Abstract — Some properties of Ca$^{2+}$ currents in hair cells isolated from frog semicircular canals by enzymatic or mechanical treatment were studied by using the whole-cell configuration of the patch-clamp technique. After blocking the large outward K$^+$ currents by substituting Cs$^+$ for K$^+$ and adding tetraethylammonium to the pipette filling solution, voltage- and time-dependent inward currents were clearly detectable in the presence of 4 mM Ca$^{2+}$ in the extracellular solution. Ca$^{2+}$ current was recruited at test potentials more positive than $-60 \text{ mV}$, showed a rapid activation, and exhibited no inactivation during 150-ms depolarizing pulses. The maximal amplitude was attained at about $-20 \text{ mV}$, with an average value of about 80 pA. When Ca$^{2+}$ in the extracellular solution was replaced with Ba$^{2+}$, the magnitude of inward currents increased about twofold. Ba$^{2+}$ currents were blocked more effectively by Cd$^{2+}$ than by Ni$^{2+}$, were suppressed by 0.5 μM ω-conotoxin, and were virtually unaffected by amiloride. The dihydropyridine Bay K 8644 caused a marked voltage-dependent increase in inward currents. The present data suggest that hair cells from frog crista ampullaris are endowed with a homogeneous population of Ca$^{2+}$ channels having several properties similar to those described for neuronal L channels. Since these channels are recruited in a range of potentials close to the resting level, it is suggested that they subserve the control of both resting and evoked transmitter release from the basal pole of the hair cells.

Keywords — frog; semicircular canals; isolated hair cells; patch-clamp; calcium currents.

Introduction

Vestibular hair cells are highly specialized transducers that are very sensitive to mechanical forces such as linear and angular accelerations of the head. Several lines of evidence indicate that these forces produce the bending of the hair bundle, which modulates an influx of K$^+$ ions from the apical surface of the cells. This current, in turn, elicits the receptor potential that controls the release of the afferent transmitter from the basal pole of the hair cell (1–4).

The different functions of the hair cell are mediated by different types of ionic channels localized in discrete areas of the cell. In fact, besides transduction channels probably localized at tips of stereocilia (4,5), hair cells are endowed with voltage-dependent Ca$^{2+}$ channels and voltage- and Ca$^{2+}$-gated K$^+$ channels, which are probably localized in the basolateral membrane (6–10).

While the different K$^+$ channels have been characterized in vestibular hair cells of several species, few investigations have been performed to evaluate the properties of Ca$^{2+}$ channels. In particular, no attempt has been made to identify the type(s) of channels involved. Because of the importance of Ca$^{2+}$ channels in virtually every aspect of hair cell function, and especially the coupling between transduction and neurosecretion, we sought...
to characterize some properties of these channels in hair cells from frog crista ampullaris.

To this purpose, Ca\(^{2+}\) currents in isolated hair cells were investigated by using the whole-cell configuration of the patch-clamp technique.

**Methods**

**Isolation of Hair Cells**

Hair cells were isolated from semicircular canals of frogs (*Rana esculenta* L.) weighing 25 to 30 g, previously anaesthetized by immersion in 0.1% MS-222 solution. The isolation procedure and the solutions used were similar to those reported by Housley and colleagues (10). After decapitation, the head was pinned to the bottom of a Perspex chamber filled with dissociation medium (Table 1). The otic capsule was opened to expose the vestibular end-organs according to a procedure described elsewhere (11). After removing the otolith organs, the ampullae of each canal were isolated and transferred to a dish containing the dissociation medium. Usually only the more easily accessible posterior semicircular canal was removed. The wall of the ampulla covering the crista ridge was removed and the preparation was transferred to a separate dish filled with dissociation medium containing the proteolytic enzyme papain (0.34 mg/mL; Calbiochem, San Diego, CA) and l-cysteine (0.6 mg/mL; Calbiochem). Enzymatic digestion was achieved at room temperature (20 to 22°C) for 5 to 8 minutes. Digestion was stopped by quickly transferring the preparation to a dish containing dissociation medium to which bovine serum albumin (0.5 mg/mL; Sigma, St. Louis, MO) was added; at least three washings, each lasting 3 minutes, were performed. The preparation was then transferred to a dish containing extracellular solution (Table 1). The hair cells were isolated from the crista ridge under a light microscope by gently twisting and pressing the preparation against the bottom of the dish. Under the most favourable conditions, about 50 to 60 cells could be isolated from two ampullae.

