the average membrane voltage (± SE, horizontal bars), calculated after correction for voltage drop on residual series resistance. Points for the inactivation curve (filled circles) are normalized means (± SE, vertical bars; n = 5) for peak current amplitude at −40 mV after conditioning at different voltages (see A). Boltzmann fit has been extrapolated to show IKA steady-state inactivation at potentials less negative than −40 mV, where it could not be directly measured because of significant overlapping with IKA tail currents. Experiments were chosen where the greatest voltage error due to residual Rm calculated at the test potential (−40 mV) after most negative conditioning voltages, was <2 mV. This would introduce an error in estimating the percentage of activated or inactivated channels <2% (well in the range of the standard deviation), as estimated from best activation and inactivation curves.

(mean ± SE) at E13–E14 (n = 11) and 0.6 ± 0.3 (n = 17) at E20–E21 (the difference is not statistically significant). Because Ih density (Ih/Cm) in the same cells did not change significantly during embryonic development (data not shown), both IKA (V) and IKA appear to increase in amplitude roughly proportionally to the increase in hair cell membrane surface area. The same is not as easy to evaluate for the other cristae zones, in which IKA is more variable (even absent).

The voltage response of hair cells to depolarizing currents was profoundly modified by the presence of IKA. This current rapidly counteracted the depolarization produced by positive-current steps, producing a very fast depolarizing peak followed by a few clear oscillations (Fig. 5D). In some cells the damped voltage oscillations could be slightly distorted by the patch-clamp amplifier (Masetto et al. 1999) (see pipette current stimulus in Fig. 5D). However, in other cells the oscillation observed was the true voltage response of the hair cell, because no pipette current artifacts were detectable (see, for example, Fig. 7). Short-lived membrane-voltage oscillations disappeared when the resting potential was depolarized by a positive-holding current (Fig. 7), which induced inactivation of KA channels. Moreover, membrane-voltage oscillations were much less pronounced in hair cells without IKA (see, for example, Fig. 9C). Similarly, in mature pigeons the quality of membrane oscillations was greater in zone 1 hair cells, which expressed the largest IKA compared with zone 3 hair cells (Weng and Correia 1999).

**Ca-activated K**

The presence of a Ca-activated K** current (IKA) was tested by perfusing the cells with a low-Ca/high-Mg extracellular solution (see slicing solution in METHODS), where Ca** was replaced by Mg** in an approximately 1:3 ratio to minimize the otherwise expected change in surface membrane potential (Blaustein and Goldman 1968).

This substitution had a complex effect: before E14 it produced a small outward current increase. This increase was most likely because of the blockade of an inward Ca current (IKA) produced by Mg** ions blocking Ca** channels (Carbone et al. 1997). Starting from E14, Ca**/Mg** substitution could produce variable effects, including either a small increase or a small decrease of the total current, or both (with current increasing for more negative potentials, and then decreasing; Fig. 8A). When both increase and decrease were observed, the currents obtained by subtracting current in the presence of...
Inward rectifying currents

At later stages of development, hyperpolarizing voltage steps from a holding potential of −60 mV activated a slow inward rectifying current in many hair cells (Fig. 9A). This current was often very small: at −120 mV it was, on average, 90.7 pA (±49.7; mean ± SE; n = 9, zones 2 and 3 hair cells at E16–E18). By comparison, in a sample of hair cells in which a time dependence of the inward current was not evident, the presumed leakage current at −120 mV was 49.5 pA (±13; n = 6, zone 1 hair cells at E16–E18), that is, not very different in amplitude. Moreover, the presence of a slight inward rectification of the inward current at more negative voltages often appeared as a consequence of hair cell deterioration at these low voltages (i.e., leakage increase); therefore, we used a combination of current-clamp (see below) and voltage-clamp experiments to increase our confidence that an inward rectifying current was actually present. In this way, we could find this slow inward rectifying current already at E14–E15, although only in 2/11 hair cells. Incidentally, of these 11 cells, 5 were from zone 1, and none of them expressed it. However, it is only from E16 that the majority of hair cells from zones 2 and 3 clearly expressed this slow inward rectifying current: from E16 up to E18, in fact, it was found in 9 of the 10 zone 2/zone 3 hair cells investigated. Conversely, it was absent in all zone 1 hair cells investigated at the same developmental stages (n = 6). This current reversed at voltages more positive than −60 mV (as indicated by the presence of inward tail currents at −60 mV at the end of hyperpolarizing steps), suggesting that it is not strongly selective for K⁺ ions.

