Calcium current in type I hair cells isolated from the semicircular canal crista ampullaris of the rat

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Abstract

The low voltage gain in type I hair cells implies that neurotransmitter release at their afferent synapse should be mediated by low voltage activated calcium channels, or that some peculiar mechanism should be operating in this synapse. With the patch clamp technique, we studied the characteristics of the Ca\(^{2+}\) current in type I hair cells enzymatically dissociated from rat semicircular canal crista ampullaris. Calcium current in type I hair cells exhibited a slow inactivation (during 2-s depolarizing steps), was sensitive to nimodipine and was blocked by Cd\(^{2+}\) and Ni\(^{2+}\). This current was activated at potentials above −60 mV, had a mean half maximal activation of −36 mV, and exhibited no steady-state inactivation at holding potentials between −100 and −60 mV. This data led us to conclude that hair cell Ca\(^{2+}\) current is most likely of the L type. Thus, other mechanisms participating in neurotransmitter release such as K\(^{+}\) accumulation in the synaptic cleft, modulation of K\(^{+}\) currents by nitric oxide, participation of a Na\(^{+}\) current and possible metabotropic cascades activated by depolarization should be considered.

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1. Introduction

IONIC conductance of vestibular hair cells varies depending on the animal species, the region where the cells originate, the developmental stage of the animal, and the hair cell type. However, in types I and II hair cells studied up to date, potassium currents dominate the basolateral membrane response [8,17,18,29].

Type I hair cells have a very peculiar afferent synapse which forms a calyceal structure surrounding the basolateral surface of the cell; many authors have wondered what the possible function of this calyx might be [9,30,31,32]. Due to the diffusional properties of the microspace formed between the calyx and the hair cell, it has been suggested that changes in this microambient may be relevant for synaptic transmission in type I hair cells. In contrast, type II hair cells have bouton afferent endings which have been thoroughly studied [10]. They correspond to a typical chemical synapse, and transmitter release has been shown to be calcium dependent [13,20,33].

The most significant electrophysiological difference between types I and II vestibular hair cells is the expression of a low voltage-activated, non-inactivating outward K\(^{+}\) current (\(g_{K,L}\)) in type I hair cells [8,26,28]. \(g_{K,L}\) is substantially activated at the resting potential, the half activation voltage \(V_{1/2}\) value of \(g_{K,L}\) can be as negative as −90 mV in physiological conditions, greatly reducing cell input resistance [28]. In fact, the low input resistance of type I hair cells determines that mechanoelectrical transducer currents seem not to be large enough to depolarize these cells to produce neurotransmitter release. Therefore, for these cells to activate the neurotransmitter release machinery, voltage-dependent Ca\(^{2+}\) channels should have a very negative activating voltage, or there should be some other mechanisms contributing to further depolarize the cell [7,9,32].

In this study, we addressed the problem of determining the characteristics and particularly the half activation voltage of the Ca\(^{2+}\) current in type I hair cells.
2. Materials and methods

Experiments were performed in young Long-Evans rats (postnatal days 14–17) supplied by the bioterium “Claude Bernard” of the University of Puebla. All efforts were made to minimize animal suffering and to reduce the number of animals used, as outlined in the “Guide to the Care and Use of Laboratory Animals” issued by the National Academy of Science, USA.

Hair cells were enzymatically dissociated from the semicircular canal crista ampullaris of the rat. Tissue pieces containing the vestibular sensory epithelia were incubated in Tyrode containing IA type collagenase (0.2 mg/ml) for 7 min at 35 °C, followed by cell incubation for 10 min at 35 °C in a Ca²⁺ and Mg²⁺-free Tyrode solution (Table 1) with trypsin (1 mg/ml). Tissue was finally washed for 10 min at 4 °C in a Ca²⁺ and Mg²⁺-free Tyrode solution containing serum bovine albumin (1 mg/ml). All enzymes were from Sigma (St Louis, MO).

Isolated cells were placed in the recording chamber (400 μl) on an inverted microscope stage. Cells settled and adhered to the bottom of the recording chamber within 10 min. The recording chamber was continuously perfused with the corresponding Tyrode solution at a constant rate (0.5 ml/min) using a peristaltic pump (Microperpex, LKB, Sweden).

Whole-cell currents were recorded at room temperature (22–25 °C), according to the method described by Hamill et al. [11] and modified for isolated rat vestibular hair cells. In brief, the recordings were obtained with the patch clamp technique in the whole-cell configuration; patch pipettes had resistances of 3–5 MΩ when filled with the corresponding intracellular solution (Table 1). Command pulse generation and data sampling (≈ every 200 μs) were controlled by pClamp 8.0 software (Axon Inst.) using a 12-bit analog to digital converter (Digidata 1200, Axon Inst.). Membrane capacitance and series resistance (80%) were electronically compensated. Voltages were corrected for a 7.9-mV liquid junction potential for the Ba²⁺ extracellular solution and Cs⁺ and TEA intracellular medium (solutions 3 and 4 in Table 1). Correction was 6.8 mV for the high Ca²⁺ extracellular solution and the intracellular Ca²⁺ solution (solutions 5 and 6 in Table 1). The junction potential was calculated with the generalized Henderson liquid junction potential equation [2] using pClamp 8.0 software (Axon Inst.). Student’s t-test was used to assess statistical significance, with P<0.05 indicating a statistically significant difference. The results are given as mean ± S.E.M.

Current evoked between −100 and −60 mV was fitted with a linear function that was subtracted assuming a linear leak throughout the whole current–voltage relation [15].

Solutions containing drugs or blockers were dissolved in the corresponding Tyrode solution and applied by micropuffusion. For this, a teflon microtube was drawn close to the cell and the desired solution was applied by pressure ejection controlled by a microsyringe (Baby Bee, BAS) with a flow rate of 20 μl/min.

To distinguish between types I and II hair cells, their morphological and electrophysiological properties were correlated. Type I hair cells presented a flask-shaped body with a highly refringent cuticular plate wider than the hair cell neck (Fig. 1A), strongly negative membrane potential, low membrane resistance, and the whole-cell currents showed a large instantaneous outward component due to g_{K,L} expression [28]. In contrast, type II hair cells exhibited more cylindrical shapes and their cuticular plate was hardly distinguishable under phase contrast optics. Fig. 1B). Electrophysiologically, they showed a less negative membrane potential, higher membrane resistance, and the whole-cell

<table>
<thead>
<tr>
<th>Solutions (in mM)</th>
<th>KCl</th>
<th>TEACl</th>
<th>CsCl</th>
<th>NaCl</th>
<th>MgCl₂</th>
<th>CaCl₂</th>
<th>BaCl₂</th>
<th>HEPES</th>
<th>NMDG⁺</th>
<th>EGTA</th>
<th>GTPNa₂</th>
<th>ATPMg</th>
<th>Glucose</th>
<th>4-AP</th>
</tr>
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<tbody>
<tr>
<td>Normal Tyrode</td>
<td>5.4</td>
<td>140</td>
<td>1.2</td>
<td>3.6</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Normal intracellular</td>
<td>140</td>
<td>–</td>
<td>10</td>
<td>2</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>0.134</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Extracellular with Ba²⁺</td>
<td>–</td>
<td>23</td>
<td>30</td>
<td>55</td>
<td>0.1</td>
<td>20</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Intracellular Cs⁺-TEA</td>
<td>–</td>
<td>23</td>
<td>69.3</td>
<td>–</td>
<td>–</td>
<td>0.1</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>0.1</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>High Ca²⁺ extracellular</td>
<td>–</td>
<td>23</td>
<td>30</td>
<td>70</td>
<td>10</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>5</td>
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<tr>
<td>Intracellular Ca²⁺</td>
<td>–</td>
<td>23</td>
<td>75</td>
<td>–</td>
<td>–</td>
<td>0.1</td>
<td>10</td>
<td>80</td>
<td>10</td>
<td>0.1</td>
<td>2</td>
<td>–</td>
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<td>–</td>
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</table>
current was dominated by outward rectifying currents without an instantaneous component [8].

3. Results

Stable recordings in normal extra and intracellular solutions (Table 1) were readily obtained. Typically, type I hair cells displayed a $g_{K,L}$ current, thus having a small membrane resistance ($R_m$) value compared with type II hair cells (Table 2). In current clamp with a membrane potential of about $-73 \pm 4$ mV, type I hair cells showed a low voltage gain with a near linear slope of $2.4 \pm 0.2$ mV/100 pA (data not shown). A current injection of as much as 600 pA depolarized the cells up to $-61 \pm 6$ mV ($n=4$).

For $Ca^{2+}$ current recording, extracellular $Ba^{2+}$ was used as a current carrier and intracellular $Ca^{2+}$-TEA solutions were used to block $K^+$ currents (Table 1). With these solutions, and since no $g_{K,L}$ was present, hair cells were differentiated on the basis of morphological criteria (see Materials and methods). Inward $Ba^{2+}$ current showed no inactivation during 200-ms voltage pulses (Fig. 2A). Application of CdCl$_2$ (30 and 300 $\mu$M; $n=3$ each) reduced $I_{Ba}$ in $74 \pm 4\%$ and $90 \pm 6\%$, respectively; perfusion with 100 $\mu$M NiCl$_2$ ($n=5$) also reduced the current $48 \pm 9\%$. Both Cd$^{2+}$ and Ni$^{2+}$ did not modify the activation kinetics nor the voltage dependence of $I_{Ba}$ (data not shown). The application of nimodipine (1, 3 and 10 $\mu$M, $n=3$, 6 and 4,

<table>
<thead>
<tr>
<th>Type</th>
<th>$V_m$ (mV)</th>
<th>$C_m$ (pF)</th>
<th>$R_m$ (MΩ)</th>
<th>$\tau_m = R_mC_m$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>$-75 \pm 1^*$</td>
<td>$6.7 \pm 1$</td>
<td>$45 \pm 10^*$</td>
<td>$302 \mu s^*$</td>
<td>7</td>
</tr>
<tr>
<td>Type II</td>
<td>$-47 \pm 3^*$</td>
<td>$6.1 \pm 1$</td>
<td>$592 \pm 62^*$</td>
<td>$3.6 \mu s^*$</td>
<td>7</td>
</tr>
</tbody>
</table>

Resting membrane potential, $V_m$; membrane capacitance, $C_m$; membrane resistance, $R_m$.

*P<0.001.

Fig. 2. Current voltage relationship for $I_{Ba}$. In A, typical recording of $I_{Ba}$ obtained for $V_H = -60$- and 10-mV voltage pulses from $-100$ to $+50$ mV. In B, current to voltage relationship for $I_{Ba}$ recorded for holding voltages of $-60 (\circ, n=13)$ and $-100$ mV (C, $n=11$), and for $I_{Ca} (\bullet, n=4)$ recorded with the same protocol and $V_H = -60$ mV. The current–voltage relationship for $I_{Ca}$ is about 50% $I_{Ba}$. Each point represents the mean ± standard error. In C and D, typical records of the current recorded using $Ca^{2+}$ as a current carrier. Slow inactivation can be observed during a 2-s pulse as shown in D (C and D are the average of three traces).
respectively) produced a reversible concentration-dependent reduction of the inward current. Nimodipine action began within the first 5 s after its perfusion reaching its maximum effect after 30 s of perfusion (Fig. 3A). It produced no apparent modification of \( I_{\text{Ba}} \) current kinetics nor modified its voltage dependence (Fig. 3B). Nimodipine (1, 3 and 10 \( \mu \)M) reduced \( I_{\text{Ba}} \) in 38\%, 57\% and 77\%, respectively (Fig. 3C).

The activation of \( I_{\text{Ba}} \) was studied at two holding levels \((V_H)\), −100 and −60 mV \((n=11\) and 13, respectively). Current was produced by 10 mV, 200-ms pulses from −100 to 50 mV. \( I_{\text{Ba}} \) was activated at potentials above −60 mV and reached its peak at −18 mV. Current inversion was above +40 mV (Fig. 2B). The mean amplitude of the current for \( V_H = -60 \) mV was 91 ± 8 pA \((n=13)\), whereas for \( V_H = -100 \) mV, it was 78 ± 11 pA \((n=11)\); differences were not significant (Student’s t-test, \( p=0.64 \)). Conductances obtained at \( V_H = -60 \) and −100 mV were normalized and approximated by a Boltzmann function:

\[
\frac{g_{\text{on}}}{g_{\text{max}}} = \frac{1}{1 + e^{\left(\frac{V_m-V_{1/2}}{s}\right)}}
\]

where \( g_{\text{on}} \) is the conductance, \( g_{\text{max}} \) is the maximal conductance, \( V_m \) is the membrane potential, \( V_{1/2} \) is the potential at which the current is half-maximally activated, and \( s \) is the exponential slope.

The \( I_{\text{Ba}} \) recorded for \( V_H = -60 \) mV had a \( V_{1/2} = -36 ± 0.4 \) mV and \( s = 5.7 ± 0.3 \) mV \((n=13)\); the \( I_{\text{Ba}} \) recorded for \( V_H = -100 \) mV had a \( V_{1/2} = -37 ± 0.8 \) mV and \( s = 5 ± 0.8 \) mV \((n=11)\,Fig. 4). There were no significant differences between them (Student’s t-test, \( p=0.94 \)). The \( g_{\text{max}} \) for \( I_{\text{Ba}} \) was 1.4 ± 0.1 nS \((n=13)\).

The inward current was also studied using \( \text{Ca}^{2+} \) as a current carrier in order to define whether any \( \text{Ca}^{2+} \)-dependent inactivation process was taking place \((n=6)\). For these experiments, extracellular high \( \text{Ca}^{2+} \) and intracellular \( \text{Ca}^{2+} \) solutions were used (Table 1). The current activated very rapidly with a \( \tau < 0.5 \) ms for pulses at −17 mV, and showed no inactivation during 200-ms current pulses. Peak current amplitude was 42 ± 10 pA, 46\% lower than \( I_{\text{Ba}} \) (Fig. 2B). The current having \( V_{1/2} = -35 ± 0.6 \) mV and \( s = 6.1 ± 0.5 \) was not significantly different to \( I_{\text{Ba}} \). The \( g_{\text{max}} \) for \( I_{\text{Ca}} \) was 0.9 ± 0.2 nS. No significant \( I_{\text{Ca}} \) inactivation was found with 200-ms pulses from −100 to +50 in 10-mV steps and for a \( V_H \) of −60 and −100 mV (Fig. 2C). However, when using 2-s pulses of the same magnitude, a slowly developing current inactivation with a \( \tau = 680 ± 133 \) ms at −17 mV was found (Fig. 2D). Current characteristics for a \( V_H \) of −60 and −100 mV were similar during the 2-s pulses.

4. Discussion

Our results are consistent with the idea that a single \( \text{Ca}^{2+} \) channel subtype may account for the major part of the macroscopic \( \text{Ca}^{2+} \) current. We found a small inward current with a peak amplitude of about 50 pA at −17 mV. Current
amplitude increased by using Ba$^{2+}$ as a current carrier, was blocked by Cd$^{2+}$ and Ni$^{2+}$, and was sensitive to nimo-
dipine. This current, activated above $-60$ mV with $V_{1/2}$ =
$-36$ mV, showed a slowly developing inactivation while
using Ca$^{2+}$ as a current carrier. Variations in the holding
temperature ($-100$ and $-60$ mV) did not significantly
modify the current properties, indicating a homogenous
current, most likely of the L type. This is in agreement
and confirms recent results obtained for semicircular canal
type I hair cells of the rat and chick, in which authors
also reported a homogenous single Ca$^{2+}$ current which
activated above $-60$ mV [1,4]. In our recordings, $V_{1/2}$
of the Ca$^{2+}$ current in type I hair cells was $-36$ mV, that is,
it was displaced $5$ mV to the right when compared to the
one reported by Bao et al. [1] which had a mean half
activation voltage of $-41.1 \pm 0.5$ mV.

Difference in the $V_{1/2}$ of the Ca$^{2+}$ current could be due
to the patch configuration used in each case. Bao et al. [1]
used the perforated patch configuration in which, there is
an incomplete solution exchange between pipette and cell,
where only the small permeant ions will be exchanged
while the larger impermeant anions will not. Therefore,
the potential difference will have a Donnan component
due to impermeant anions and, previous to a complete
equilibration, a diffusion potential dependent on the ion
selectivity of the ionophore will be present. The Donnan
potential across the membrane causes the pipette interior
to differ in voltage from the cell interior by an amount
depending upon the extent of ionic redistribution [23]. At
the same time, perforated patch will allow the cell interior
to retain various active molecules which may modulate
Ca$^{2+}$ channels.

Compared to the results previously reported by Bao et al.
[1], our records also showed a higher efficacy of nimo-
dipine. Since a dihydropyridine block is enhanced by depo-
larization, this difference could be due to a more depolarized
$V_{II}$ and longer test pulses used in our records. It is also
worth to note that we obtained hair cells from animals with a
much narrower age range (P14–17 as compared to P4–20
in Ref. [1]), thus assuring in our experiments that hair cell
phenotype and innervation were completely established
[29].

Previous studies have reported that in guinea pig type I
hair cells, there is a transient Ca$^{2+}$ inward current, probably
T type [25]. By using immunohistochemical techniques, it
has also been reported that chinchilla semicircular canal hair
cells expressed $\alpha$1B, $\alpha$1C and $\alpha$1D subunits [14], thus
suggesting that various Ca$^{2+}$ channel subtypes may be
functionally assembled in the system. Pharmacological
studies have also suggested that L, T and R type Ca$^{2+}$
channels remain the most feasible to be expressed in hair
cells [5]. In type II vestibular hair cells, it has been shown
that calcium current related to neurotransmitter release from
hair cells is a high voltage activating current, probably L
type [1,16,20,21,22]. However, in frog semicircular canals
and sacculus, it seems that non-L type Ca$^{2+}$ channels may
also be involved in hair cell response and transmitter release
[16,27]. In our experimental conditions, the macroscopic
Ca$^{2+}$ current did not show any component suggesting the
presence of an inactivating Ca$^{2+}$ current (R or T type) in
type I hair cells. Furthermore, our results suggest that Ca$^{2+}$
current activation in types I and II hair cells appears to be
similar, as reported in the chick [4].

Our results leave open the question about the role of
Ca$^{2+}$ current in afferent synaptic transmission in hair cells.
In the turtle crista, it has been reported that less than 10% of
type I hair cells do not have a $g_{\text{K,L}}$ activated at $V_{II}$ > 70
mV [6]. Also, $g_{\text{K,L}}$ half activation in the turtle crista ($-55$
to $-60$ mV) has been reported to be shifted to the right as
compared to previous reports in mammals and birds
[8,18,28]. This implies that type I hair cells very probably
do not constitute a homogenous population, and more
probably that the activation of the dominant $g_{\text{K,L}}$ current
spans a whole range and is highly sensitive to cyclic GMP
modulation [3,7]. In the case of type II hair cells, at least two
sub-types have been reported: fast and slow. They have
significantly different input resistances and voltage gains,
although both are capable of generating receptor potentials
of an amplitude enough to activate the Ca$^{2+}$ current [6,32].
The question is whether in some type I hair cells, the Ca$^{2+}$
activation threshold could be reached without implying
other mechanisms. At least in those type I hair cells in
which $g_{\text{K,L}}$ shows the more negative half activation value
($>-70$ mV), some additional mechanism is needed to boost
synaptic transmission. Cells displaying no such hyperpolar-
ized $g_{\text{K,L}}$ activation may have receptor potentials within the
range of Ca$^{2+}$ current activation.

Among the proposed mechanisms participating in type
I hair cell afferent synapses, K$^{+}$ accumulation in the
synaptic cleft that may depolarize both hair cell and afferent
terminal, increasing transmitter release, and facil-
itating the afferent neuron spike discharge seems the most
feasible [9,32]. This hypothesis has been reinforced by
recent results showing that afferent calyceal synapses in
type I hair cells also express a low voltage activated K$^{+}$
current [12,24]. Another mechanism operating in this
synapse may involve the modulation of $g_{\text{K,L}}$ by cyclic
GMP [3,7]. In addition, in the chick, a Na$^{+}$ current could
reinforce, at least in a subpopulation of hair cells, the
membrane depolarization which will boost synaptic output
[4,19].

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