In several cases the hair cells were isolated by the mechanical procedure described above without using enzymatic treatment. Under these conditions, typically only 8 to 10 cells could be isolated from two ampullae.

Following isolation, hair cells were observed by using a Nikon inverted microscope equipped with phase contrast optics. In agreement with the observations made by Housley and colleagues (10), the majority of hair cells had a club-like shape, some were cylindrical, and only a few occasional cells were small and pear shaped. In order to select cells with a good viability, only cells with an evident kinocilium, smooth surface, and apparent opacity were used. These cells excluded the vital dye Trypan blue added to the external medium. No discernible difference was observed be-

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Note: the osmolality of all solutions was about 275 mOsm/kg as determined by freezing point osmometry (type 5B, Camlab, Cambridge, UK). The pH of each solution was adjusted to 7.25 by adding the hydroxide of the cation present at the highest concentration.
Calcium Currents in Vestibular Hair Cells

between the viable hair cells isolated enzymatically and those obtained by mechanical treatment only.

Recordings of Electrical Currents and Data Analysis

Macroscopic current measurements from hair cells were performed at room temperature by the whole-cell configuration of the patch-clamp technique (12) using an L/M EPC-7 amplifier (LIST Electronics, Darmstadt, Germany). Unless specified otherwise, the bandwidth of the amplifier was set at 3 kHz during measurements. Command potentials to the amplifier were applied after D/A conversion by using a Vic-20 Commodore computer. Patch pipettes made from borosilicate glass capillaries (Drummond, Broomall, PA) and showing 2 to 3 MΩ resistance when filled with the intracellular solutions (Table 1) were used. To improve the quality of patch conditions, the osmolality of the internal media was reduced by 20%.

Before establishing the seal, the junction potential between the pipette filling solution and the bath was cancelled. After formation of a high-resistance seal and cancellation of the residual capacitance of the pipette, the membrane patch was broken by gentle suction. In the whole-cell condition, input capacitance, clamp time constant, and series resistance were evaluated as described by Marty and Neher (13). To measure the capacitive transient, the amplifier was set at a wide bandwidth from DC to 100 KHz, and hyperpolarizing pulses of 10 mV amplitude and 150 ms duration were delivered from a holding potential of -80 mV. The signal was sampled at a frequency of 100 kHz by using an 8 bit A/D converter and displayed on an X-Y plotter. In agreement with Housley and colleagues (10), in 12 cells the series resistance was 8.6 ± 0.6 MΩ, computed from 60.4 ± 8.8 μs clamp time constant and 7.1 ± 0.4 pF input capacitance. We compensated electronically at 50% for the series resistances during the recording of the ionic currents. However, since the maximal current was 300 pA, the maximal potential error in the calculation of the I/V relationships was less than 2 mV. The leakage currents were very small and were linear over the range from -60 to -100 mV. In 38 cells, average input resistance was of 2.1 ± 0.82 GΩ.

Recordings of ionic currents were stored on a videotape after A/D conversion through a digital audio processor (Sony PCM-701/ES), modified to obtain a bandwidth of 0 to 20 kHz (14). The signals from the videotape were digitized off-line at a sampling frequency of 2 kHz and processed by using a HP 9816 personal computer. Recordings were corrected by subtracting the linear components of leakage and capacitive currents.

The selective Ca²⁺ channel blockers Cd²⁺, Ni²⁺, ω-conotoxin, amiloride (all purchased from Sigma) and the Ca²⁺ channel agonist Bay K 8644 were dissolved in external medium and delivered through a multibarrel pipette positioned close to the hair cell (15). The perfusion rate was of 4 to 10 μL/s.

For each set of experiments, number of cells (n) and mean values ± SE are reported.

Results

Isolation of Ca²⁺ Currents

Immediately after establishing the whole-cell condition, viable hair cells perfused with the intracellular solution I (Table 1) showed zero-current membrane potentials ranging from -42 to -74 mV (-48.2 ± 2.3, n = 52). These cells invariably produced a complex outward rectifying K⁺ current when depolarized. The characteristics of this current have been described by Housley and colleagues (10).