These features resemble those of the Iₜ described in adult pigeon semicircular canal hair cells (Masetto and Correia 1997). According to Hestrin (1987), Iₜ could be well fitted by a sum of two exponential functions (Eq. 3). In a sample of cells τₛ (the slow-time constant) was 81.9 ms (±18; mean ± SE; n = 5) at −120 mV, a value very close to that measured in mature canal hair cells of the pigeon (92.4 ms at −120 mV) (Masetto and Correia 1997), and decreased with increasing hyperpolarization. As anticipated, current-clamp experiments helped to reveal the presence of Iₜ. This current in fact produced a clear sag in the voltage response to hyperpolarizing currents steps (Fig. 9B). Moreover, because its reversal potential is more positive than average Vₑ of hair cells (likely close to −40 mV) (Holt and Eatoek 1995), Iₜ also produced a transient afterdepolarization at the end of hyperpolarizing current steps. The afterdepolarization produced by deactivating Iₜ could be emphasized by hyperpolarizing the cell resting membrane potential. In the example of Fig. 9C, the cell was hyperpolarized from −69 to −90 mV by a holding current of −60 pA. At the end of the negative-current step, a large afterdepolarization devel-

![Figure 8](http://jn.physiology.org/)
larization was most likely the result of the slow deactivation of \( I_{K(V)} \) and/or \( I_{KCa} \), because \( I_{K(A)} \) was not evident in this cell.

From \( E19 \) \( I_h \) was often obscured by a larger and faster inward rectifying current, \( I_{K1} \) (Fig. 10A). As for \( I_h \), this current did not appear abruptly from \( E19 \); in fact, it was found in 3/16 hair cells from \( E16 \) to \( E18 \) (of these 16 cells, 5 were from zone 1, and none of them clearly expressed \( I_{K1} \)). However, from \( E19 \) this fast inward rectifying current was found in most zone 2 and zone 3 hair cells (13/20 cells expressed \( I_{K1} \), plus possibly a small \( I_h \), whereas the remaining 7 cells displayed \( I_h \) only). Although in very few zone 1 hair cells (2/15 expressed a small \( I_{K1} \), plus possibly a small \( I_h \) and 2/15 expressed only a very small \( I_h \)).

The time-dependent activation of this current could be well fitted by a single-exponential function (Eq. 1); the activation time constant was 3.1 ms (±0.8; mean ± SE; \( n = 3 \)) at −120 mV (i.e., about 20 times smaller than that of \( I_h \)) and decreased by increasing hyperpolarization. The preceding value is very close to that of \( I_{K1} \) described in mature pigeon vestibular hair cells (2.9 ms at −120 mV) (Masetto and Correia 1997). \( I_{K1} \) accelerated and compressed the voltage response to hyperpolarizing-current steps (Fig. 10B). For example, from \( E10 \) to \( E15 \) negative-current steps of 40 pA amplitude drove the hair cell to the current stimulus shown earlier. Hair cell resting potential was evident for the small afterdepolarization at the end of the negative current steps, more evident for the −100 pA step. C: current-clamp response of an \( E20 \) zone 2 hair cell to the current stimulus shown earlier. Hair cell resting potential was hyperpolarized by a 60-pA holding current to emphasize the action of \( I_h \). Note that when the conditioning current was −120 pA, a clear afterdepolarization was present on stepping back to the holding current, whereas when the cell was depolarized before stepping back, an afterhyperpolarization was generated. \( R_s = 10 \) MΩ; \( C_m = 7.4 \) pF.

![Image 9. \( I_h \) and its effect on hair cell voltage response. A: \( I_h \) recorded from an \( E17 \) zone 3 hair cell (voltage protocol shown above current traces). Note that the tail currents, obvious immediately after the residual artifact, are inward. \( R_s = 5 \) MΩ; \( C_m = 6.2 \) pF. B: current-clamp responses of the same cell to 150-ms hyperpolarizing current steps of −50 pA (thin line) and −100 pA (thick line) applied from the cell resting potential (protocol not shown). Note the small afterhyperpolarization at the end of the negative current steps, more evident for the −100 pA step. C: current-clamp response of an \( E20 \) zone 2 hair cell to the current stimulus shown earlier. Hair cell resting potential was hyperpolarized by a 60-pA holding current to emphasize the action of \( I_h \). Note that when the conditioning current was −120 pA, a clear afterdepolarization was present on stepping back to the holding current, whereas when the cell was depolarized before stepping back, an afterhyperpolarization was generated. \( R_s = 10 \) MΩ; \( C_m = 7.4 \) pF.](image9.png)

![Image 10. Inward rectifying K⁺ current \( I_{K1} \) and its effect on hair cell voltage response. A: inward currents activated by hyperpolarizing voltage steps (holding potential −60 mV; step increment −10 mV; last step −150 mV) in two different hair cells from \( E20 \) chick embryos. Top: cell with \( I_{K1} \) as the dominant (if not the sole) inward rectifying current; bottom: cell where both \( I_{K1} \) and \( I_h \) are evident. Note that in the top panel \( I_{K1} \) tails at −60 mV are clearly outward. Vertical bars = 0.25 nA. \( R_s = 10 \) MΩ and 7.5 MΩ, \( C_m = 6.4 \) pF and 6.7 pF (top and bottom, respectively). Residual artifact in top traces has been blanked (0.5 ms). B: current-clamp response of same cell as in A. Bottom, \( I_{K1} \) as shown above voltage responses. C: current/voltage plots for negative voltages. Peak inward current was measured and plotted versus step voltage before \((E10-E18)\) and after \((E19-E21)\) the appearance of \( I_{K1} \) in the different crista zones (zone 2 and zone 3 have been grouped given their similarity). Inward rectifying currents are very small or negligible in zone 1 hair cells at all developmental stages, whereas they increase considerably from \( E19 \) in zone 2 and zone 3 hair cells. Mean values for current amplitude (± SD = vertical bars) and membrane voltage (± SD = horizontal bars) corrected for voltage drop across residual \( R_s \) are shown.](image10.png)
cell membrane potential from its resting value up to an average value of \(-149.9\) mV (\(\pm 37.6; n = 22\), all zones pooled); from \(E19\) to \(E21\) the same current step injection applied to zone 2/zone 3 hair cells produced a significantly smaller hyperpolarization: \(-85.5\) mV (\(\pm 10.7; n = 16\). Probably because of the low expression of \(\mathrm{K}_\text{V}\) in zone 1 hair cells even at \(E19\)–\(E21\), these cells generated relatively large voltage responses to negative-current steps: for example, 40 pA negative-current steps hyperpolarized zone 1 hair cells from their resting value up to an average value of \(-118.1\) mV (\(\pm 34.8; n = 8\)).

\(I_{\text{K1}}\) was accompanied by a more negative \(V_s\); from \(E19\) to \(E21\), \(V_s\) was on average \(-63.1\) mV (\(\pm 13.5\); mean \(\pm SE; n = 17\)) in hair cells lacking \(I_{\text{K1}}\), and \(-73.7\) mV (\(\pm 11.1; n = 15\)) in hair cells displaying \(I_{\text{K1}}\) (\(P < 0.05\); Student’s \(t\)-test).

Inward currents through voltage-dependent \(\text{Ca}^{2+}\) channels

In the presence of blockers of \(\text{K}^+\) currents and \(I_h\) (see Methods), depolarization evoked voltage-dependent inward currents in the majority of hair cells tested. The inward current was better recorded with \(\text{Ba}^{2+}\) instead of \(\text{Ca}^{2+}\) in the extracellular medium, because with \(\text{Ca}^{2+}\) it was smaller and largely contaminated by unblocked residual outward currents (not shown). \(\text{Ba}^{2+}\) currents \((I_{\text{Ba}})\) were present from the earliest developmental stage investigated \((E10)\), and increased considerably with development (Fig. 11, A and B). For example, \(I_{\text{Ba}}\) peak amplitude measured at \(-25\) mV was 12.3 pA (\(\pm 12.2\); mean \(\pm SE; n = 7\)) at \(E10\)–\(E12\), and 44.4 pA (\(\pm 24.9\); \(n = 7\)) at \(E19\)–\(E21\). The difference in \(I_{\text{Ba}}\) amplitude between earlier and later developmental stages was statistically significant (\(P < 0.01\); Student’s \(t\)-test); however, if the \(\text{Ba}^{2+}\) current density is computed \((I_{\text{Ba}}/C_m)\), no significant differences are found. \(I_{\text{Ba}}\) activated rapidly, and did not show evident time-dependent inactivation, at least for the 160-ms depolarizing-step duration (Fig. 11A). No voltage- or Ba-dependent inactivation of \(I_{\text{Ba}}\) was observed conditioning the cell membrane voltage between \(-30\) and \(-110\) mV for 250 ms before step to \(-10\) mV (\(n = 2\); not shown). No significant differences were observed in \(I_{\text{Ba}}\) voltage-dependent parameters (Fig. 11B) between early and late developmental stages: on average, \(I_{\text{Ba}}\) activated at \(-50\) mV (\(\pm 0.9; n = 7\)) and peaked at \(-23.4\) mV (\(\pm 1.9; n = 7\)) in \(E10\)–\(E12\) embryos, versus \(-53\) mV (\(\pm 1.8; n = 7\)) and \(-26.4\) mV (\(\pm 4; n = 7\)), respectively, in \(E19\)–\(E21\) embryos. \(I_{\text{Ba}}\) was completely blocked (in a partially reversible fashion) by adding 200 \(\mu\)M \(\text{CdCl}_2\) to the external medium (Fig. 11B). This feature was used to isolate in some cells the net \(\text{Ba}^{2+}\) current from the contaminating leakage and outward current, by subtracting from the control current the current remaining after \(\text{Cd}^{2+}\) block. In seven cells in which \(\text{Cd}^{2+}\) subtraction was compared with the usual leakage subtraction (see Methods), \(I_{\text{Ba}}\) obtained after \(\text{Cd}^{2+}\) was found to peak 3 mV less negative, and to reverse more positive [more positive than 40 mV, vs. an average value of 11.9 mV (\(\pm 10.3; n = 7\))]. No differences were observed in voltage threshold of \(I_{\text{Ba}}\) activation, consistently with the observation that the \(\text{Cd}\)-insensitive outward current just started activating around \(-50\) mV, thereafter increasing monotonically with depolarization (Fig. 11B). The preceding results are consistent with previous reports of \(\text{Ba}^{2+}\) current flowing through voltage-dependent \(\text{Ca}^{2+}\) channels in mature (Lang and Correia 1989) and developing (Sokolowski et al. 1993) avian hair cells.

**Fig. 11.** \(\text{Ba}^{2+}\) currents at different developmental stages. Hair cells were internally perfused with \(N\)-methyl-D-glucamine (NMDG), and externally perfused with a solution containing \(\text{Ba}^{2+}\), tetraethylammonium (TEA)\(^+\), 4-aminopyridine, and \(\text{Cs}^+\) (see Methods), to block \(I_h\) and most \(K^+\) currents. A: \(\text{Ba}^{2+}\) currents evoked by voltage stepping hair cells from \(-60\) mV to \(-25\) mV. The smaller inward current was recorded from an \(E10\) embryo zone 2 hair cell \((R = 4.8 \mu\Omega; C_m = 3.1 \mu F)\), the larger one from an \(E20\) embryo zone 3 hair cell \((R = 6.5 \mu\Omega; C_m = 10 \mu F)\). Ionic currents were subtracted for leakage as described in the Methods section. B: \(\text{Ba}^{2+}\) currents evoked by ramp voltage protocols (from \(-75\) mV to 45 mV in 250 ms; slope = 0.48 mV/ms). Thin traces: current recorded in an \(E10\) zone 2 hair cell \((R = 9.0 \mu\Omega; C_m = 2.9 \mu F)\); Cont. = control current; \(\text{Cd}^{2+}\) 200 \(\mu\)M = current recorded after perfusing the same cell with the \(\text{Ba}^{2+}\) extracellular solution added with \(\text{Cd}^{2+}\) 200 \(\mu\)M, which blocked the inward \(\text{Ba}^{2+}\) current. Thick trace: current recorded in an \(E21\) embryo zone 2 hair cell \((R = 4.4 \mu\Omega; C_m = 9 \mu F)\); current shown \((\text{net} I_{\text{Ba}})\) has been obtained by subtracting from the control current the current after \(\text{Cd}^{2+}\) block. Note that the net \(I_{\text{Ba}}\) reverses more positive than 40 mV. Filled squares, net \(I_{\text{Ba}}\) peak amplitude recorded from a different hair cell \((E20, \text{zone 2})\) by the use of 250-ms voltage steps from a holding potential of \(-60\) mV, in 5-mV increments up to 5 mV. Step-current amplitudes were normalized to the peak of the net \(I_{\text{Ba}}\) recorded with the ramp protocol, and superimposed in the figure, to show that the two different voltage protocols produce similar current–voltage relationships.

Ionic currents in type I hair cells

Starting from \(E15\), some hair cells in the slices displayed morphological features typical of mature type I hair cells: they had a large apical region bearing a short bundle of stereocilia, and a very constricted neck (Fig. 12D, left panel), compared with a typical type II hair cell (Fig. 12D, right panel). We were able to record from cells showing the aforementioned morphology from \(E17\); all these cells \((n = 11)\) displayed a large outward current considerably active at \(-60\) mV, as shown by the presence of inward tail currents in response to hyperpolarizing voltage steps and of substantial instantaneous currents in response to depolarization (Fig. 12A). This outward current was almost completely deactivated at the holding potential of \(-80\) mV (Fig. 12B). This low-voltage-activated outward current resembles \(I_{K1,L}\) described in avian and mammalian mature
vestibular type I hair cells (Rennie and Correia 1994; Rüsch and Eatock 1996). In most cells we found it difficult to fit with a single Boltzmann function the activation curve of the total outward current, because it appeared to reach a first plateau around $-50 \text{ mV}$, and then to increase again to reach a second plateau around $-10 \text{ mV}$ (Fig. 12C, diamonds). A second outward current component, activating at less negative voltages than $I_{K,L}$, but partially overlapping to it, can explain the...
observed inflection in the current–voltage relationship. However, in one cell $I_{K,1}$ activated more negative, and its activation curve could be better resolved (variability of $I_{K,1}$ activation curves among type I hair cells has already been reported) (Rüsch and Eatock 1996). The activation curve of $I_{K,1}$ for this single cell is shown in Fig. 12C (triangles); Boltzmann fit (Eq. 4) gave a $V_{1/2}$ value of $-82 \text{ mV}$ and a slope factor ($S$) of 8.4 mV. $I_{K,1}$ started activating around $-110 \text{ mV}$, and was almost completely activated at $-60 \text{ mV}$, when outward current began to increase again, presumably because of the activation of a different channel population. For this reason, saturation for $I_{K,1}$ activation curve is shown. We did not investigate the nature of the second outward current component, although the absence of a fast inactivation allowed us to rule out that it was $I_{K(A)}$. Additional outward rectifying currents besides $I_{K,1}$ and activating less negative than $-55 \text{ mV}$ have been reported in type I hair cells (Rennie and Correia 1994; Rüsch and Eatock 1996).

As far as type I hair cell outward current kinetics is concerned, a comparison with type II hair cells is possible only at potentials less negative than $-60 \text{ mV}$, because in type II hair cells outward currents just start activating at $-60 \text{ mV}$. From this comparison, type I hair cell outward currents appear to activate significantly more slowly than type II hair cell outward currents. The average time-to-peak for total outward currents elicited at $-20 \text{ mV}$ was, in fact, 164.8 ms $(\pm 33; n = 9)$ in zone 2 $E19$–$E21$ type I hair cells, and 55.5 ms $(\pm 40; n = 13)$ in zone 2 $E19$–$E21$ type II hair cells ($P < 0.0001$). The average time-to-peak for total outward currents in the same type I hair cells measured at $-60 \text{ mV}$ was 161.6 ms $(\pm 38.2; n = 9)$.

Among type I–like hair cells we recorded from, 10/11 were located at zone 2, and 1/11 at zone 1. In zone 2, therefore, there appears to be a maximal density of type I–like hair cells, intermingled with type II hair cells. Because of $I_{K,1}$, zone 2 type I–like hair cells display many differences once compared with zone 2 type II hair cells of the same embryonic age ($E17$–$E21$). For example, the average peak chord conductance, measured at $-60 \text{ mV}$ (stepping from a holding potential of $-80 \text{ mV}$) was 18.7 nS $(\pm 13.5; n = 10)$ in type I–like hair cells, versus 2.2 nS $(\pm 1; n = 9)$ in type II hair cells ($P < 0.01$; Student’s $t$-test). The difference in the average peak chord conductance between the two cell samples was significant also when measured at $-20 \text{ mV}$ $(55.8 \text{nS} \pm 1.9$ in type I–like hair cells vs. $9.9 \text{nS} \pm 1.7$ in type II hair cells; $P < 0.001$; Student’s $t$-test). Because the average membrane capacitance (see Fig. 2A) of the two previous samples was not significantly different $(5.8 \text{pF} \pm 1.4; n = 10$ for type I–like hair cells vs. $5.2 \text{pF} \pm 1.8, n = 9$ for type II hair cells), the difference observed in chord conductance is not the result of differences in type I hair cell size versus type II hair cell size.

Given its low-voltage-activation range, $I_{K,1}$ is likely to contribute to the negative $V_z$ of type I–like hair cells ($-73.6 \text{ mV} \pm 5.1; n = 11$).

**DISCUSSION**

In the present work we describe for the first time the electrophysiological properties of semicircular canal hair cells during in vivo embryonic development. Our results show that developing hair cells acquire different types of ion channels, following a precise temporal sequence. Each appearing ion current shapes hair cell voltage response in a specific manner. The main findings are discussed in this section, also in relation to other developmental parameters.

**Hair cells’ electrophysiological properties change along with development**

During embryonic development, from $E10$ up to $E21$, crista hair cells increased in size, ion current amplitude, and ion channel variety. Because at $E10$ most chick cochlear hair cells are postmitotic (Katayama and Corwin 1989), and provided that the same is true for crista hair cells, then ionic currents expressed after $E10$ mostly should reflect their acquisition by preexisting hair cells, and not the appearance of new hair cells with different electrophysiological profiles. For the same reason it should be assumed that, although at early stages all hair cells morphologically resemble mature type II hair cells, some of these cells will differentiate as type I.

At the earlier stages here examined ($E10$–$E12$), all hair cells expressed only $I_{Ca}$ and $I_{K(V)}$. As far as $I_{Ca}$ is concerned, in mature hair cells this current is mainly involved in afferent transmitter release, most likely of glutamatergic nature (Otersen et al. 1998). However, it is not known when inner ear afferent synapses become functional during development. Interestingly, in the chick cochlea developing cochlear ganglion neurons express functional glutamate receptors even before connections between cochlear hair cells and cochlear neurons exist (Jiménez and Núñez 1996). In the chick semicircular canal, the earliest afferent synapselike contacts are observed around $E7$ (stages 28 and 29), although these early contacts could represent either synaptic precursors or nonsynaptic adherent junctions (Ginzberg and Gilula 1980). On the basis of morphological criteria, afferent innervation can be considered maturelike from $E14$–$E15$ (stage 39) (Meza and Hinojosa 1987). Therefore Ca$^{2+}$ channels, here reported to be functionally expressed from (at least) $E10$, could also play additional roles during the development and maturation of the crista afferent innervation; this is interesting in light of the recognition that transmitters play a role in shaping the developing pattern of neuronal connectivity (Spitzer 1991).

$I_{K(V)}$ is likely to be important for counteracting the regenerative depolarizing action of $I_{Ca}$; in fact, both currents activate between $-60$ and $-50 \text{ mV}$, and $I_{K(V)}$ inactivates very slowly and incompletely up to $0 \text{ mV}$.

In principle immature hair cells could be depolarized by the transducer current. At $E10$ hair cells already display short hair bundles, on which tip links have been observed (Tilney et al. 1986), but it is not known whether the mechanotransduction apparatus is actually working. If this is the case, mechanotransduction currents would be able to strongly affect hair cell membrane potential: in fact, present current-clamp experiments show that positive-current steps of 100 pA (in the range of the receptor current in newborn chicks) (Ohmori 1985) can steadily depolarize the hair cell membrane voltage to $-20 \text{ mV}$. Depolarization by transducer current and/or Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels may be important during development: for example, sustained membrane depolarization has been suggested to facilitate insertion of membrane proteins, such as receptors for neurotransmitters (Ohmori and Sasaki 1977). At later stages of development, with the acquisition of $I_{K(A)}$ (at $E12$) and $I_{KCa}$ (at $E14$), hair cells’
voltage response to depolarizing current becomes more rapid and compressed: for example, 100-pA positive-current steps depolarize hair cells between −50 and −40 mV.

Voltage responses to negative current steps were purely passive until the appearance of $I_h$, followed by $I_{K1}$. Both inward rectifying currents were also likely to contribute to the membrane resting potential, although differently, given their different reversal potential. It is well known that mature hair cells can be hyperpolarized by negative deflection of the hair bundles and by the activation of the inhibitory efferent system (Guth et al. 1998). In the chick semicircular canal, the latter is presumably functional from stage 43 [judging from choline acetyltransferase (ChAT) activity, corresponding here to E18] (Meza and Hinojosa 1987), that is, at a time when hair cells have already started expressing $I_h$ and $I_{K1}$, which therefore could play a role in the efferent action.

From E17 an additional ion current, termed $I_{K, L}$, can be found in some hair cell, which is particularly important because it is considered peculiar for type I hair cells (Correia and Lang 1990; Eatock and Hutzler 1992; Rennie and Correia 1994). From a morphological point of view, chick crista type I hair cells have been previously reported to differentiate at stage 39 (our E14–E15), when nerve afferent terminals shaped as half-chalices also appear (Meza and Hinojosa 1987). In close agreement, we could distinguish type I–like hair cells and the associated calyx synapse from E15. We also found that these cells typically expressed (at least from E17) a low-voltage-activated delayed rectifier current resembling $I_{K, L}$, thus further supporting their type I nature (Correia and Lang 1990; Eatock and Hutzler 1992; Rennie and Correia 1994). The precocious differentiation of type I hair cells in chick vestibular hair cells compared with mammals (where it occurs around the 4th postnatal day with the acquisition of $I_{K, L}$) (Eatock and Rüschi 1997; Rüschi et al. 1998) is not surprising because, as already recalled, inner ear development in mammals lags behind that of avians.

The chicken sensory epithelia are essentially mature at hatching (Forge et al. 1997), even regading the nervous afferent activity (Jones and Jones 1995, 1996; Manley et al. 1991). In our experiments, we found that, close to hatching, the assortment of ion channels is very similar to that of mature avian vestibular hair cells (at least for type II) (Lang and Correia 1989; Masetto and Correia 1997). Nonetheless, ion current kinetics, voltage-dependence, and pharmacology (Griguer and Fuchs 1996), might still be different from those of adult animals. Unfortunately, comparable data on adult chicken are not available, so the closest counterpart would be the pigeon. When compared with mature pigeon crista hair cells (Masetto and Correia 1997; Weng and Correia 1999), chick embryo crista ion currents do show some differences (e.g., total ionic currents for the chick embryo are slower for all crista zones); however, this could be the result of either developmental or interspecies differences.

A schematic drawing summarizing our findings about type I and type II hair cells differentiation during chick crista embryonic development is shown in Fig. 13. At E10 all hair cells are quite small, display a short and immature hair bundle, and have already been reached by immature afferent nerve fibers; when recorded from, these cells express a voltage-dependent Ca$^{2+}$ current ($I_{Ca}$) and a voltage-dependent slow outward K$^+$ current ($I_{KNa}$). From E12, most zone 1 and zone 3 hair cells additionally express a fast and transient outward rectifying K$^+$ current ($I_{KA}$). By this time, morphological features typical of true synaptic contacts (pre- and postsynaptic membrane thickenings, synaptic bars surrounded by vesicles and facing the afferent nerve terminals) are commonly observed throughout the sensory crista. By E14, when afferent synaptic contacts appear to be morphologically mature and efferent contacts start to be observed in type II hair cells, some hair cells acquire a Ca-dependent K$^+$ current ($I_{KKc}$).

It is difficult to precisely set the timing of appearance of inward rectifying currents: in fact, a small minority of hair cells started expressing $I_h$ at E14, and $I_{K1}$ at E16 (see Results). However, only by E16 and E19 functional expression of $I_h$ and $I_{K1}$, respectively, was observed in most if not all zones 2/3 hair cells. By E19 a rise in ChAT activity indicates the ability of afferent fibers to produce acetylcholine (Ach), which appears to play a major role in the efferent transmission of the inner ear end organs (Guth et al. 1998).

Starting from E15, hair cells morphologically resembling type I (large cell body and apical surface, with a very constricted neck) and contacted by large afferent terminals shaped as half-chalices were observed, preferentially in zone 2. We currently do not know which ionic currents are expressed by these immature type I–like cells. By E17 clear synaptic contacts between efferent endings and the afferent calyx, which now completely surrounds the basolateral region of type I hair cells, are found. By this time type I–like hair cell express $I_{K, L}$, the signature current of type I hair cells, in addition to $I_h$ (at least some cells) and to a second unidentified outward rectifying current ($I_{K(V)}$), activating less negative than −60 mV, and possibly analogous to $I_{KNa}$ described in avian and mammalian crista type I hair cells (Rennie and Correia 1994) or to $I_{DR}$ described in mouse utricle type I hair cells (Rüschi and Eatock 1996; Rüschi et al. 1998).

So far it is not known when type I hair cells commit to their fate, and little is known also on the possible factors regulating the expression of diverse ion currents by hair cells. Neurotrophic factors recently have been described to modulate the expression of hair cell potassium currents in vitro (Sokolowski et al. 1999). Because multiple signaling molecules affect different stages of inner ear development (Don et al. 1997; Montcouquiol et al. 1998; Pirvola et al. 1997; Ylikoski et al. 1993), it is conceivable that regionally and sequentially expressed factors contribute to both regional and developmental expression of hair cell ionic currents. The present results provide a baseline for future studies intended to investigate the role of altered environmental conditions on hair cell electrophysiological profile, for example, by blocking the expression/function of specific factors at given developmental stages.

**Topographical distribution of ionic currents**

From the time of their first expression, some ionic currents were preferentially, although not exclusively, expressed in different positions in the crista: for example, zone 1 hair cells always expressed a large $I_{KNa}$, a small $I_{KNa}$, and small or no inward rectifying currents. Hair cells from zone 2 and zone 3 always expressed a large $I_{KNa}$, and at least one inward rectifying current ($I_h$ and/or $I_{K1}$). $I_{K(V)}$ was also expressed by a significant fraction of zone 3 hair cells, but very few zone 2 hair cells. No comparable data are available for mature chickens;
previous data from another avian show strong similarities (Masetto and Correia 1997; Masetto et al. 1994; Weng and Correia 1999), but in which the main difference is the lower incidence of $I_{K1}$ we observed in zone 1 hair cells (Weng and Correia 1999). However, because zone 1 appears not to be homogeneous with respect to inward rectifier expression (Marcotti et al. 1999), cell-sampling biases may be responsible for this difference.

From the preceding discussion it comes out that, similarly to what was observed in the chick developing cochlea for $I_{K(A)}$ and $I_{K(V)}$ (Fuchs and Sokolowski 1990; Griguer and Fuchs 1996), chick crista hair cell ionic currents during development are expressed at similar positions as in the mature animal; therefore, those factors determining the mature topographical distribution of ion channels in the inner ear operate early during hair cell differentiation.

**In vivo versus in vitro development**

In a previous in vitro study of the whole chick otocyst (Sokolowski et al. 1993), the electrophysiological properties of hair cells after 3 wk in culture resembled those of crista type II hair cells isolated from 2- to 3-wk-old chicks. When compared with the present results, in both studies $I_{Ca}$ and $I_{K(V)}$ were already present in the earliest record, and were followed by $I_{K(A)}$ and $I_{KCa}$ (which appeared with a few days lag in vitro). Conversely, $I_{K1}$ appeared in vitro significantly earlier (at E12 vs. E19 here); this might suggest that some hair cells from the cultured otocyst develop with an utricular-like rather than with a canal-like pattern (in the mouse utricular hair cells $I_{K1}$ appears earlier than $I_h$) (Eatock and Rüsch 1997).

$I_h$ was not reported in vitro (possibly because of the too-short voltage protocol used) as $I_{K1}$, although hair cells morphologically resembling type I hair cells were occasionally noted. On the other side, some hair cells from the chick cultured otocyst and 2- to 3-wk-old chick cristae (Sokolowski et al. 1993) expressed a $Na^+$ current, not reported here. If hyperpolarizing prepulses were necessary to remove $I_{Na}$ inactivation, as reported for developing mammalian outer hair cells (Oliver et al. 1997), then we could have missed this current because in our experiments on $Ba^{2+}$ current (i.e., in the presence of $K^+$ channel blockers) depolarizing-voltage steps were usually delivered from a holding potential of $-60\ mV$. At the same time, no $Na^+$ currents active in the range of the cell resting membrane potential, like those recently described in the
Development versus regeneration

After the discovery that hair cells in higher vertebrates can regenerate after noise or ototoxic trauma (Corwin and Cotanche 1988; Forge et al. 1993; Ryals and Rubel 1988; Warchol et al. 1993; Weisleider and Rubel 1993), considerable interest has focused on the modalities of hair cell regeneration. Shortly after injury, new hair cells appear in the neuroepithelium, bearing a striking resemblance to immature hair cells, and their sequence of hair bundle differentiation parallels the embryonic one (Cotanche 1987). Type I hair cells reappear only at a later stage. As far as type II hair cell properties are concerned, in the pigeon crista early-regenerating hair cells display lower membrane capacitance and smaller and slower total outward currents compared with control and fully regenerated hair cells (Masetto and Correia 1997). These findings could be explained, assuming that cell and ion current sizes involved in K(V) and Na half inactivation were delayed in respect to that of the slower I(K(A)). The present results show that the preceding sequence of events is also observed during chick crista embryonic development; it is therefore possible that hair cell damage could trigger this same differentiation program in the adult animal.

We thank M. J. Correia, PhD, for valuable comments on the manuscript. This work was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Rome, Italy.

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Received 21 September 1999; accepted in final form 4 January 2000.

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