Electrophysiological Properties of Vestibular Sensory and Supporting Cells in the Labyrinth Slice Before and During Regeneration

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Masetto, Sergio and Manning J. Correia. Electrophysiological properties of vestibular sensory and supporting cells in the labyrinth slice before and during regeneration. *J. Neurophysiol.* 78: 1913–1927, 1997. The whole cell patch-clamp technique in combination with the slice preparation was used to investigate the electrophysiological properties of pigeon semicircular canal sensory and supporting cells. These properties were also characterized in regenerating neuroepithelia of pigeons preinjected with streptomycin to kill the hair cells. Type II hair cells from each of the three semicircular canals showed similar, topographically related patterns of passive and active membrane properties. Hair cells located in the peripheral regions (zone I, near the planum semilunatum) had less negative resting potentials ([current voltage in current-clamp mode]($V_{h}$) = $-62.8 \pm 8.7$ mV, mean ± SD; $n = 13$) and smaller membrane capacitances ($C_m$ = 5.0 ± 0.9 pF, $n = 14$) than cells of the intermediate (zone II; $V_h = -79.3 \pm 7.5$ mV, $n = 3$; $C_m = 5.9 \pm 1.2$ pF, $n = 4$) and central (zone III; $V_h = -68.0 \pm 9.6$ mV, $n = 17$; $C_m = 7.1 \pm 1.5$ pF, $n = 18$) regions. In peripheral hair cells, inward rectifying currents were dominated by a rapidly activating/inactivating outward $K^+$ current, presumably an A-type $K^+$ current ($I_K\text{A}$). Little or no inwardly rectifying current was present in these cells. Conversely, inward rectifying currents of central hair cells were dominated by a slowly activating/inactivating outward $K^+$ current resembling a delayed rectifier $K^+$ current ($I_{K\text{D}}$). Moreover, an inward rectifying current at voltages negative to $-80$ mV was present in all central cells. This current was composed of two components: a slowly activating, noninactivating component ($I_{K\text{N}}$), described in photoreceptors and saccular hair cells, and a faster-activating, partially inactivating component ($I_{K\text{I}}$) also described in saccular hair cells in some species. $I_{K\text{N}}$ and $I_{K\text{I}}$ were sometimes independently expressed by hair cells. Hair cells located in the intermediate region (zone II) had higher inward rectification currents than those of central hair cells than peripheral hair cells. Outward currents in intermediate hair cells activated only slightly more quickly than those of the cells of the central region, but much more slowly than those of the peripheral cells. Additionally, intermediate hair cells, like central hair cells, always expressed an inward rectifying current. The regional distribution of outward rectifying potassium conductances resulted in macroscopic currents differing in peak-to-steady state ratio. We quantified this by measuring the peak ($G_p$) and steady-state ($G_s$) slope conductance in the linear region of the current-voltage relationship ($-40$ to $0$ mV) for the hair cells located in the different zones. $G_p/G_s$ average values ($4.1 \pm 2.1$, $n = 15$) were sometimes independently expressed by hair cells. Hair cells located in intermediate hair cells ($2.3 \pm 0.8$, $n = 4$) and central hair cells ($1.9 \pm 0.8$, $n = 21$). The statistically significant differences ($P < 0.001$) in $G_p/G_s$ ratios could be accounted for by $K_A$ channels being preferentially expressed in peripheral hair cells. Hair cell electrophysiological properties in animals pretreated with streptomycin were investigated at ~3 wk and ~9–10 wk post injection sequence (PIS). At 3 wk PIS, hair cells (all zones combined) had a statistically significantly ($P < 0.001$) lower $C_m$ ($4.6 \pm 1.1$ pF; $n = 24$) and a statistically significantly ($P < 0.01$) lower $G_p$ ($48.4 \pm 20.8$ nS, $n = 26$) than control animals ($C_m = 6.2 \pm 1.6$ pF; $n = 36$; $G_p = 66 \pm 38.9$ nS; $n = 40$). Regional differences in values of $V_h$, as well as the distribution of outward and inward rectifying currents, seen in control animals, were still obvious. But, differences in the relative contribution of the expression of the different ionic current components changed. This result could be explained by a relative increase in $I_K\text{A}$ compared with $I_{K\text{D}}$ during that interval of regeneration, which was particularly evident in peripheral hair cells. At 9–10 wk PIS, hair cells of all zones had membrane properties not statistically different ($P > 0.5$) from those in untreated normal animals. $C_m$ was $6.1 \pm 1.3$ pF ($n = 30$) and $G_p$ was $75.9 \pm 36.6$ nS ($n = 30$). Thus it appears that during regeneration, avian semicircular canal type II hair cells are likely to recover all their functional properties. At 9–10 wk PIS, regenerated hair cells expressed the same macroscopic ionic currents and had the same topographic distribution as normal hair cells. Measurements obtained at 3 wk PIS suggest that regenerated hair cells come from smaller cells (smaller mean values of $C_m$) endowed with fewer potassium channels (smaller mean values of $G_p$). In addition, differences observed in peripheral hair cells’ kinetics and $G_p/G_s$ ratios at 3 wk PIS suggest that different ionic channels follow different schedules of expression during hair cell regeneration. We recorded from nine supporting cells both in normal ($n = 5$) and regenerating ($n = 4$) epithelia. These cells had an average negative resting potential of $V_h = -49.5 \pm 14.1$ mV ($n = 9$), but no obvious sign of voltage- and time-dependent ionic currents, except for a very weak inward rectification at very negative potentials, both in normal and streptomycin-recovering animals. Therefore, if all semicircular canal supporting cells are like the small sample we tested and if supporting cells are actually the progenitors of regenerating hair cells, then they must change shape, develop hair bundles, become reinnervated, and also acquire a complete set of ionic channels ex novo.

INTRODUCTION

During the past decade, considerable interest has focused on the repopulation of the sensory regions of the vestibule or cochlea by sensory cells (hair cells) that, in some species, reappear either continuously throughout life (Corwin 1981; Jorgensen and Mathiesen 1988; Popper and Hoxter 1990; Roberson et al. 1992) and/or following sudden death after acoustic or ototoxic trauma (Corwin and Cotanche 1988; Forge et al. 1993; Ryals and Rubel 1988; Warchol et al. 1993; Weisleder and Rubel 1992, 1993). These results have raised hope of finding a way to stimulate hair cell replacement in human inner ear organs, but it is still controversial whether all types of hair cells regenerate in mammals (Rubel et al. 1995; Warchol et al. 1993). Regenerated hair cells have the typical anatomic features of normal hair cells; and some of the newly produced hair cells in the avian cochlea
(Rylas and Westbrook 1994) and semicircular canals (K. R. Blumberg, G. A. Kevetter, and M. J. Correia, unpublished observations) become reinnervated. These morphological data suggest a true functional recovery of the damaged sensory epithelia. The few electrophysiological recordings, made in peripheral (Boyle et al. 1994) and central (Carey et al. 1996; Jones and Nelson 1992) vestibular pathways, suggest that regenerated hair cells participate in the observed recovery of function, but no data directly assessing their functional properties have been reported.

Sensory transduction in hair cells involves a mechanoelectric transduction current that results from hair bundle deflection (Hudspeth 1983). This current modulates the resting membrane potential of the hair cell and this change in the receptor potential is encoded at the postsynaptic membrane of the innervating afferent terminal as a change in action potential discharge. The time course and intensity of the receptor potential depends on the interplay of various ionic conductances, which vary in different types of hair cells in different inner ear organs (Correia 1992) and even in different regions of the same organ (Art and Fettiplace 1987; Baird 1994; Griguer and Fuchs 1996; Masetto et al. 1994; Prigioni et al. 1996; Sugihara and Furukawa 1989).

Our understanding of the mechanisms by which vestibular hair cells regenerate is incomplete. Because only supporting (nonsensory) cells remain in the vestibular neuroepithelium after the death of all the hair cells, it has been hypothesized that through direct or indirect transdifferentiation (Baird et al. 1996; Balak et al. 1990; Raphael et al. 1994; Tsue et al. 1994; Weisleder et al. 1995) some supporting cells become regenerated hair cells. Only very recently, recordings have been made from isolated supporting cells (Sugihara and Furukawa 1996). These authors reported the presence, in supporting cells of the goldfish saccule, of only one current, called $I_{\text{r}}$. This current is different from all other ionic currents described in hair cells. However, no data exist concerning supporting cell electrophysiological properties in higher vertebrates or during sensory epithelium regeneration.

To address these issues, we recorded current and voltage responses from type II hair cells and supporting cells in control animals and in animals after streptomycin otoxicity. We recorded from these cells in situ from the semicircular canal of the pigeon. We used the conventional whole cell configuration of the patch-clamp technique in voltage- and current-clamp mode (Hamill et al. 1981) in combination with a slice of the sensory neuroepithelium (Masetto and Correia 1997). The slice approach allowed us to record for the first time from in situ supporting and sensory cells, in different regions of the neuroepithelium, during regeneration. We found that type II hair cells in control pigeons, as in frog (Masetto et al. 1994), showed topographically related passive and active membrane properties. We noted that these regionally distributed properties returned within $\sim$3 wk after streptomycin otoxicity. The conductances underlying the macroscopic currents in regenerating hair cells seemed to recover between 3 and 9 wk in a sequential order resembling that noted during the first few weeks of embryological development. At 10 wk, the macroscopic currents in regenerated hair cells were not different from those of control animals. We recorded from a few supporting cells believed to be the precursor cells of regenerated hair cells. We were unable to record any voltage- and time-dependent currents other than those noted in normal goldfish saccule (Sugihara and Furukawa 1996).

Preliminary results have been published in abstract form (Masetto and Correia 1996).

METHODS

Streptomycin injection sequence and post-injection sequence testing

Young (6–10 wk) white king pigeons (Columbia livia) were injected with streptomycin (10 days, 250 mg·kg\(^{-1}\)·day\(^{-1}\) im, $n = 28$) to kill hair cells; 22 age-matched pigeons were used as controls. Previously, we (Masetto and Correia 1997) reported anatomic studies showing the absence of almost all hair cells and hair bundles at 5 days post injection sequence (PIS) in fixed tissue and at 6 days in fresh sliced tissue. Type II hair cells with normal-looking hair bundles consistently repopulated the neuroepithelium in 2–3 wk PIS, whereas type I hair cells (with single and multiple hair cells in a calyx) consistently reappeared after 9/10 wk PIS. The above pattern of events parallels that observed in young post-hatched chickens (Weisleder and Rubel 1993). Therefore we studied the electrical properties of type II hair cells in untreated 3 and 9–10 wk PIS animals.

Slice preparation

Semicircular canals were harvested as previously reported (Correia et al. 1989) and subsequently incubated for up to 6 h (Ricci et al. 1996) in Dulbecco’s Modified Eagle’s Medium (Gibco, catalog number 31600-034) augmented with 24 mM NaHCO\(_3\), 15 mM piperazine-N,N’-bis-(2-ethanesulfonic acid), 50 mg/l ascorbate, and 1.5% fetal calf serum (Sigma) at 37°C in a saturated 5% CO\(_2\) environment. During the incubation period, between 30 min and 6 h, a membranous ampulla with an attached part of the semicircular duct was removed from the culture medium and embedded in 4% agar (wt./vol.) in a slicing solution containing 145 mM NaCl, 3 mM KCl, 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 0.1 mM CaCl\(_2\), 7.5 mM MgCl\(_2\), 50 mg/l ascorbate, and 10 mM glucose, pH adjusted to 7.4 with NaOH.

The technique used for obtaining slices of pigeon semicircular canal neuroepithelium (cristae) closely followed that reported for slicing the posterior semicircular canal of the frog (Masetto et al. 1994). Briefly, an agar block containing the ampulla and the semicircular duct was sliced in the slicing solution (partially frozen) with the use of a vibratome (Campden, Silby, Loughborough, UK). The thickness of the longitudinal slices through the neuroepithelium varied between 150 and 250 $\mu$m. The slices were transferred to a dish with a glass coverslip bottom. The dish contained an extracellular solution (composition, in mM: 145 NaCl, 3 KCl, 15 HEPES, 2 CaCl\(_2\), 1 MgSO\(_4\), and 10 glucose, pH adjusted to 7.4 with NaOH). The slices were immobilized with the use of a weighted nylon mesh. The tissue and microelectrode were viewed with the use of differential interference contrast optics employing an upright microscope (Zeiss Axioskop) equipped with a $\times 40$ water immersion objective.

Figure 1A shows a portion of the neuroepithelium from the posterior ampulla of a control animal. A type I hair cell with surrounding calyx, two type II hair cells, and two type I hair cells in a calyx can be seen. These cell types and calyces were generally distinguishable by shape alone. Where necessary, the cell types were identified by shape measurements previously described (Correia et al. 1989; Masetto and Correia 1997; Ricci et al. 1997a,b). Figure 1B shows a supporting cell with its nucleus and apical process from a control animal’s posterior ampulla.

Recordings were made from hair cells and supporting cells in selected regions or zones of the neuroepithelium of the posterior,
horizontal, and anterior semicircular canals. To maintain consistent nomenclature, we named the zones the same as those that were initially established for the bullfrog crista (Myers and Lewis 1990) on the basis of regional differences in hair bundle morphology, hair cell density, and primary afferent arborizations. We adapted the location of these zones to the pigeon neuroepithelium and chose the zones so that the areas were clearly distinct (Masetto and Correia 1997). In the vertical canals (Fig. 2, Bb and Ca), we chose as zone I regions that extended ~60 μm from the planum semilunatum and represented the peripheralmost regions of the long axes of the cristae. We chose as zone III regions about the center of the cristae (~160 μm on either side): these regions represent the apex of the cristae. We chose as zone II a region (~135 μm) at the base of the cristae that is intermediate between the peripheral and the central zones. In the pigeon, as in the frog, the horizontal crista, sectioned longitudinally, is like one of the symmetrical sides of the anterior or posterior semicircular canal crista. Therefore, as in the case of the vertical semicircular canals, we identified zones I, II, and III as regions extending from the planum semilunatum (Fig. 2Aa).

Electrical recordings

Glass blanks (Garner Glass, type 7052) were pulled to tip diameters between 0.5 and 1.0 μm, fire-polished, and partially covered with Sylgard (Dow Corning, 184). The micropipettes were filled with intracellular solution composition, in mM: 140 KCl, 2 MgCl₂, 10 HEPES, 1 CaCl₂, and 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH adjusted to 7.4 with KOH and had a resistance in the bath of 2–3 MΩ. The patch-clamp amplifier was the Axopatch 200 (Axon Instruments). A few experiments were conducted with an Axopatch-1C amplifier (Axon Instruments). Series resistance (Rₛ) and cell membrane capacitance (C_m) were read in voltage-clamp mode directly from the amplifier’s compensation dials. Series resistance was electronically compensated between 70% and 90%. The mean residual value was 2.98 ± 4.63 (SD) MΩ (n = 99). The bandwidth of the amplifier’s filter (Bessel) was set at 2–10 kHz depending on the experimental protocol. Digital sampling frequency was 2–5 times the analog bandwidth of the signal recorded. Current and voltage were measured and controlled through a DigiData 1200 Interface (AD/DA converter; Axon Instruments) that was connected to a microcomputer (486 PC) running pClamp software (version 6.0, Axon Instruments). Resting membrane potential was measured as the zero-current voltage in current-clamp mode (V_C). Cell input resistance (Rᵢ) was calculated by measuring the average current evoked by a series of seven hyperpolarizing pulses (10 mV in amplitude and 250 ms in duration) from a holding potential of ~60 mV. This voltage series yielded the largest Rᵢ. Because no time-dependent currents were evident, we considered the current measured in response to the above protocol to be leakage current. This leakage current was subsequently subtracted from the current traces. Voltages were not corrected for the liquid junction potential (3 mV negative inside the pipette). Although current-voltage (I-V) plots were not corrected for the voltage drop across the residual series resistance, all reported values of peak (Gₚ) and steady-state (Gₛ) slope conductance were corrected for the residual series resistance.

Statistical analysis

Statistical comparisons of means were made with the use of a one-way analysis of variance test (Sigma Stat, Jandel or SPSS, SPSS). If any of the variables deviated from a normal distribution, a nonparametric test (Kruskal-Wallis test) was used. Statistical significance was set at the 0.05 level unless otherwise specified.

![FIG. 1. Differential interference contrast light photomicrographs of longitudinal slices through the posterior ampullary neuroepithelia from untreated (control) animals. A: open white arrows show type I hair cells; black arrows show their calyces; black asterisks show type II hair cells; solid white arrow shows the nucleus of a supporting cell. Bar: 10 μm. B: black arrows show the nucleus and apically projecting process of a supporting cell on the left that is in the plane of focus. Open black arrows: nuclei and another supporting cell process (on right). Asterisk: type II hair cell out of the plane of focus. Bar: 10 μm.](http://jn.physiology.org/)

![FIG. 2. Representative ionic currents recorded from hair cells in a longitudinal neuroepithelial slice from each of the 3 semicircular canals: (A) a horizontal canal; (B) a posterior canal; and (C) an anterior canal. Arrows: locations of the hair cells (Aa, Ba, and Ca) within the zones from which the recordings were made. Traces of outward (Ab, Bb, and Cb) and inward (Ac, Bc, and Cc) ionic currents are also presented. Voltage protocols (Ab and Ac, insets) and calibrations are the same for all 3 cell responses. Current calibration bar: 2 nA (outward currents), 0.2 nA (inward currents). Differences in the outward and inward ionic currents between zone I (A), zone II (B), and zone III (C) can be noted by comparing the amplitude and the activation/inactivation kinetics of the inward and outward currents. PS, planum semilunatum.](http://jn.physiology.org/)
Generally, in the text, means are expressed as means ± SD (n, number of cases) unless otherwise stated.

RESULTS

Table 1 summarizes mean ± SD and n values for parameters measured from the complex ionic currents of type II hair cells in the three zones in control, 3 wk PIS, and 9–10 wk PIS animals.

Passive membrane properties of normal pigeon hair cells

All type II hair cells showed a negative resting membrane potential ($V_r$). When $V_r$ values were grouped in relation to hair cell location, a statistically significant difference ($P < 0.01$, Kruskal-Wallis Exact test) was evident. Mean values were as follows: $V_r = -62.8$ mV in cells of the periphery (zone I); $V_r = -79.3$ mV in cells of the intermediate region (zone II); and $V_r = -68$ mV in cells of the central region (zone III). The same was true for $C_m$, which was 5.9, 7.1, and 7.1 pF for zones I, II, and III, respectively. No statistically significant differences were found for $R_m$, which appeared to be quite variable (See Table 1) among hair cells located in different regions of the neuroepithelium.

Voltage-dependent ionic currents in normal pigeon type II hair cells

Figure 2 presents typical ionic currents recorded from type II hair cells in different regions of the crista in response to depolarizing and hyperpolarizing voltage steps about a holding potential of −60 mV. The hair cell location (↓) and canal of provenance are indicated for each cell. These representative currents have been chosen to illustrate that the current response depended on hair cell location in the crista, but not on the semicircular canal. The parameters of the macroscopic currents in hair cells located in zone I, II, or III of the horizontal canal ampulla were not statistically significantly different from those in hair cells similarly located in either the anterior or posterior ampullae. Thus from here on the semicircular canal of origin will not be specified and results will be grouped in relation to hair cell position in the crista only (Table 1).

Hair cells located in the three different regions produced outward ionic currents that apparently are carried by the same ion. Preliminary measurements of tail current reversal potentials (Masetto and Correia 1997) indicated that both peak and steady outward currents were mostly carried by K+ ions. Otherwise, important differences in current responses both to depolarizing and hyperpolarizing voltage steps were noted in the different regions. A clear difference, related to hair cell location, was seen for outward current activation and inactivation, which gradually became slower in hair cells, moving from the periphery (zone I) toward the center of the crista (zone III). This can be seen by comparing the outward current traces in Fig. 2, Ab, Bb, and Cb.

During hyperpolarizing steps, all type II hair cells of the central and intermediate regions expressed a significant inward rectifying current for potentials negative to −80 mV (Fig. 2, Bc andCc), whereas hair cells of the periphery expressed little (Fig. 2Ac) or none. This is further illustrated in the I-V plot shown in Fig. 3B. In a sample of eight peripheral hair cells, a small inward rectifying current was ob-

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<th>TABLE 1. Comparison of type II hair cells in different regions of the crista in normal and streptomycin-treated pigeons</th>
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Values are means ± SD; number of hair cells in parentheses. $V_r$, 0-current membrane potential; $C_m$, membrane input capacitance; $R_m$, membrane input resistance; $G_e$, total outward current peak slope conductance; $G_i$, total outward current steady-state slope conductance.
ward ionic currents showing voltage- and time-dependent current, current evoked at 20 mV after a conditioning pulse in peripheral hair cells.

Outward currents activate close to $-50$ mV, inward currents close to $-90$ mV. Calculated Nernst K⁺ equilibrium potential: $-97$ mV.

We observed at a membrane potential of $-120$ mV, it had an average peak current ($I_{\text{peak}}$) amplitude, after leakage subtraction, of $-71.8 \pm 52.6$ pA ($n = 8$). In another sample of peripheral hair cells, almost no inward current ($I_{\text{peak}}$ less than $-10$ pA at $-120$ mV, $n = 8$) could be measured. In contrast, in all hair cells of the intermediate and central regions, a much larger inward rectifier current was present. $I_{\text{peak}}$ at the same test potential was $-190.3 \pm 98.8$ pA ($n = 3$) and $-150.8 \pm 68.1$ pA ($n = 17$), in zones II and III, respectively.

We observed two different types of inward rectifying currents: one had a rapid time-dependent activation and showed partial inactivation for potentials more negative than $-120$ mV [Fig. 3B, peak ($\triangle$) and steady-state current values ($\triangle$), for the cell in Fig. 2Cc]. A second current component activated much more slowly in a sigmoidal fashion and did not inactivate at any of the potentials tested [Fig. 3B, peak currents for the cell shown in Fig. 2, Ac ($\bullet$) and Bc (■)]. The fast and the slow inward rectifying currents, respectively, strongly resemble $I_{K_1}$ (a fast K⁺-selective inward rectifying current) and $I_{K_2}$ (a slow K⁺-preferring inward rectifying current), which have recently been described in type II hair cells (see DISCUSSION). We therefore keep the same nomenclature.

**Voltage and time dependence of outward potassium currents**

Type II hair cells in all the three regions expressed outward ionic currents showing voltage- and time-dependent inactivation. Outward currents in all cells activated close to $-50$ mV and increased in amplitude with further depolarization (Fig. 3A). No inflections in any I-V curves suggestive of a possible initial N shape and thus of the presence of an $I_{K_{1(2)}}$ (Meech 1978) were found in the voltage range examined (i.e., up to 60 mV). The macroscopic currents in peripheral hair cells were dominated by a rapidly activating/rapidly inactivating conductance, whereas the macroscopic currents in central hair cells were dominated by a slowly activating/slowly inactivating conductance.

Consistent with the differences in kinetics of the dominant currents shown in Fig. 2, inactivation of fast-activating outward potassium currents in peripheral hair cells (zone I) could be strongly influenced with the use of very short conditioning potentials, whereas for zone III hair cells whose macroscopic currents were dominated by a current with slower kinetics, much longer conditioning potentials were necessary. In the peripheral hair cell whose currents are illustrated in Fig. 4Aa, short conditioning pulses of 20 ms in duration in the voltage range from $-90$ to $0$ mV were able to affect 74% of the peak current relative to that elicited with the use of a $-100$-mV conditioning voltage. Thus the percent current affected was calculated by subtracting the peak current at the test potential (20 mV) following a 0 mV conditioning voltage from the peak current at the test potential (20 mV) following a $-90$-mV conditioning voltage and then dividing this result by the peak current at the test potential (20 mV) following a $-100$-mV conditioning voltage.

By comparison, only 28% of the peak total outward currents elicited in a central hair cell (Fig. 4Ba) were affected by the same voltage manipulation. In the latter cell, conditioning pulses 250 ms in duration were necessary to affect comparable fractions (78%) of the current (Fig. 4Bb). Increasing the duration of the most negative conditioning voltages removed inactivation of even more channels, apparently without saturation in either zone I (Fig. 4Ad) or zone III (Fig. 4Bd).

Conversely, longer positive conditioning voltages were able to produce almost complete inactivation of the outward current; this is shown in Fig. 4, Ac and Bc, which illustrates the responses to 1- and 4-s conditioning voltage protocols, respectively.

With the use of similar measurements on a group of cells, the percentage of outward currents evoked at 20 mV that were affected by 20-ms conditioning voltage manipulation was on average 66.5 ± 5.5% ($n = 7$) in peripheral hair cells and 31.3 ± 5.4% ($n = 3$) in central hair cells. In hair cells of zone II an intermediate value was found: 39.8 ± 8.3% ($n = 4$). In zones III and II, conditioning steps of 250 ms affected 78.2 ± 3.8% ($n = 4$) and 75.5 ± 3.8% ($n = 6$) of the outward currents at the test potential, respectively. Thus outward currents expressed in hair cells of all crista zones inactivate as a function of time and voltage, but conditioning pulses 10 times longer are required to affect comparable fractions of potassium currents in more central hair cells compared with peripheral ones. The slope of the inactivation curves was very steep at voltages around the resting membrane potential in both peripheral hair cells (Fig. 4Ad) and central hair cells (Fig. 4Bd), indicating that small voltage changes from $V_r$ can produce large changes in current in all hair cells.

To isolate the fast component of the outward complex current, current evoked at 20 mV after a conditioning pulse...
FIG. 4. Voltage- and time-dependent inactivation of outward currents in a cell from zone I (A) and from zone III (B). Cells were conditioned at different potentials from a holding potential of −60 mV before a step to a test potential of 20 mV. Duration of the conditioning potentials: 20 ms (Aa and Ba; see insets), 250 ms (Ab and Bb), and 1 or 4 s (Ac and Bc). Duration of the test potential: 250 ms. Intertrial intervals for these 3 protocols: 5 s when the conditioning pulses were 20 or 250 ms, 20 s when the conditioning pulses were 1 or 4 s. Normalized (mean ± SE) peak current measured at the test potential vs. the conditioning voltage is presented for zone I in Ad and for zone III in Bd.
of 20 ms at −50 mV was digitally subtracted from current evoked at the same test potential but after a conditioning pulse of 20 ms at −100 mV (Fig. 5A). The difference current (Fig. 5B) was uncontaminated by the slow component, because 1) a 20-ms conditioning voltage at −100 mV did not recruit additional slow current with respect to −60 mV (Vc) but removed enough steady-state inactivation of the fast component permit it to be isolated; and 2) outward current only began to activate close to −50 mV (see Fig. 5A), so that time-dependent activation of outward current at 20 mV was the same from −100 and −50 mV. Time-dependent inactivation of current isolated as above was well fitted with a single-exponential function (Fig. 5B), whereas the original trace could not (compare the best-fit same single-exponential function with peak appropriately scaled, presented in Fig. 5A, and best-fit function to the original complex current in the inset). The average inactivation time constant (τi) of the fast outward current isolated as above was 15.3 ± 3.3 ms (n = 7) for peripheral hair cells and 17.9 ± 0.6 ms (n = 2) for intermediate hair cells. In central hair cells the fast current isolated with the same procedure was, when present, too small to be well characterized. This evidence was used in turn to characterize the slow outward current. In fact, in those central hair cells in which the above protocol did not influence the outward current, it was possible to fit complex current traces’ inactivation with a single exponential. This result implies that the slow component was the only current or at least the largely dominant current in these cells. This impression was supported by the observation that the whole complex trace could be fitted reasonably well by a sum of two exponentials, one describing activation, the other describing inactivation (Fig. 5C). Both activation time constant (τa) and τi were voltage dependent. The time constants decreased from 191 ms (τa) and 12.8 ms (τi) at −40 mV to 75 ms (τa) and 2.7 ms (τi) at 20 mV. At 20 mV, τi was on average 107 ± 28.2 ms (n = 6, i.e., ~7 times greater than the faster τa of the peripheral cells.

Times-to-peak of the complex current were used to characterize the speed of the activation kinetics of the outward currents in the different zones. The time-to-peak at a test voltage level of −20 mV from a holding level of −60 mV was on average 5.8 and 17.3 ms in peripheral and central hair cells, respectively. Intermediate values were found for hair cells of the intermediate regions (Table 1). Although time-to-peak values increased from zone I to zone III, they were not statistically significantly different. The variance of these means illustrates that the activation kinetics of the major components of the complex currents within each zone are not completely homogenous.

The predominance of a fast-activating/fast-inactivating current in zone I peripheral hair cells, implicit in the above time-to-peak regional distribution, was also suggested by the greater values of Gp/Gs ratio measurements in peripheral hair cells. Gp and Gs were calculated as peak and steady-state slope conductance of the total outward current in the linear range from −40 to 0 mV of the F-V relationship (Fig. 3A). As indicated in Table 1, Gp was on average 30% of Gp in peripheral zone hair cells, 46% of Gp in intermediate zone hair cells, and 60% of Gp in central zone hair cells. Because of the very rapid inactivation kinetics of the fast-activating current (Figs. 4 and 5) and because it has been shown that in pigeon dissociated semicircular canal hair cells there are two dominant currents (Lang and Correia 1989), Gp can be considered mostly an expression of the slowly activating/slowly inactivating current. Accordingly, a higher percentage of Gp would indicate a smaller contribution of the fast-activating/fast-inactivating current to the total outward current.

The regional distribution of Gp/Gs ratios, particularly between peripheral and more central hair cells, appears to be influenced by a differential contribution of the fast-activating/fast-inactivating current to the cell’s complex currents and, to a lesser extent, by regional variations of the slowly activating/slowly inactivating current. In fact, the amplitude of Gs was less variable between the two zones than the
amplitude of $G_p$. The value of $G_p$ decreased from 87 nS with a coefficient of variation (CV) of 0.5 in peripheral hair cells to 50 nS with a CV of 0.6 in zone III hair cells, whereas $G_s$ in the same cells was 23 nS (CV = 0.5) and 25 nS (CV = 0.3), respectively. Zone II hair cells, possibly because of the low number of cells investigated, showed intermediate $G_s$ values but higher $G_p$ values (see Table 1).

Kinetics of inward rectifying currents

As stated above, intermediate and central zone hair cells expressed two types of $K^+$ inwardly rectifying currents with different activation and inactivation kinetics. One current showed rundown and inactivation, whereas the other did not. Another difference between these two currents was the kinetics of activation. The current that showed inactivation activated rapidly and the current that did not inactivate activated more slowly. The latter current’s activation kinetics could be best fitted either by a second-order power function of the form $A (1 - \exp[-(t - k)/\tau_1])^2 + B$ (see fit in Fig. 6A, top trace) or by a sum of two exponentials: $A_1 \exp[-(t - k)/\tau_1] + A_2 \exp[-(t - k)/\tau_2] + C$, as in Fig. 6A, bottom trace. In the latter equation, $\tau_1$ (fast time constant) 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 (see Hestrin 1987). By contrast, the fast-activating/inactivating current was best fitted by a single-exponential function (Fig. 6B). Comparison of the $\tau_s$ led to values that differed by ~30 times. $\tau_s$ measured at −120 mV were 2.9 ± 1.3 ms ($n = 7$) and 92.4 ± 30.6 ms ($n = 7$) for the putative $I_{K1}$ (fast inward current) and $I_h$ (slow inward current), respectively (Fig. 6C). For both inward currents, activation kinetics were voltage dependent; $\tau_s$ decreased exponentially with increasing hyperpolarization, as shown by the linear regression of the semilogarithmic plots in Fig. 6C. Moreover, activation kinetics of the two inward rectifying currents had quite similar voltage dependence, reflected in the similar slope of the best fitted lines.

The fast-activating/inactivating component, but not the slowly activating component of the complex current, often ran down 20–30 min after membrane rupture. Thus in those hair cells that expressed both currents over time only the slowly activating current remained (Fig. 8B, inset). Presumably, the activation of the fast-activating/inactivating current depends on some intracellular molecule that is dialyzed out of the cell over time.

Electrophysiological properties of regenerating pigeon type II hair cells

Electrophysiological properties of type II hair cells in streptomycin-recovering animals were investigated at two different intervals: 3 and 9–10 wk PIS. Figure 7 summarizes the general qualitative results for outward currents. Not only do hair cells recover their ionic currents by 3 wk PIS, but regenerated hair cells at 3 wk PIS show the same topographic zonal distribution of complex currents as seen in control animals. Hair cells have been chosen from different semicircular canals to demonstrate that even during recovery the pattern of outward ionic current expression is related to hair cell location but not semicircular canal type. Quantitative comparisons of certain parameters of membrane properties are shown in Table 1, and the trends and statistical comparisons are presented in Fig. 9. The resting membrane potential ($V_r$) was not statistically significantly different in hair cells

![Fig. 6](http://jn.physiology.org/)
in any of the zones during regeneration (Fig. 9A), but the regional distribution seen in control animals was maintained. \( C_m \), in zones II (\( P < 0.05 \)) and III (\( P < 0.001 \)) and in all zones combined (\( P < 0.001 \)), was statistically significantly smaller at 3 wk PIS when compared with control and with 9–10 wk PIS animals (Fig. 9B). As in the normal epithelium, regenerating peripheral hair cells (zone I) had no (69%, \( n = 11 \)) or very small inwardly rectifying currents (mean \( I_{\text{peak}} = -88 \pm 63.7 \) pA; \( n = 5 \)) at \(-120 \) mV. But all intermediate and central hair cells had inward rectifying currents. Mean \( I_{\text{peak}} \) at \(-120 \) mV was \(-243 \pm 140 \) pA (\( n = 12 \)) and \(-244 \pm 123 \) pA (\( n = 21 \)) for cells in zones II and III, respectively. No significant differences were noted in the amplitudes of the inward rectifying currents between the 3 wk and 9–10 wk PIS animals. Two examples from central hair cells at 3 and 9–10 wk PIS are shown in Fig. 8. Traces in Fig. 8A show the fast-activating/inactivating component, whereas traces in Fig. 8B show a current consisting of both components. The \textit{inset} shows the response of the same cell during a \(-110\)-mV hyperpolarizing pulse at the beginning and 30 min after membrane rupture.

The \( G_p \) for total outward current (Fig. 9C), when all zones were combined, was similar in hair cells from normal and 9–10 wk PIS animals but statistically significantly (\( P < 0.01 \)) reduced at 3 wk PIS (Fig. 9C). This suggests that at 3 wk PIS, provided that the single-channel properties were not changed, the absolute number of ionic channels was less. When, however, the peak conductance was normalized relative to the input capacitance, no statistical difference between control cells and regenerating cells existed. This could imply that as hair cells continue to regenerate from 3 wk to 9–10 wk, ionic channels are added proportionally as the cell enlarges. \( G_p \) decreased more in hair cells located in zone I (32% reduction) when compared with zone II (29%) or III (23%). \( G_c \) was statistically significantly decreased in zone III hair cells (\( P < 0.05 \); Fig. 9D). Both \( G_p \) and \( G_c \) values reacquired their normal values at 9–10 wk PIS. The time-to-peak statistically significantly increased in zone III hair cells, where it was twice that of control animals or 9–10 wk PIS animals (Fig. 9E). These results suggest that in each zone at 3 wk PIS there is a change in the distribution of the contribution of the fast- and more slowly activating currents. That is, in each of the zones, at 3 wk PIS either the relative contribution of slowly activating/slowly inactivating component to the total current is increased or the fast-activating/fast-inactivating component makes less of a contribution to the total current. Thus a decrease in \( G_p \) would be more evident in zone I hair cells, where the complex current amplitude is strongly determined by the fast-activating/fast-inactivating component, and time-to-peak would increase especially in zone III hair cells, where the kinetics of the total current would now reflect more closely that of the slowly activating/slowly inactivating component alone. The general decrease of \( G_p \)/\( G_c \) ratio at 3 wk PIS (\( P < 0.05 \) in all zones, Fig. 9F) also corroborates this hypothesis, because a small ratio would indicate the dominance of the slowly inactivating...
current over the fast-inactivating current. Finally, during regeneration, $R_m$ did not vary systematically (Table 1). Average values of $R_m$ for cells from all zones during 3 and 9–10 wk PIS did not differ statistically from the mean for all cells in zones from the control animals ($1.9 \pm 1.7 \text{ G}\Omega$, $n = 36$).

**Electrophysiological properties of supporting cells**

A photomicrograph of a supporting cell in a slice of the semicircular canal neuroepithelium has been presented in Fig. 1B and elsewhere (Fig. 3b in Masetto and Correia 1997). Figure 10 presents representative traces from supporting cells from control (A), 21 day PIS (B) and 68 day PIS (C) animals. The nine supporting cells studied had a mean $V_r$ of $-49.5 \pm 14.1$ mV ($n = 9$), $R_m$ of $0.8 \pm 0.6 \text{ G}\Omega$ ($n = 8$), and $C_m$ of $3.7 \pm 1.7 \text{ pF}$ ($n = 6$). Compared with the type II hair cells studied here, the supporting cells had a lower $C_m$ (smaller cell body), a less negative zero-current potential, and a lower $R_m$. Moreover, supporting cells in the control animals (Fig. 10A) and animals at 3 wk (Fig. 10B) and 9–10 wk (Fig. 10C) PIS showed little if any voltage- or time-dependent inward or outward ionic currents compared with (see I-V plot in Fig. 10D) normal or regenerating hair cells, which showed strong rectification of the outward currents and consistent inward rectifying currents (if they were located in zones II and III).

An I-V plot for the supporting cell whose traces are shown in Fig. 10B is presented in Fig. 10E. Inward and outward steady-state current data points were each fit with a linear regression equation. The different slopes of these best fitted lines indicate the presence of a weak inward rectification in the current response of this cell. $G_{in}$ was $3.3$ and $2 \text{ nS}$ for the inward and outward rectification phases of the plot, respectively. However, an inward rectification was not always appreciable. Figure 11 shows, for example, a current-clamp response of a supporting cell from a normal animal, where neither the voltage traces nor the V-I plot present strong evidence for inward rectification except at the most negative voltage responses. Although not shown, the I-V plot for the same cell leads to the same conclusion. The $G_{in}$ for the cell was $0.9 \text{ nS}$.

**DISCUSSION**

In the present work, using the slice preparation, we present new data on the ionic currents in normal hair cells and supporting cells in the neuroepithelium of the pigeon’s semicircular canals. A topographic distribution of ionic currents for normal and regenerating hair cells is described in this species for the first time. Inwardly rectifying currents in the pigeon are characterized for the first time. The first records of ionic currents of vestibular supporting cells in a warm-blooded animal are presented. And this paper provides the first description in any species of type II hair cell functional properties during regeneration.

Previous anatomic studies have provided clear evidence that hair cells regenerate after ototoxic death (Corwin and Cotanche 1988; Cruz et al. 1987; Lippe et al. 1991; Ryals and Rubel 1988; Weisleder and Rubel 1993). Results from previous electrophysiological studies of eye movement responses (Carey et al. 1996), brain evoked potential responses (Jones and Nelson 1992), and preliminary recordings from primary afferent responses (Boyle et al. 1994) have suggested that hair cells recover their physiological function after regeneration. But it remained to be demonstrated that regenerated hair cells actually participated in the observed recovery of function and that hair cells recovered their basolateral ionic currents. To discuss the properties of regenerated hair cells in comparison with normal hair cells, the latter topic is examined first.

**Electrophysiological properties of type II hair cells**

The use of the slice preparation allowed us to record from hair cells of different regions of the crista in situ. We found that type II hair cells differ in their electrophysiological properties in relation to their location in the crista. More specifically: $V_r$ is less negative, $C_m$ is smaller, and a rapidly activating/rapidly inactivating current presumed to be dominated by $I_{KA}$ (Lang and Correia 1989) characterizes hair cells of the periphery (zone I) compared with more central regions (zones II and III), which have complex outward currents dominated to a greater extent by a slowly activating/slowly inactivating current presumed to be the delayed rectifier cur-
FIG. 10. Traces of current measured in supporting cells from (A) a control animal, (B) a 21 day PIS animal, and (C) a 68 day PIS animal. Recording in B was not compensated for $R_s$ and $C_m$. Corresponding voltage protocols and calibration bars are presented in each panel as insets. D: comparison between $I$-$V$ relations for 3 different hair cells (Control, 3 wk, and 9–10 wk PIS) and 1 supporting cell (3 wk PIS). Difference in outward rectification between hair cells and supporting cells is particularly evident. E: $I$-$V$ relationship for the same supporting cell whose $I$-$V$ plot has been isolated to be shown at higher magnification. Data points in the diagram: steady-state outward and inward current. Negative and positive values were separately fitted with 2 linear regression equations. Best-fit lines were extrapolated to emphasize the difference in slopes between the outward and inward regions of the $I$-$V$ plot. As indicated by parameters of the equations shown in the figure, current showed a weak inward rectification at negative potentials.

rent $I_{KD}$ isolated in pigeon semicircular canal type II hair cells (Lang and Correia 1989). Taken as a whole, the voltage dependence of activation (Fig. 3) and inactivation (Fig. 4) and the kinetics (Fig. 5) of outward currents in the slice preparation closely resemble those of outward currents described in pigeon dissociated type II semicircular canal hair cells (Lang and Correia 1989). The present results suggest that ionic currents dominated by $I_{KA}$ were expressed by hair cells dissociated from the periphery, whereas those dominated by $I_{KD}$ were expressed by hair cells coming from more central regions of the crista.

Moreover, in the slice preparation, anomalous (inward) rectifying currents are almost exclusively expressed by intermediate and central hair cells. Although generally intermediate hair cells were not very different from central cells, we preferred to keep them separate for the following reasons: First, although we found no peripheral hair cells showing the pattern of ionic currents typical of central hair cells, or vice versa, some intermediate hair cells did express outward currents not appreciably different from those of peripheral hair cells, or alternatively from those of central hair cells, and in many cases the intermediate hair cells had current kinetics that appeared to be intermediate between those of zone I and zone III (See Fig. 7). Second, we did not study as many type II hair cells in zone II as in zone III, because there appeared to be a concentration of type I hair cells in the zone II region. It was our general impression that there is a more gradual change in the kinetics of the outward currents in hair cells as they were tested in locations moving from the periphery toward the center of the crista than would characterize a strict regional segregation. Conversely, inwardly rectifying currents appeared more rigorously confined to nonperipheral regions.

The above results from peripheral and central hair cells...
feature of $I_{K_1}$ in leopard frog saccular hair cells (Holt and Eatock 1995). Besides $I_{K_1}$, a second inward rectifying current, characterized by a slower sigmoidal activation, was present in many cells of zone II and III. This current, which resembles the current $I_h$ described in frog and goldfish saccular hair cells (Holt and Eatock 1995; Sugihara and Furukawa 1996), did not undergo rundown, and therefore remained the only inward rectifying current after $I_{K_1}$ rundown after 20–30 min of whole cell recording. In other cells, $I_h$ was apparently the only inward rectifying current present, although extremely rapid $I_{K_1}$ rundown after membrane rupture cannot be excluded.

The possible role of the different $K^+$ conductances in frequency tuning has received much attention in the recent years. For example, $I_{K(Ca)}$ has been found to play a critical role in tuning the response of cochlear and saccular hair cells in lower vertebrates (Art and Fettiplace 1987; Hudspeth and Lewis 1988). This current does not appear to be expressed frequently in pigeon semicircular canal hair cells (Lang and Correia 1989; present results) or saccular canal hair cells from other species (Housley et al. 1989).

However, it cannot be dismissed that the low incidence of $I_{K(Ca)}$ might be a result of exchange of molecules between the hair cell cytoplasm and the internal solution of the micropipette, because the ruptured patch variant of the patch clamp method was used in these studies. In cells dissociated from the pigeon’s semicircular canals, only a resonance of modest quality has been observed in the physiological range of membrane potentials (Angelaki and Correia 1991; Correia et al. 1989).

In contrast to saccular hair cells (Hudspeth and Lewis 1988), at $V = -56 \text{ mV}$, 10–14% of $I_{K_{A}}$ is not inactivated in pigeon type II semicircular canal hair cells (Lang and Correia 1989). This current may play a role in the resonance even at rest and in response to small perturbations.

It has been suggested that $I_{K_{A}}$ could rapidly and transiently stabilize the membrane potential (Housley et al. 1989). It has also been suggested that $I_{K_{A}}$ could compensate for adaptation of the mechanoelectric transduction (Fuchs 1992). If this was true, then it would fit with $I_{K_{A}}$ being preferentially expressed by peripheral hair cells, which in mammals are contacted by those afferent fibers characterized by scarce adaptation (i.e., demonstrating tonic response dynamics) (e.g., Goldberg et al. 1992).

Up to now no specific role has been posited for $I_{K_{D}}$, which is $\sim 60\%$ not inactivated at $-56 \text{ mV}$ (Lang and Correia 1989) in pigeon semicircular canal hair cells. It has been suggested (Correia et al. 1989) that this current could participate in the low-quality resonance seen in dissociated pigeon type II hair cells. This current could be the principal repolarizing current for semicircular canal hair cells, especially during prolonged stimuli, when $I_{K_{A}}$ has inactivated.

It has been suggested (Holt and Eatock 1995; Sugihara and Furukawa 1996) that $I_{K_1}$ might be associated with moving the value of the resting membrane potential more negatively toward potassium reversal potential. In the present study, we have found that those hair cells in zones II and III, i.e., those more centrally located, which always expressed at least one of the inward rectifying $K^+$ currents, had more negative resting membrane potentials than peripheral hair cells, where both of the inwardly rectifying currents were smaller or missing. Moreover, in the hair cells of this study,
those expressing the rapidly activating/rapidly inactivating inward current, either in conjunction with the slowly activating inward current or alone, had on average more negative $V_h$ ($-73.6 \pm 10.3$ mV, $n = 38$) than hair cells only expressing the slowly activating inward current ($V_h = -64.4 \pm 7.1$ mV, $n = 18$) and hair cells without inward rectifying currents ($V_h = -60.7 \pm 6.2$ mV, $n = 19$).

In turtle cochlea hair cells, inward rectifying currents have been implicated in membrane resonance (Art and Goodman 1996). An active role for $I_{K1}$ has been hypothesized for hair cells in the leopard frog saccular macula, where only with $I_{K1}$ had smaller, faster responses to small current steps from rest compared with cells only expressing $I_h$. Moreover, cells with $I_{K1}$, in contrast to those with $I_h$ only, did not resonate at rest (Holt and Eatock 1995).

Because it is conceivable that different ion channels contribute in a specific manner in shaping the receptor potential, it is also conceivable that hair cells located in different regions of the crista, by expressing different arrays of K$^+$ channels, as observed in the present study, could subserve different functions. It may be that this regional distribution can eventually be related to the corresponding anatomic and functional organization noted for vestibular primary afferents (Baird et al. 1988; Boyle et al. 1991; Brichta and Goldberg 1996; Fernández et al. 1988, 1995; Goldberg et al. 1990; Honrubia 1989; Lysakowski et al. 1995; O’Leary 1976) and efferents (Boyle et al. 1991; Brichta and Goldberg 1996) in several species.

### Type II hair cell regeneration

Pigeon semicircular canal type II hair cells, at 9–10 wk after the end of the streptomycin treatment, express all the passive and active electrophysiological properties of normal cells. Therefore these cells are likely to fully recover their physiological functions. In fact, by 3 wk PIS, the ionic currents were present. This is different from type I hair cells, several of which lacked $I_{K1}$, the signature current of type I hair cells (Correia and Lang 1990; Rennie and Correia 1994), even after 9–10 wk PIS (Masetto and Correia 1997).

The topographic zonal expression of K$^+$ currents in type II hair cells was maintained during regeneration. This suggests no major migration of regenerated type II hair cells during regeneration, consistent with what was recently observed for regenerating cells in the lateral line system (Jones and Corwin 1996). At 3 wk PIS, although the zonal expression of the outward and inward K$^+$ currents was maintained, some significant differences were noted: $C_m$, for example, was on average smaller, and this was more evident for the bigger, centrally located hair cells. This result is consistent with the anatomic observation (Corwin and Cotanche 1988; Forge et al. 1993) that regenerating hair cells increase in size (at least at the apical surface) as regeneration proceeds, and parallels an analogous observation in normally developing hair cells of the bird cochlea (Tilney et al. 1986).

This result, taken together with the observation that $G_h$ was lower during this interval of regeneration, leads us to speculate that new hair cells come from smaller hair cells endowed with proportionally less K$^+$ channels and that channels are added as the cell enlarges. It is difficult for us to speculate that these precursor cells are supporting cells, because we did not observe in supporting cells any of the time- and voltage-dependent ionic channels seen in regenerating and mature hair cells. However, we sampled currents in supporting cells late in regeneration (3 wk PIS) and sampled only a small number.

At 3 wk PIS, on average $G_h$ was statistically significantly lower, $G_{Vr}$, was smaller, and time-to-peak was larger than in control and 3–10 wk PIS animals. If the fast-activating/fast-inactivating component of the current is $I_{K1}$ and the slowly activating/slowly inactivating component is $I_{K2}$, as is likely, then $I_{K2}$ was expressed before $I_{K1}$ in these hair cells during this time period. The inward rectifying currents were recorded in central hair cells during 3 wk PIS. These results suggest that the emergence of the delayed rectifier conductance and inward rectifier conductances occur before the A-type potassium conductance in regenerating hair cells. This sequence of expression resembles that noted in embryologically developing cells in the chick otocyst (Sokolowski et al. 1993). Those authors, in fact, found that K$^+$ currents appeared in the following order: $I_h$ (delayed rectifier) and $I_{Vr}$ (inward rectifier) after embryologic day nine, $I_{K1}$ after embryologic day 12, $I_{K2}$, and $I_{Vr}$ after embryologic day 13, and $I_{K2}$ after embryologic day 17. The similarity of these sequences and the similarity of the regional variation of the ionic currents in regenerating neuroepithelia suggests that the molecular cues that set up regional variation and the expression of ion channels during development are still present or are reestablished during regeneration in mature tissue.

On the basis of the present results, the interval between the expression of the different ion channels in regenerating hair cells takes weeks whereas in embryologically developing cells the events occur over the course of days. This decelerated time scale may provide a tool for studies of the molecular mechanisms of regulation of ion channel expression.

### Supporting cells

We recorded for the first time from vestibular supporting cells in situ and during regeneration. All supporting cells investigated, both in control and regenerating epithelia, showed a similar pattern of responses. The supporting cells showed no voltage- or time-dependent ionic currents in voltage-clamp recording mode and had an almost ohmic response both in voltage- and current-clamp recording modes, except that in some cells a very weak inward rectification at very negative voltages was present. The ionic currents in supporting cells in the pigeon cristae differ from dissociated nonsensory cells in the cochlea described by Santos-Sacchi (1991), which showed outward rectification, but are almost identical to dissociated vestibular supporting cells in the goldfish saccular macula recently described by Sugihara and Furukawa (1996).

Because semicircular canal supporting cells in the slice preparation had a relatively negative resting membrane potential (about $-50$ mV), their membrane should be preferentially permeable to potassium ions, whose theoretical equilibrium potential was negative enough (i.e., $-97$ mV) to provide a driving force. As suggested for fish saccular supporting cells (Sugihara and Furukawa 1996), the small inward rectification we observed could be the expression of potassium channels that are already open at $V_h$ but that were further activated at very negative potentials. Conversely,
these channels apparently did not deactivate with depolarization, because we did not observe current rectification in the outward region of the I-V relationship (at least for steps up to 80 mV and lasting 500 ms). These results support the conclusions of Sugihara and Furukawa (1996) that, as in goldfish saccular hair cells, supporting cells in the pigeon semicircular canals are most likely involved in controlling the ionic environment around hair cells' basolateral membranes and probably do not have a sensory function.

These findings also imply that if regenerating hair cells are progeny or arise by direct transdifferentiation from supporting cells (Adler and Raphael 1996; Baird et al. 1996; Raphael et al. 1994; Tsue et al. 1994), they must develop, aside from hair bundles and synaptic connections with primary afferents, a complete set of ionic channels ex novo. Moreover, this must happen after supporting cells migrate from the basement membrane, because our data, which showed no time- or voltage-dependent ionic conductances in supporting cells from control (n = 5) or streptomycin-treated animals (n = 4; 3 at 3 wk PIS and 1 at 9–10 wk PIS), come from supporting cells attached to the basal lamina. We did not record from supporting cells in the early stages of regeneration or in regions of the neuroepithelium between the basal lamina and the apical surface. If so, we might have found some intermediate cell type that showed voltage- and time-dependent conductances.

In conclusion, we studied normal and regenerating hair cells and supporting cells in a slice through the crista of the semicircular canals. In untreated controls, we demonstrated outward currents that were like those of dissociated cells but that had a zonal distribution of complex currents. We also characterized inward rectifying currents and their zonal distribution. Our results present strong support for the view that type II hair cells in the regenerating avian vestibular epithelia are able to recover their functionality. Newly produced hair cells, in fact, not only show a pattern of progressive recovery of membrane properties and ionic currents finally approaching those of control hair cells, but the regenerating type II hair cells already, by 3 wk PIS, show the normal topographic organization of ionic currents found in untreated animals. Our results from measurements of ionic currents in supporting cells led us to conclude that, as in goldfish saccule, these cells probably do not have a sensory function but maintain K+ ionic equilibrium around the base of the hair cells. Finally, the absence of voltage- and time-dependent currents in supporting cells attached to the basal lamina leads us to speculate that if these cells are the precursors of regenerated hair cells, they (or their progeny) must develop the ion channels seen in regenerated hair cells ex novo and as they migrate toward the apical surface of the neuroepithelium.

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