After blocking the outward current by substituting Ca²⁺ for K⁺ and adding TEA to the pipette filling solution (Table 1: intracellular solution II), voltage- and time-dependent inward currents were clearly detectable in the presence of 4 mM Ca²⁺ in the extracellular solution. Conversely, inward currents disappeared when Ca²⁺ was removed and substituted with an equimolar concentration of Mg²⁺.
In order to evaluate a possible contribution of Na\(^+\) ions to the inward currents, in 4 experiments Na\(^+\) in the extracellular solution was replaced with choline. Since this ionic condition did not affect the magnitude of the inward current, we could exclude the occurrence of a Na\(^+\) ion influx through Ca\(^{2+}\) channels as well as the presence of Na\(^+\) channels.

In the whole-cell condition, Ca\(^{2+}\) currents showed a clear cut run-down, decaying by about 50% in a few minutes as previously observed for inner hair cells (6,7) and several other cells (16,17). Therefore, measurements were performed during the first 5 min of the recording, before the onset of the run-down.

**General Properties of Ca\(^{2+}\) Currents**

In preliminary experiments, inward currents were recorded at holding potentials ranging from -70 to -110 mV; no difference was observed either in their amplitude or in their time course over this range of holding potentials. Therefore, a holding potential of -80 mV was chosen to recruit the inward current. Figure 1A shows typical Ca\(^{2+}\) currents recorded from the same hair cell in response to 4 depolarizing 150-ms command voltage pulses. The currents show a rapid activation and no evidence of inactivation during the test pulses. As demonstrated by the I/V relationship shown in Figure 1B (mean of 8 experiments), Ca\(^{2+}\) current activation could be detected already at potentials more positive than about -50 mV, the maximal amplitude being attained at around -20 mV. Maximal magnitude of Ca\(^{2+}\) current ranged in different hair cells from 50 to 160 pA. The reversal potential was close to 20 mV, a very low value compared to the Ca\(^{2+}\) equilibrium potential estimated by the Nernst equation (+161 mV), suggesting incomplete blockade of outward currents at positive potentials, as already reported in the inner ear (6,18). The contribution of outward currents to the total membrane currents was assessed by blocking Ca\(^{2+}\) currents. In the presence of 100 \(\mu\)M Cd\(^{2+}\), inward Ca\(^{2+}\) currents were fully blocked and

![Figure 1](image-url)
outward currents became apparent at potentials close to 0 mV, increasing in an approximately linear fashion at more positive test potentials (Figure 1C). The same effect was observed in the presence of the organic Ca$^{2+}$ blocker ω-conotoxin (0.1 mM). Therefore, contamination of Ca$^{2+}$ currents by unblocked outward components could be excluded at negative test potentials. Figure 1C shows the true I/V relationship for Ca$^{2+}$ current, defined as the difference between the currents recorded before and after Cd$^{2+}$ administration.

As clearly shown by the tracings in Figure 1A, the activation rate of Ca$^{2+}$ currents is voltage dependent. Though no attempt was made to calculate the time constants, an estimation of the rate of activation was obtained in 12 cells by measuring the time to reach the steady-state level at membrane potentials ranging from -40 to 0 mV. We observed that at -40 mV the inward current reached its maximum in 5.2 ± 0.3 ms. This time decreased about two-fold (3.1 ± 0.3 ms) at a test potential of -20 mV and thereafter remained constant (3.2 ± 0.3 ms) when the potential was increased further to 0 mV.

**Pharmacological Properties of Ca$^{2+}$ Currents**

In 10 experiments, Ca$^{2+}$ was replaced with Ba$^{2+}$ in the extracellular solution. As shown in the representative tracings in Figure 2A, the inward current approximately doubled when 4 mM Ba$^{2+}$ was substituted for 4 mM Ca$^{2+}$. Ba$^{2+}$ currents, like Ca$^{2+}$ currents, showed a rapid activation and no evidence of inactivation during 150-ms test pulses. Figure 2B shows the I/V relationships for Ca$^{2+}$ and Ba$^{2+}$ currents calculated by subtracting the contaminating outward currents unmasked by Cd$^{2+}$, as described previously. Both currents are recruited at potentials close to -60 mV, reach a maximum at about -20 mV, and decrease at more positive membrane potentials.

The sensitivity of inward Ba$^{2+}$ currents to some inorganic and organic compounds commonly employed to characterize Ca$^{2+}$ channels was tested in 30 hair cells. As shown in Figure 3A and 3B, