Ionic currents in hair cells dissociated from frog semicircular canals after preconditioning under micro-gravity conditions

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ABSTRACT
The effects of micro-gravity on the biophysical properties of frog labyrinthine hair cells have been examined by analyzing calcium and potassium currents in isolated cells by the patch-clamp technique. The entire, anaesthetized frog was exposed to vector-free gravity in a “random positioning machine” (RPM) and the functional modification induced on single hair cells, dissected from the *crista ampullaris*, were subsequently studied in vitro. The major targets of microgravity exposure were the calcium/potassium current system and the IA kinetic mechanism. The amplitude of $I_{Ca}$ was significantly reduced in micro-gravity conditioned cells. The delayed current, $I_{KD}$ (a complex of $I_{KV}$ and $I_{KCa}$), was drastically reduced, mostly in its $I_{KCa}$ component. Micro-gravity also affected $I_{KD}$ kinetics by shifting the steady-state inactivation curve towards negative potentials and increasing the sensitivity of inactivation removal to voltage. As concerns the fast, transient potassium current, IA, the I-V and steady–state inactivation curves were indistinguishable under normo- or micro-gravity conditions; conversely, IA decay systematically displayed a two-exponential time course and longer time constants in micro-gravity, thus potentially providing a larger K$^+$ charge; furthermore, IA inactivation removal at -70 mV was slowed down. Stimulation in the RPM machine under normo-gravity conditions resulted in minor effects on $I_{KD}$ and, occasionally, incomplete IA inactivation at -40 mV. Reduced calcium influx and increased K$^+$ repolarizing charge, to variable extents depending on the history of membrane potential, constitute a likely cause for the failure in the afferent mEPSP discharge at the cytoneural junction observed in the intact labyrinth after micro-gravity conditioning.

Key words: labyrinth - hair cells - microgravity - ionic currents
INTRODUCTION

The importance of gravity in influencing basic mechanisms of cell and organism physiology has been overlooked until the development of a life-supporting milieu in space has required understanding cellular and organismic dysfunction and adaptation to conditions of altered gravity. In this report we intended to explore the effects of exposing the vestibular organ, which is typically devoted to sensing rotational and gravitational information, to vector-free gravity and to subsequently study the functional modification induced by this treatment on the sensory hair cells, isolated in vitro from the crista ampullaris. The Desktop Random Positioning Machine (10) provides the closest approximation, as a biological Ground-based model, to the conditions occurring during experiments in space. The entire animal can be maintained for many hours under simulated micro-gravity conditions, preserving the sensory organ intact; this procedure spares the crucial three-dimensional structural relations, and the unique fluid conditions, of the labyrinth, thus providing a more attractive experimental model than cell-tissue cultures.

A plethora of ionic currents has been described in type II hair cells of amphibian, avian and mammalian semicircular canals (1, 4, 9, 11, 12, 13, 14, 15, 17, 18, 19, 21, 26). Functionally, the most important components are: the transduction current, mostly carried by K⁺, inward in the native cell, given the apical E_K close to 0 mV; Ca²⁺ currents, which have been described in detail at normal frog vestibular hair cells (14) and are likely to influence transmitter secretion at the cytoneural junction (14, 25); and basolateral voltage-dependent K⁺ currents. The latter are comprised of: a fast, large and rapidly inactivating current, I_A (mostly relevant in rapidly counteracting dynamic depolarizations); and a delayed current, generated by a mix of I_KV and a Ca²⁺-dependent current, I_KCa (these currents are less sensitive to inactivation by the previous history of membrane potential). Literature on the basic mechanisms involved in micro-gravity-induced electrical dysfunction of excitable cells, and hair cells in particular, is lacking. Using intracellular recordings from single afferent fibers, we have observed that the quantal and spike afferent discharge of frog labyrinthine receptors were strongly depressed following a 4 h exposure to micro-gravity (M.L. Rossi,
manuscript in preparation). Therefore, a complementary analysis has been performed on the hair cells of the *crista ampullaris*, where the sensory information is generated, with the aim of analyzing in detail the hair cell conductances potentially affected by micro-gravity. We have focussed our attention on the currents that are expected to control, by their intensity and velocity, the hair cell membrane potential migrations or the secretory activity at the cytoneural junction, thereby contributing to sensory information genesis. Thus, in this initial phase of the study, K⁺ and Ca²⁺ currents have been considered in particular.

Hair cells isolated from control and micro-gravity-exposed semicircular canals were examined to investigate whether: 1) micro-gravity by itself persistently modifies the overall current complement of the hair cell; 2) the K⁺ and Ca²⁺ currents, which interact in controlling secretory activity at the cytoneural junction, are specifically involved in the observed synaptic failure.
MATERIALS AND METHODS

The experiments were performed on wild frogs (*Rana esculenta*, 25-50 g body weight) at 20-22 °C. All procedures for animal handling and surgery were approved by the Animal Care and Use Committee of the University of Ferrara. Frog labyrinth preparation, stimulation and recording procedures have been described in detail previously (13, 14). Briefly, the animals were anaesthetized by immersion in tricaine methane sulfonate solution (1 g/l in water; Sigma) and subsequently decapitated: immediately to obtain control hair cells, or after 4h conditioning in the presence or absence of gravity. The head was pinned down at the bottom of the dissection chamber and submerged in a dissection solution of the following composition (in mM): NaCl 120, KCl 2.5, EGTA 0.5, Hepes 5, glucose 3, sucrose 20. The final pH was 7.2 and the osmolality 260 mOsmol/kg. The ampullae were isolated from both labyrinths and treated for a period of 20-30 s with subtilisin A, type VIII (50 µg/ml, Sigma); thereafter, they were transferred, in the presence trypsin inhibitor type II-S (Sigma) (final concentration 0.7 mg/ml), into the experimental chamber (500 µl volume), submerged in the standard extracellular solution (mM): NaCl 120, KCl 2.5, CaCl₂ 2, MgCl₂ 1, glucose 5, Tris buffer (pH 7.3) 5; 245 mOsmol/kg. The hair cells were mechanically dissociated from the ampullae by gently scraping the epithelium with fine forceps. Some 30 min elapsed between the end of preconditioning and the start of electrophysiology. Recordings were carried out by using the patch-clamp recording technique in the "whole-cell" configuration. Pipettes were pulled from 50 µl glass capillaries, and fire-polished to a final resistance of 3-4 MΩ. The pipette was filled with the following solution (mM): KCl 110, MgCl₂ 2, ATP (K salt) 1, GTP (Na salt) 0.1, EGTA-NaOH 5, Hepes-NaOH 10 (pH 7.2; 235 mOsmol/kg). When calcium currents were recorded, the composition of the chamber solution was (mM): 100 NaCl, 6 CsCl, 20 TEACl, 4 CaCl₂, 10 HEPES, 6 glucose (pH 7.2); 260 mOsmol/kg. Pipette solution was as follows (mM): CsCl 90, TEACl 20, MgCl₂ 2, ATP (K salt) 1, GTP (Na salt) 0.1, EGTA-NaOH 5, Hepes-NaOH 10, (pH 7.2; 235 mOsmol/kg).
Isolated hair cells exhibited high membrane resistance (> 1 GΩ) at –70 mV. These values indicate that no major conductances, such as hyperpolarization-activated currents or current through transduction channels (possibly open at rest in the native hair cell), were significantly active. Series resistance ranged between 8 and 22 MΩ. The cell capacitance and series resistance were electronically compensated (50-75%) before each voltage-clamp protocol was run. The uncompensated series resistance component introduced an error in the applied voltage command, which was limited to 10-12 mV when the largest currents were recorded. Leak was measured near resting potential with a 10 mV × 15 ms hyperpolarizing pulse; this leakage current was subsequently subtracted, assuming a linear behavior, in correcting current recordings off-line.

Cd^{2+} (200 µM) was used as blocker of voltage-dependent calcium channels. It was applied by rapidly (typically < 50 ms) changing the external solution by horizontally moving (with a computer-controlled stepping motor) a multi-barrelled perfusion pipette positioned in front of the recorded cell.

Cells were viewed through a TV monitor connected to a contrast enhanced video camera (T.I.L.L. Photonics, Planegg, Germany). The camera was coupled to an inverted microscope (Olympus IMT-2, Tokyo, Japan) equipped with a 40× Hoffman-modulation contrast objective. The current was recorded with a commercial patch-clamp amplifier (EPC-7, List-Electronic, Darmstadt, Germany); the command protocol and data acquisition were performed with a Digidata 1322A computer interface and a pClamp package (Version 9.0) running on a Pentium computer. The recordings, filtered at 2, 6, or 10 kHz via an eight-pole Butterworth filter, were acquired at 5, 12.5, or 40 kHz, respectively, and stored on disk. Mathematical procedures were implemented with Matlab (Version 5.3, The Math Works, Natick, MA, USA).

Voltage-dependent parameters were fitted throughout by Boltzmann equations of the form

\[ B(V) = A \cdot \left[ 1 + \exp(-b \cdot (V - V_{50}) \cdot F/RT) \right]^{-1} \]

where \( A \) is maximum amplitude, \( b \) is the slope.
coefficient and $V_{1/2}$ is the value of potential for which the equation has half its maximum value ($B(V_{1/2}) = A/2$).

The differences among various experimental conditions were examined by two-way ANOVA. When necessary, logarithmic transformation of data was performed to homogenize variances. This was necessary for current amplitudes, which markedly varied with membrane potential and generally displayed, at each value of potential, a variability among cells roughly proportional to the average value. Values of $F$ and $P$ are reported in the text for significance of treatment effect; contrasts between pairs of treatments were examined by Student’s $t$-test; significant differences ($P < 0.05$, two tails) are reported as either absolute or percentage change with 95% confidence limits (CL95). In the figures, data are presented by pooling the results obtained from several cells under each experimental condition. Average values and SEM are plotted for each condition.

Micro-gravity simulation

The anaesthetized frog was exposed to micro-gravity treatment by using a Desktop Random Positioning Machine (RPM). The frog was mounted on a custom-made tablet fixed to the internal frame of the apparatus so that the rotation axis of the inner frame was close to the axis of the posterior canal (antero-posterior or cranio-caudal vector). This way, rotation axes cross the midline point of the line that connects the centres of the acoustic membranes of the frog (both labyrinths are positioned about 5 mm apart from the precise centre of rotation). Micro-gravity can thus be simulated by producing a pseudo-random three-dimensional pathway of rotation. This is achieved by coupling two square frames: the outer frame rotates around a horizontal axis; the inner frame rotates within the outer one, around a perpendicular axis. The combined rotation of both frames produces a continuous repositioning of the preparation with respect to the gravity vector (vertical axis), and a condition of virtual absence of gravity in the geometrical centre of rotation. The frog was mounted on the table so that (i) the rotation axis of the inner frame was coincident with the antero-posterior (cranio-caudal) midline vector (thus, parallel and close to the axis of the posterior canals), and (ii) the rotation axis of the outer frame passed through the centres of the two acoustic
membranes. Under this experimental condition, the labyrinths were exposed to simulated absence of gravity. The average rotational velocity contributed a relative centrifugal force $\leq 0.001 \times g$. This treatment will be referred to as “micro-gravity” (4-h stimulation in simulated zero-gravity).

In order to isolate the effect of gravity from generic mechanical stimulation, a set of control preparations were obtained by exposing frogs to random-path rotations (time-varying angular velocity = a constant + bandwidth limited pseudo-random noise) in the sole horizontal plane, with the same overall angular velocity variance (and acceleration variance as well, i.e. an equivalent intensity of overall mechanical stimulation). Thereafter, the stimulated canals were used to isolate hair cells. This control treatment will be referred to as “gravity” (4-h stimulation in normal gravity). Untreated animals were also examined (no stimulation: “control”).
RESULTS

Hair cell passive properties

Hair cells of several different morphologies have been observed in the frog crista ampullaris, and position-specific differences have been reported for some bioelectrical properties of hair cells (15, 26). In dissociated hair cells no indication could be obtained about the original position within the crista ampullaris; only the cell type could be recognized. The cells used in this study were classified, and their passive parameters measured, as indicated here:

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell input Resistance GΩ</th>
<th>Cell input Capacitance pF</th>
<th>Zero current membrane potential mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 98)</td>
<td>1.6 ± 0.1</td>
<td>9.8 ± 0.4</td>
<td>-72.3 ± 2.3</td>
</tr>
<tr>
<td>Cylinder-like</td>
<td>2.0 ± 0.1</td>
<td>8.7 ± 0.7</td>
<td>-81.6 ± 1.6</td>
</tr>
<tr>
<td>Micro-gravity (n = 50)</td>
<td>1.9 ± 0.2</td>
<td>10.8 ± 0.7</td>
<td>-73.5 ± 3.3</td>
</tr>
<tr>
<td>Control (n= 40)</td>
<td>1.4 ± 0.2</td>
<td>9.9 ± 0.6</td>
<td>-67.1 ± 3.4</td>
</tr>
<tr>
<td>Pear-like</td>
<td>2.1 ± 0.4</td>
<td>8.2 ± 0.8</td>
<td>-71.8 ± 4.2</td>
</tr>
<tr>
<td>Micro-gravity (n= 14)</td>
<td>1.9 ± 0.8</td>
<td>9.0 ± 1.9</td>
<td>-70.2 ± 1.2</td>
</tr>
<tr>
<td>Control (n= 9)</td>
<td>0.9 ± 0.5</td>
<td>9.0 ± 0.4</td>
<td>-72.1 ± 3.6</td>
</tr>
<tr>
<td>Club-like</td>
<td>1.9 ± 0.4</td>
<td>10.1 ± 0.5</td>
<td>-73.5 ± 7.4</td>
</tr>
<tr>
<td>Micro-gravity (n=5)</td>
<td>2.9 ± 0.3</td>
<td>8.8 ± 0.1</td>
<td>-68.7 ± 4.0</td>
</tr>
</tbody>
</table>

These data indicate that the basic resting properties of the isolated hair cells that survived the dissection procedures were relatively homogeneous and unaffected by the previous treatments under micro-gravity or normal gravity conditions. As previously reported (12), the magnitude and kinetic properties of single ionic currents did not display any consistent differences and/or correlations in the different cell types. No further attention has been therefore paid to the specific cell type in subsequent analyses and comparisons among the experimental conditions.
**Distinctive current properties**

Compound currents are evoked by depolarizing the hair cell. The mix of currents that are actually activated, however, strictly depends on the membrane potential level prevailing before the depolarization; this is particularly evident for the potassium currents. A pivotal role is sustained by the transient IA; its inactivation properties rule out any IA participation when currents are evoked from holding levels positive to -40 mV, whereas its contribution becomes progressively larger at more negative potential, as inactivation is removed.

In control hair cells, command steps from a holding potential of -40 mV (as in Fig. 1A) evoked summated currents comprised of inward calcium current ($I_{Ca}$) and two outward potassium currents (the slow $I_{KV}$ and the calcium-dependent $I_{KCa}$). The compound current rapidly built-up in the tracings to a maximum value, that was monotonically related with the amplitude of the command voltage step, $V_C$ (Fig. 1A). At later times, a steady level was reached and maintained over time. This steady level increased with $V_C$ up to +40mV, but its magnitude decreased for more positive voltages, giving rise to an “N-shaped” I-V relation (Fig. 1A and 2A). At very positive command potentials a component of $K^+$ currents rapidly set off and vanished; this component might be sustained by $Ca^{2+}$ dependent channels, and its time course related to the kinetics of $Ca^{2+}$ inflow, characterized by an increasingly fast onset and rapid decrease and fading as the command membrane potential approaches the $Ca^{2+}$ equilibrium potential. In the presence of Cd$^{2+}$ (200 μM, a powerful blocker of any voltage-dependent $Ca^{2+}$ channels), a major fraction of the current was cancelled and the initial transient peaks at highly positive potentials were abolished, thereby confirming the role of $Ca^{2+}$ inflow in controlling this particular feature of $K^+$ currents. Representative current tracings in the presence of external Cd$^{2+}$ are shown in Fig. 1C. The current time courses became homogeneous, the initial transient peak virtually disappeared, a monotonic relation of current to voltage became apparent with no sign of the “N”-behavior of control cells. The current variance, which paralleled the steady-state I-V curve in control cells, was drastically
reduced by Cd\(^{2+}\), suggesting that a major contribution to current variance arose from Ca\(^{2+}\)-activated channels (presumably BK type, in particular, due to their large conductance: the variance/mean ratio is proportional to single-channel conductance).

The hair cells extracted from preparations previously exposed to micro-gravity exhibited an overall behavior reminiscent of the normal untreated cells in the presence of Cd\(^{2+}\) (Fig. 1B): the I-V plot was monotonic, a small transient initial peak was present in single current tracings but no large peaks occurred at very positive potentials (Fig. 2B), the “N” shape of the I-V curve was cancelled (Fig. 2A), and the current variance increase with progressively positive voltage steps was small (Fig. 2C).

The hair cells dissociated from semicircular canals stimulated in the presence of gravity exhibited intermediate current properties between control and micro-gravity-conditioned cells: the “N” profile of the I-V curve was still present but less pronounced compared to controls, the early current peak at positive potentials was attenuated, and the current variance was similarly reduced.

From these data, it appeared that labyrinth stimulation produced marked changes in steady-state current amplitude. In general, for each group of cells, the standard deviation of steady state current was roughly proportional to the average, at any value of potential; thus, ANOVA was performed after homogenizing variances by logarithmic transformation of the data. The difference among treatments was confirmed (two-way ANOVA: \(F = 31.0, P < 0.01\)). In particular, stimulation under normo-gravity produced a 28% decrease (CL95 = −9-43%), compared to controls; micro-gravity conditioning resulted in a 68% decrease (CL95 = −58-76%) compared to controls, and in a 56% decrease (CL95 = −44-65%) with respect to normo-gravity. Similar results were obtained when either the amplitude of early peak (\(F = 49.0, P < 0.01\)) or the steady-state current variance (\(F = 19.9, P < 0.01\)) were considered. The transient peak amplitude was reduced by 26% (CL95 = −8.2-40 %) in gravity-conditioned hair cells, compared to controls; stimulation performed under micro-gravity conditions produced a decrease by 75% (CL95 = −67-81%) with respect to controls, and by
67% (CL95 = −58-73%) with respect to normo-gravity stimulation. The current variance also decreased with stimulation under either normo-gravity (by 81%, CL95 at least −58%) or micro-gravity (by 88%, CL95 at least −55%), with respect to control, but in this case the difference between the two stimulation conditions did not reach statistical significance. The voltage dependence of current variance also displayed a significant difference among treatments (F = 2.2, P<0.01), and in particular it was reduced after normo-gravity and even more after micro-gravity conditioning.

The calcium current

Attention was thereafter focussed on the Ca$^{2+}$ current, which is expected to play a central role in controlling both quantal release at the cytoneural junction and the potassium conductance of the hair cell basolateral membrane. Calcium currents were readily recorded in control and micro-gravity-conditioned hair cells (two typical I$_{Ca}$ families are illustrated in Fig. 3A). They usually exhibited a large variability in amplitude and shape (14), so that a relatively large number of observations had to be collected. An initial transient current peak was in fact discernible in 17/44 control hair cells and only in 7/48 of the cells exposed to micro-gravity (statistically different frequency; chi squared test: $P < 0.001$). In all the tracings, the inward current settled to a plateau level: 78.0 ± 9.5 pA in control hair cells and significantly lower (47.0 ± 7.0 pA, $P < 0.01$) in micro-gravity-exposed cells. The histograms of the I$_{Ca}$ amplitude distributions in control and micro-gravity-conditioned hair cells are shown in Fig. 3B. The two distributions proved statistically different (Kolmogorov-Smirnov test: $D = 0.28$, $P = 0.05$), confirming that the micro-gravity conditioning produces a decrease in I$_{Ca}$. The average I-V plot of the nine largest I$_{Ca}$ measured in control hair cells (circles) was compared (Fig. 3C) with that of the eight smallest currents observed in micro-gravity-conditioned hair cells (triangles); in Fig. 3D the same curves are normalized to the maximum current amplitude in each cell: it clearly appears that, although I$_{Ca}$ current is much reduced, its voltage-dependence is unaffected by the micro-gravity treatment, especially in the voltage range of physiological interest.
Some differences did appear for the largest voltage commands, suggesting that control hair cells might exhibit a more positive equilibrium potential for calcium ions ($E_{Ca}$). This would be consistent with lower intracellular Ca$^{2+}$ levels. It should be considered, however, that with large positivity some unspecific caesium ion permeation might occur through the calcium channels (14), so that the experimental null point of Fig. 3C actually reflects the balance between Ca$^{2+}$ and Cs$^+$ flows. The decrease in calcium current might thus contribute to make the apparent $E_{Ca}$ less positive in micro-gravity-conditioned hair cells.

The activation and deactivation properties of $I_{Ca}$ have been analyzed to better define possible kinetic modifications. The mono-exponential activation time constant of the current at -10 mV was $1.23 \pm 0.07$ ms ($n = 6$) in control preparations and was not altered ($1.18 \pm 0.15$ ms) in micro-gravity-conditioned hair cells ($n = 6$). Current deactivation ensued with a two-exponential time course: in the same cell sample, with -10 mV command potential the fast time constant was $1.58 \pm 0.30$ ms in controls and $1.18 \pm 0.15$ ms in micro-gravity-exposed hair cells, while the slow time constant was $8.33 \pm 0.65$ in controls and $8.42 \pm 2.19$ ms after micro-gravity treatment. It would thus appear that micro-gravity results in a decrease of $I_{Ca}$ amplitude, which is simply scaled down with respect to controls, without any relevant modification of the basic activation-deactivation processes.

**Transient and persistent potassium current dissection**

Hair cells express a fast transient potassium current, $I_A$, which is rapidly and completely inactivated at potentials above -40 mV. Hyperpolarizing conditioning pulses remove this inactivation. Following such conditioning, positive voltage pulses evoke a mix of currents in which delayed currents summate to $I_A$. A simple procedure to isolated the $I_A$ component consists in subtracting the tracings without preconditioning to those obtained after preconditioning at negative potentials. This current subtraction procedure was successfully applied in the past (2, 3), but it is valid only provided that $I_{KD}$ does not display any inactivation. We have recently shown that the delayed current actually exhibits a slow inactivation mechanism in frog hair cells; both the development and
the removal of inactivation display a slow kinetic component (time constants of several seconds) and, in addition, the slow recovery mechanism ensues with a substantial delay (12). If this aspect is not taken into account, the slow time- and voltage-dependent inactivation of \( I_{KD} \) complicates the usual procedure of IA dissection (12). However, an efficient voltage protocol can be devised to dissect and study the IA and delayed currents in isolation, over a wide voltage range in the same cell, in the absence of any pharmacological treatment (12). An example of this procedure is illustrated in Fig. 4 for a cell previously exposed to micro-gravity conditioning. The protocol is based on applying pairs of identical voltage steps, separated by a 300 ms sojourn at –40 mV, and exploring the range of \( V_C \) from –20 to +80 mV (Fig. 4). In A the cell was held for 30 s at -40 mV, to fully inactivate IA and the fraction of delayed currents that is susceptible to inactivation; the series of paired steps was subsequently applied (a and b). In Fig. 4B (a different cell), a 1-s conditioning pulse at -100 mV was applied before each pair of steps, to remove inactivation of both IA and delayed currents; the two currents were thereafter evoked together in each trial (Fig. 4B,cd). The interval between the two test pulses in each cycle features a sufficiently positive voltage (–40 mV) to maintain full IA inactivation and a duration which minimizes the development of slow current inactivation (due to its significant delay in onset): thus the second test pulse only evokes pure and virtually unaffected \( I_{KD} \) components. In the cell shown in A, the difference current (a–b) is almost zero for any current pair. In some cells, however, a marginal amount of \( I_{KD} \) inactivation is detectable during the protocol, as revealed by a slight decrease in current amplitudes at the end of the second test pulse (Fig 4B,f) with respect to the end of the first one (Fig. 4B,e). The second pulse currents (Fig. 4B,d) can be corrected for this slight amplitude drift, so that the difference currents (c-d(f-e) in Fig. 4B) dissect out the complete family of IA curves in isolation.

**Comparison of IA general properties**

By applying the procedure just described, separate I-V curves were obtained in control and micro-gravity-conditioned hair cells for peak IA and maximum \( I_{KD} \) over the -50/+80 mV voltage range;
they are shown in Fig. 5, A and B (control: circles; micro-gravity: triangles). As concerns IA, the current appeared to be significantly activated in all cells at membrane potentials more positive than -40 mV; above this value, its amplitude increased monotonically with increasing depolarization. The I-V plots of Fig. 5A are virtually coincident in control and micro-gravity-conditioned hair cells; From these curves a rough estimate of the gA conductance vs. voltage relationship was obtained by using the relation \( g = I/(V - V_K) \), with \( V_K = -96 \) mV, as is the case for pure potassium currents. Conductance data points were fit in control hair cells by a Boltzmann-type equation with the following parameters: size, \( g_{A_{max}} = 19.3 \) nS; centre voltage, \( V_{1/2} = -7.7 \) mV; slope factor, \( b = 1.1 \). The corresponding values in micro-gravity-conditioned cells were \( g_{A_{max}} = 21.1 \) nS; centre voltage, \( V_{1/2} = -14.1 \) mV; slope factor, \( b = 1.2 \).

A first-order kinetics could be systematically detected in the current rising phase; at -40 mV the rise time constant was 2.5 ± 0.4 ms in controls (n = 9) vs. 2.3 ± 0.4 ms in micro-gravity (n = 11); its value became 1.1 ± 0.3 ms vs. 1.3 ± 0.5 ms at 0 mV; 0.8 ± 0.3 ms vs. 0.6 ± 0.1 ms at +40 mV; 0.6 ± 0.2 ms vs. 0.4 ± 0.1 ms at +80 mV.

Once activated, the IA rapidly declined to zero, but the decay pattern was different depending on command voltage and preconditioning: the results are summarized in Fig. 7A. In control and normo-gravity-conditioned cells the decay time course of the current was mono-exponential for command potentials in the range -40/+0 mV, whereas it was clearly bi-exponential at positive voltage levels in 10 out of 12 examined cells, i.e. a late slow phase of decay ensued. In micro-gravity-conditioned cells the decay of IA was bi-exponential at all tested voltages (though the -40 mV point was ill-defined) and the mean values of the slow decay time constant were larger compared to controls (Fig. 7A, upper panel). When the slowly decaying current component was present, the total charge it carried (\( Q_s \)) was always larger than that carried by the fast component (\( Q_f \)). In particular, the \( Q_s/Q_f \) ratio (indicated by the numbers in Fig. 7A) was systematically larger after micro-gravity treatment than in control hair cells. In the voltage range in which the slow component was present in both groups (+20/+60 mV), ANOVA analysis showed that the slow decay time constants were
statistically different \((F = 5.76, P < 0.05)\), and in particular they increased in micro-gravity by 44\% (CL95 = +10-79 \%).

**Comparison of \(I_{KD}\) general properties**

The delayed currents were investigated either when evoked from holding potentials sufficiently positive to inactivate the IA \((-40\text{ mV} \text{ in the examples of Fig. 1})\) or from any more negative holding voltage level \((-100\text{ mV} \text{ in Fig. 4B})\), after applying the isolation procedure described in Fig. 4. It should be noted, however, that the -100 mV preconditioning removes not only IA inactivation but also \(I_{KD}\) inactivation (though fractionally less relevant). As reported above, the delayed current, which was originally thought to be exempt of inactivation, exhibited a well detectable process of slow recovery from inactivation; in control hair cells such recovery only ensued after a delay of about 300 ms and developed following a single exponential time course with a time constant in the order of seconds, thereby producing a consistent final increase of \(I_{KD}\) amplitude after prolonged sojourns at well polarized potentials (12). By comparing the \(I_{KD}\) current amplitude presented in Fig. 2A (holding potential \(-40\text{ mV}, \text{steady state } I_{KD} \text{ inactivation present}\)) with those in Fig. 5B (1s sojourn at \(-100\text{ mV}, \text{partial inactivation removal}\)), it turns out that that the typical “N” shape of steady state current vs. voltage (illustrated for control cells in Fig. 2A) was attenuated by negative preconditioning, and that inactivation removal had limited effects on the amplitude of currents in control hair cells. Conversely, in microgravity-conditioned cells, inactivation removal brought about a dramatic increase in \(I_{KD}\) amplitude, bringing it up to values comparable to control cells (an increase by a factor of 3.1 at +0 mV, and 3.7 at +40 mV, with respect to cells held at \(-40\text{ mV}\)). It appears therefore that the steady-state \(I_{KD}\) amplitude is markedly reduced in microgravity-conditioned hair cells, at \(-40\text{ mV}, \text{but } I_{KD} \text{ inactivation mechanism becomes so sensitive to high membrane potential negativity that 1s at -100 mV is sufficient to produce a recovery of steady state current amplitude to values close to those of controls.\text{.}**
For mild depolarizations the current onset was usually so fast that it was hardly resolved from the capacitive transients; the peak amplitude was rapidly attained and was maintained constant during the whole voltage step, giving a virtually rectangular shape to the tracings evoked in the range -40/+0 mV (Figs. 1, 4, 6). For large positive pulses the current time course was complicated with increasing frequency by the onset of a transient peak (Figs. 1, A and B) and/or the occurrence of a mild current decay during long-lasting pulses. This general description regards both control and microgravity-conditioned cells, although in the latter group six out of 22 cells exhibited a slow onset over the -20/+80 mV voltage range, with a single time constant little sensitive to voltage (9.5 ± 4.1 ms at 0 mV, 7.3 ± 1.1 ms at +80 mV).

The I-V plots of Fig. 5B yield an estimate of the $g_{KD}$ conductance-voltage relationship, which is described by a Boltzmann-type equation with the following parameters: $g_{KD_{max}} = 7.9 \text{nS}$, $V_{1/2} = -29.3 \text{mV}$ and $b = 2.2$ in control; $g_{KD_{max}} = 9.1 \text{nS}$, $V_{1/2} = -16.6 \text{mV}$ and $b = 1.6$ in microgravity-exposed hair cells.

*Isolation and comparison of $I_{KCa}$ amplitudes*

The possible involvement of the $I_{Ca}/I_{KCa}$ system in the microgravity-induced dysfunction urged a more detailed analysis of these current components. The data presented in Fig. 3B indicate a significant reduction of $I_{Ca}$ peak amplitude after microgravity conditioning; the differences versus the controls, however, were relatively limited, and the cell sample required to reliably quantify them so large to discourage any extension of this type of analysis to the hair cells stimulated in the presence of gravity. Thus, as an alternative approach, we preferred to consider the effect of calcium movements on the calcium-dependent potassium conductance under control-, normogravity- and microgravity-conditions, rather than directly studying the calcium inflow. The calcium-dependent fraction of $I_{KD}$ in single hair cells was evaluated in a new set of experiments by applying 200 µM Cd$^{2+}$ by fast superfusion: the difference current recorded before and after blockade of Ca$^{2+}$ influx isolates $I_{KCa}$ at each command potential. Considering that the apparent equilibrium potential for
calcium ion is about +40 mV in these cells (14) and that the N-shaped behaviour of the compound current above this membrane potential level was not systematically observed in all the units, we analysed the $I_{KCa}$ families in the limited range from –40 to +40 mV, with the aim of focussing on the direct priming effect of calcium inflow on the $I_{KCa}$, rather than on the subsequent voltage-dependent $I_{KCa}$ development. The mean I-V curves for the pure $I_{KCa}$ are shown in Fig. 6A for the three experimental groups.

ANOVA was performed after homogenising variances by logarithmic transformation of the data. The difference among treatments was analysed (two-way ANOVA: $F = 25.2$, $P < 0.01$). In particular, stimulation under normogravity produced a 21% decrease of $I_{KCa}$, compared to controls, but the difference between the two conditions did not reach statistical significance; micro-gravity conditioning resulted in a significant 76% decrease (CL95 > 44%) compared to controls, and in a 69% decrease (CL95 > 23%) with respect to normogravity. The distributions of the individual $I_{KCa}$ amplitudes measured in the three groups are shown in Fig. 6B; they proved statistically different (Kolmogorov-Smirnov test: $D = 0.6$, $P = 0.01$) only when comparing control and microgravity-conditioned hair cells, confirming that microgravity produced a rather specific impairment in the $I_{Ca}/I_{KCa}$ system.

**Steady-state inactivation and IA inactivation removal**

The steady-state value of the inactivation process was studied by the conventional method of varying the holding potential and depolarizing the cell to a fixed test voltage. The double step procedure illustrated in Fig. 4 dissects pure IA tracings (Fig. 7B) by using the subtraction protocol and yields the corresponding families of $I_{KD}$. Steady-state inactivation is classically expressed by an “$h_\infty$” parameter comprised between 0 (full inactivation) and 1 (inactivation completely removed). The steady-state inactivation curves ($h_{IA}$ for IA and $h_{KD}$ for $I_{KD}$, vs. holding potential) are illustrated in Fig. 5, A and B (open circles: control; open triangles: microgravity-exposed cells) and show the mean values measured for each of the two currents in individual cells ($n = 6$-7). To obtain
peak IA currents were normalized to their largest value; for \( I_{KD} \) only the fraction subject to inactivation was considered, and \( h_{kd} \) was therefore computed considering only the increments of current amplitude above the values observed at -40 mV. The continuous lines through data points depict normalized Boltzmann-type equations; the coefficients of the curves fitting \( h_{ae} \) were \( V_{\frac{1}{2}} = -76.8 \text{ mV} \), \( b = -2.2 \) in control vs. \( V_{\frac{1}{2}} = -75.1 \text{ mV} \), \( b = -2.6 \) in microgravity-exposed cells; for \( h_{kd} \) the corresponding values were \( V_{\frac{1}{2}} = -80.9 \text{ mV} \), \( b = -2.3 \) in control, vs. \( V_{\frac{1}{2}} = -88.7 \text{ mV} \), \( b = -3.1 \) in microgravity-exposed cells. ANOVA analysis indicated that the curves for microgravity-exposed hair cells were not different from control as regards \( h_{ae} \); conversely, the difference was statistically significant for \( h_{kd} \) curves (\( F = 7.33, P < 0.01 \)), the curve for microgravity-exposed hair cells being shifted to the left and inactivation removal being apparently more sensitive to voltage.

The time course of removal of IA inactivation was investigated in double-pulse experiments by holding the cells at -40 mV (IA fully inactivated) and stepping to +20 mV after a conditioning pulse (at -70 or -100 mV) of variable duration. Peak IA was computed by subtracting to these current recordings the current measured without pre-pulse. A fast process of IA recovery (inactivation removal) was apparent, with a single exponential time course (see Fig. 8A). The mean value of its time constant, \( \tau_{hA} \), was 18.2 ± 1.6 ms (n = 6) in control vs. 21.2 ± 2.0 ms (n = 8) in microgravity-exposed cells at -100 mV (12 ms and 14 ms in the particular examples in Fig. 8, C and D, filled circles); fast recovery was complete within about 0.5 s in all tested hair cells, and was stable thereafter. In this same range of preconditioning pulse durations, no changes were observed in \( I_{KD} \) amplitude (see Fig. 8, A and B), thereby indicating that possible inactivation of delayed K\(^+\) currents was not removed by brief preconditioning pulses.

IA recovery was studied also at -70 mV, a membrane potential close to the zero-current level of the isolated cells. Again, inactivation removal ensued in controls with a single-exponential time course; the mean value of \( \tau_{hA} = 18.3 ± 4.6 \text{ ms} \) was virtually identical to the value observed with pre-pulses at -100 mV. Quite a different behavior was observed in microgravity-exposed hair cells: IA inactivation removal became systematically slower, displaying a mean time constant of 130.9 ±
Furthermore, the time course of recovery often became more complex: in 4 out of 8 cells of the sample a second fast time constant became discernible, with a mean value of $11.7 \pm 3.4$ ms (an example in Fig. 8D, open circles). In addition, a small rapidly inactivating current (therefore identifiable as IA) was observed in 5 out of 8 cells even starting from $-40$ holding potential with no or very brief hyperpolarizing pre-pulse, indicating incomplete inactivation of IA at $-40$ mV in these cells (see the example in Fig. 8D). This unexpected behavior, however, appears to be related to stimulation rather than to microgravity per se; in fact, similar results were observed (with $-70$ mV pre-pulses) in 3/8 hair cells which had been stimulated under normo-gravity conditions.

*Sinusoidal voltage commands mimic the physiological membrane potential shifts*

The zero current membrane potential, about $-70$ mV in our experiments, can be considered close to the cell membrane potential of the “silent” hair cell, i.e. in the absence of active ionic conductances and receptor current flow. This membrane potential value, however, would be obtained in the native cell only by closing the transduction channels, that are instead open at rest and maintain the cell sufficiently depolarized (in the range $-50/\text{-}40$ mV) to sustain a basal calcium influx which is responsible for transmitter release at the resting cytoneural junction. The membrane potential of the hair cell is thus expected to fluctuate physiologically from a ‘resting’ status close to $-40$ mV (cilia in an intermediate position, a fraction of the receptor transduction channels open), towards $-70$ mV, when all channels are closed by the inhibitory deflection of the cupula, or towards a more positive voltage level after deflection of the sensory hair bundle and activation of additional transduction channels, during the excitatory movements of the cupula.

We mimicked the receptor potential time course by driving the hair cell membrane potential with a sinusoidal waveform that fluctuated from $-70$ to $-10$ mV, to simulate the excitatory/inhibitory deflections of the hair bundle, or from $-40$ to $-10$ mV to simulate the excitatory effects only (Fig.9). The currents evoked by the voltage swing down to $-70$ mV exhibited clear-cut asymmetries during
the sinusoidal voltage shifts: the hyperpolarizing portion of the command effectively removed IA inactivation, causing an initial excess in outward current during depolarization, due to IA activation, that appears as a shoulder in the current profile (Fig. 9, A - C). We measured the ratio between the total outward charges evoked at membrane potential levels positive to -50 mV during the depolarizing vs. the repolarizing phase of the command (we indicated this ratio as ‘hysteresis index’, h.i.). Conversely, when membrane potential fluctuated within the -40/-10 mV voltage range, active currents operated during the whole cycle and the on-off phases became virtually symmetrical, since IA was inactivated all over.

We evaluated the hysteresis index in control, either in the absence or in the presence of external Cd²⁺, in microgravity and in normogravity-exposed hair cells. Representative tracings are shown in Fig. 9 (only -70/-10 mV cycles) and the results are summarized in Table 1. Direct comparison of the h.i. values shows that significant asymmetries arose systematically and exclusively during the -70/-10 mV cycles, independent of the experimental condition tested. Hysteresis was particularly prominent in control hair-cells exposed to external Cd²⁺ and in microgravity-conditioned hair cells. In particular, the hysteresis index in the microgravity group was significantly higher than in controls as well as in the gravity group (P values reported in Table 1).

In control cells some hysteresis was clearly evident during the first cycle, but appeared to vanish during the subsequent cycles of rotation, suggesting that at 1 Hz the 0.5 s inhibitory half cycle (and the very brief sojourn close to −70 mV) was not sufficient to remove IA inactivation produced by the excitatory phase of the previous rotation cycle. The same occurred when stimulation in the RPM machine was performed in normal gravity conditions, whereas control cells in the presence of Cd²⁺ and cells exposed to microgravity displayed some hysteresis during the second and third cycle of rotation as well; this suggests that under these conditions IA is at least partly recovered with faster kinetics. This would be in agreement with the observations discussed above and illustrated in Fig. 8D, i.e. with a more complex behavior of IA recovery in micro-gravity exposed cells (a fraction of IA not inactivated at −40 mV, in some cells, and the detection of a second, fast recovery time
constant that accounts for rapid recovery of about 30% of total IA current). ANOVA analysis of the h.i. values of the three -70/-10 mV cycles in each group demonstrated a statistical difference among treatments ($F = 12.2$, $P < 0.01$) and among successive cycles ($F = 12.4$, $P < 0.01$). In particular hysteresis was more evident in micro-gravity (h.i. = 1.21) than either in control (h.i. = 1.06; difference = +0.15; CL95 = +0.07-0.22) or in normo-gravity (h.i. = 1.04; difference = +0.17; CL95 = +0.10-0.23). Hysteresis generally decreased from the first cycle (average h.i. = 1.19) to the second and third (average h.i. = 1.04; difference = −0.15; CL95 = −0.09-0.21).

When starting from -70 mV holding potential, hysteresis was evident also at command potentials that did not reach -10 mV, namely during -70/-40 mV voltage cycles (which produce minimal $I_{Ca}$ activation and a slight but noticeable IA activation), and when calcium movements were blocked by external $Cd^{2+}$. 
DISCUSSION

In order to generate the microgravity environment, rotational accelerations of variable intensity had to be persistently applied to the frog head, and to its vestibular organ, which therefore was continuously stimulated. The effects of stimulation and microgravity thus partially overlapped, so that experimental conditions had to be devised to isolate the single effects. We report results which are specifically due to exposure to microgravity, while others appear to be more generally due to vestibular stimulation.

The major targets of microgravity exposure appear to be the calcium/potassium current system and the IA kinetic mechanisms. The interplay between calcium entering the hair cell and the consequent activation of the basolateral potassium calcium-dependent conductance is a well known mechanism governing the hair cell activation-deactivation cycle. We show here that the calcium current amplitude is significantly decreased in hair cells conditioned under microgravity conditions and that the compound delayed current $I_{KV}$ is drastically reduced. The latter effect mirrors the actions of Cd$^{2+}$ previously reported and confirmed here: namely, a marked decrease of the steady-state current and cancellation of the typical N-shaped behavior of the I-V curve and of the initial, transient outward peak of the current at very positive command potentials. In this concern, the data presented in Fig. 6AB provide direct evidence that the $I_{KCa}$ current fraction is actually involved in the hair cell dysfunction; they are however unable to discern between the two possibilities: the reduced calcium inflow directly generates decreased $I_{KCa}$ activation, or the $I_{KCa}$ failure is due to an independent basolateral cell impairment. The kinetics of $I_{Ca}$ is apparently unaffected by exposure to microgravity, so that the decreased $I_{Ca}$ might be sustained by a reduced number of calcium channels susceptible to activation by the depolarizing steps. We have no direct estimate of the relationship between amount of calcium inflow and amount of $I_{KCa}$ activated; in auditory hair cells, it was reported that Ca$^{2+}$ and Ca$^{2+}$-activated potassium channels are colocized in such a way that the same Ca$^{2+}$ signal that triggers transmitter release also activates BK channels (22). Should this be case, the present suggestion that the $I_{Ca}/I_{KCa}$ system is directly involved appears to be reasonably
self-consistent. It should be noted, however, that, at variance with \( I_{Ca} \), the \( I_{KD} \) system typically exhibits a strong inactivation mechanism, whose removal/development greatly affects the actual \( I_{KD} \) amplitude. In the hair cells exposed to microgravity, \( I_{KD} \) amplitude evoked from the -40 mV holding level (\( I_{KD} \) inactivation completely developed) exhibits a strong reduction (about -72% at +30 mV, compared to controls; Fig. 2A). After conditioning at -100 mV (inactivation removed), however, \( I_{KD} \) amplitude becomes comparable to that of control hair cells (Fig. 5B), suggesting that the kinetics of inactivation, and not the number of BK present in the membrane, are primarily involved in controlling the momentary delayed current amplitude. Additional effects of microgravity on \( I_{KD} \) kinetics were the negative shift of the steady-state inactivation curve and a larger sensitivity of inactivation removal to voltage.

Kinetics modifications of individual currents following microgravity treatment appeared to be prominent in our experiments. For \( I_{A} \), although its I-V and steady–state inactivation curves were indistinguishable between control and microgravity conditions, the current systematically decayed in microgravity according to a two exponential time course with longer time constants than in control, thus potentially providing a larger potassium charge carried by \( I_{A} \). Specific dysfunctions in \( I_{A} \) inactivation removal were revealed in microgravity treated hair cells by the longer recovery time constant measured at -70 mV, further complicated in some units by the development of a two-exponential removal time course. Moreover, whereas \( I_{A} \) inactivation was usually fully developed at -40 mV in normal hair cells, incomplete \( I_{A} \) inactivation was detected in some stimulated hair cells at -40 mV. This appears to be an unexpected, but unspecific behavior, since it was related to stimulation per se rather than to microgravity exposure.

In summary, in labyrinthine hair cells, after stimulation under microgravity conditions, the following appear to be the most significant biophysical persistent effects: impairment of the \( I_{Ca}/I_{KCa} \) system, most likely sustained by reduced calcium inflow; larger fraction of \( I_{KD} \) susceptible to inactivation with a consequent drastically reduced \( I_{KD} \) fraction activatable from the -40 mV holding level; prolonged duration of \( I_{A} \) due to slower and biexponential decay time constants; slower
removal of IA inactivation at -70 mV. Stimulation in normo-gravity results in a minor involvement of $I_{KD}$ and, occasionally, in the incomplete IA steady-state inactivation at -40 mV.

The functional role sustained by each individual conductance present in the hair cell membrane, and their interactions, are far from understood. Similarly undetermined is the time course of the transduction current, either in normo- or microgravity conditions, and its crucial interaction with the outward current balancing its inflow. As reported above, we believe that a sinusoidal voltage command migrating in the -40/-10 mV voltage range might simulate the effects of the receptor transduction current during the excitatory deflection of the sensory hair bundle, while a -70/-10 mV range might more properly reproduce the entire excitatory/inhibitory cycle of cupula movements in the native hair cell (12).

Reduced calcium entry via voltage-dependent calcium channels is clearly linked to neurotransmitter release at the cytoneural junction; therefore, the present results adequately reflect, in principle, the electrophysiological observation of reduced quantal emission when recording from the afferent fibers of the intact frog labyrinth, at rest or during sinusoidal 0.1 Hz stimulation (M.L. Rossi, manuscript in preparation). This conclusion requires some caution, as in the latter case the hair cell is not depolarized by the artificial voltage-clamp step, but rather by the interplay between the transduction current and the voltage-dependent $K^+$ currents. The absence of any hysteresis during the -40/-10 mV sinusoidal cycles suggests that the reduced transmitter release at the cytoneural junction might result simply from the interaction between a decreased calcium current and an $I_{KD}$, reduced in turn, but still of sufficient intensity to counteract the transduction current, limiting its depolarizing effect. When wider membrane potential excursions are experienced (the -70/-10 mV range, or, in other words, during full excitatory/inhibitory actions on hair cell bundle) the additional contribution by IA should be considered, since its activation-inactivation kinetics are now operated by voltage. During the excitatory phase of the cycle, an IA of similar peak amplitude is likely activated after either normo- or microgravity conditioning (the I-V curve is virtually unaffected by microgravity); under the latter condition, however, an enduring contribution is expected from IA,
because of the much longer decay time constant ($\tau_c$) of this current, exclusively observed in microgravity-exposed cells. Consistently, hysteresis was present in all the conditions tested during the -70/-10 mV cycles, but became much more evident in the microgravity exposed hair cells, compared to control and normo-gravity stimulated hair cells. Thus, the repolarizing power of the hair cell was reinforced by a stronger IA contribution in microgravity-treated cells, which presumably more than compensated the decreased amplitude of the intrinsically small $I_{KD}$. This effect shunted the transduction current, shortened the opening time of the calcium channels and eventually decreased the transmitter release at the synaptic pole of the hair cell. Reduced calcium influx via voltage-dependent channels and increased $K^+$ repolarizing charge, in a variable mix according to the momentary membrane potential shifts, might thus be responsible for the failure in the afferent sensory discharge observed after microgravity conditioning.

**Perspectives and significance**

The properties of the vestibular system of vertebrates are readily responsive to microgravity. There is a growing body of evidence that significant morphological (7, 8, 23, 24, 27), biochemical (20), genetic (for a review see [16]), behavioral (5), developmental (6) modifications occur in the labyrinth in response to prolonged exposure to microgravity. Those findings, however, were never correlated with functional features of the vestibular structures at cellular level, which remain poorly understood. The present results indicate, for the first time, that also the biophysical behavior of the hair cells of the semicircular canals is modified after cancellation of the gravitational vector, with consequent failure in the processing of the afferent sensory information. This is particularly intriguing because the semicircular canals are not the primary target of gravitational stimulation. It is unclear whether these changes are simply the result of the dysfunction of a peripheral end organ or whether they might be involved in a wider adaptive response. However, our data unequivocally provide evidence that the plasticity mechanisms in the vestibular responses operate also where the
sensory information is generated, contrary to the common view favouring an exclusively central nature.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. A-C, representative families of K⁺ currents evoked in the -20/+80 mV membrane potential range recorded in a control, unstimulated hair cell (A), in a control hair cell in the presence of 200 μM Cd²⁺ (C), and in a cell dissociated from a preparation stimulated for 4 h under microgravity conditions (B, notice the amplified vertical scale). Holding potential was -40 mV throughout to inactivate IA.

Figure 2. A, I-V plots of steady-state current amplitude of hair cells dissociated from control labyrinths (circles) and from preparations preconditioned under microgravity (triangles) or gravity conditions (diamonds). Mean values ± SEM in a 10-18 cell sample. B, I-V plots of peak current amplitude (arrows in Fig.1A-B) measured in the same groups shown in A. C, steady-state current variance at different membrane potential levels in the same cells shown in A.

Figure 3. A, typical ICa families recorded in control (a) and microgravity-conditioned hair cells (b) at the command potentials indicated close to each trace. Holding potential was -70 mV throughout. B, histograms show the distribution of the ICa maximum amplitude measured at -10 mV in control (white) and microgravity conditioned hair cells (grey). Continuous lines depict the cumulative distributions, that indicate a significant difference in microgravity vs. control (Kolmogorov-Smirnov test). C, I-V plots of the ICa measured in control hair cells (circles; mean of the nine largest currents of the sample) or in microgravity-exposed cells (triangles; mean of the eight smallest currents). D, normalized ICa values (the same as shown in C) to demonstrate the overlap of the I-V curves virtually over the whole membrane potential activation range.

Figure 4. Dissecting out IA. A, ionic currents elicited in the -40/+80 mV voltage range from a holding potential of -40 mV (IA fully inactivated). Two 300-ms pulses at the same test potential, separated by a 300 ms at -40 mV, were applied in each trial (a and b). The difference current is
almost zero \((a - b)\) for each current pair. After 1 s conditioning at -100 mV, the two-pulse series as in A were applied. The first pulse in each pair elicited both IA and \(I_{KD}\) (c), whereas the second only evoked the \(I_{KD}\) component (d), because IA had fully inactivated during the first pulse and the interposed 300 ms at -40 mV; (e) and (f) indicate the final values of current at the end of periods (c) and (d), respectively. Successive trials were separated by 30 s intervals to reset the inactivation mechanism. Subtraction of the records in Bd (after multiplying by the ratio e/f, to compensate for the onset of partial inactivation of \(I_{KD}\) during 300 ms at -40 mV) from the corresponding ones in Bc dissects out the pure IA currents.

Figure 5. A, mean IA activation curve in control (filled circles, \(n = 9\)) and microgravity-exposed hair cells (filled triangles, \(n = 10\)). Mean steady-state inactivation curve, \(h_{ae}\), in control (open circles, \(n = 7\)) and microgravity-exposed hair cells (\(n = 6\)). B, mean \(I_{KD}\) activation curves in control (filled circles, \(n = 10\)) and microgravity-exposed hair cells (filled triangles, \(n = 10\)). Mean steady-state inactivation curve, \(h_{kd}\), in control (open circles, \(n = 6\)) and microgravity-exposed cells (open triangles, \(n = 7\)). IA was isolated from difference tracings according to the procedure illustrated in Fig. 4. Steady-state inactivation parameters were obtained from experiments similar to that illustrated in Fig. 7B, by normalizing peak IA currents, or the increment of \(I_{KD}\) above its value at -40 mV holding potential, to their respective maximum values. See text for the Boltzmann-type equations best fitting the underlying conductances and steady-state inactivation curves (continuous lines).

Fig. 6. A, I-V plots of \(I_{ KCa}\) steady-state amplitude of hair cells dissociated from control labyrinths (circles) and from preparations preconditioned under microgravity (triangles) or gravity conditions (diamonds). Holding potential was -40 mV throughout. Mean values ± SEM in 10-16 cell samples. B, distribution of the \(I_{ KCa}\) maximum amplitude measured at +40 mV in the same groups shown in A.
Lines depict the cumulative distributions; a significant difference in microgravity vs. control is indicated by the Kolmogorov-Smirnov test.

Figure 7. A, IA decay time constants in control (circles, n = 10) or microgravity-treated hair cells (triangles, n = 10). IA was isolated from difference tracings according to the procedure illustrated in Fig. 4. The late (slow) decay time constant (τs) and the initial (fast) decay time constant (τf) are illustrated in the upper and lower panel, respectively. Note that the slow phase of decay was present in control cells only for command voltages >0 mV. Qf and Qs respectively indicate the total charge flowing during the fast and slow phases of current decay, and the numerical value of the ratio Qs/Qf is indicated close to each τs data point, in control and microgravity conditioned cells. B, measurement of steady-state inactivation in a microgravity-conditioned cell. Tracings illustrate currents evoked by pairs of pulses to +20 mV, separated by 0.3 s at –40 mV (to fully inactivate IA), following 1 s conditioning in the -40/-120 mV voltage range. Both IA and IKD contribute to tracings in (a), whereas the sole IKD contributes to tracings in (b). The subtraction procedure illustrated in Fig. 4 is applied to isolate the pure IA family (not shown).

Figure 8. A, time course of fast IA recovery at -70 mV. Starting from a –40 mV holding potential (IA fully inactivated), the currents were repeatedly elicited in a control hair cell at +20 mV following a conditioning pulse of variable duration at –70 mV. B, same as in A, in a microgravity-conditioned hair cell, to demonstrate the more prolonged time course of inactivation removal (time constant = 110 ms in B vs. 20 ms in A). C, IA peak amplitude, recorded in the same microgravity-exposed cell, is plotted vs. the conditioning period duration either at -100 mV (filled circles) or -70 mV (open circles). D, same as C to show, in a different cell, the bi-exponential time course of IA inactivation removal, together with the persistent fraction, incompletely removed at -40 mV. The best fitting lines, and the corresponding time constants, are reported close to each curve.
Figure 9. Typical currents elicited by sinusoidal voltage commands mimicking the effects of a receptor potential generated by bidirectional (excitatory-inhibitory) hair bundle movements. Cells were subjected to a sinusoidal voltage command oscillating at 1 Hz from $-70 \text{ mV}$ to $-10 \text{ mV}$. $A$, control hair cell; $B$, cell exposed to microgravity; $C$, cell stimulated under gravity conditions. Dotted vertical lines are centred at the positive peak of the voltage command, to help visualizing asymmetries in the rising and falling phases of the cycle (hysteresis).
Table 1. Hysteresis index evaluated in single hair cells during sinusoidal voltage command oscillating at 1 Hz in the -70/-10 mV range (three cycles) or in the –40/-10 mV range.

<table>
<thead>
<tr>
<th>Sinusoidal stimulation: -70/-10 mV</th>
<th>-40/-10 mV</th>
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<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; cycle</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; cycle</td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>1.17 ± 0.03*</td>
</tr>
<tr>
<td>Control + Cd&lt;sup&gt;2+&lt;/sup&gt; (n=3)</td>
<td>1.43 ± 0.22</td>
</tr>
<tr>
<td>Microgravity (n=6)</td>
<td>1.37 ± 0.09**§</td>
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<tr>
<td>Gravity (n=10)</td>
<td>1.10 ± 0.05§</td>
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Hair cells were obtained from labyrinths dissected in untreated controls, in controls exposed to 200µM Cd<sup>2+</sup>, in animals previously stimulated for 4h either under microgravity or under gravity conditions. The magnitude of the observed hysteresis during the -70/-10 mV cycles (1<sup>st</sup> cycle) vs. the -40/-10 mV cycle are statistically different in control and microgravity conditioned hair cells (*P<0.01; ** P<0.05). The hysteresis index values during the first cycle in microgravity vs. gravity experiments are also statistically different (§ P<0.05). Values are means ± SEM.
REFERENCES


Fig. 6

A

- **CONTROL** (n=16)
- **CONDITIONED IN GRAVITY** (n=10)
- **CONDITIONED IN MICROGRAVITY** (n=12)

B

Steady-state $I_{KCa}$ amplitude vs. Membrane potential (mV)

Cumulative frequency vs. $I_{KCa}$ amplitude (pA)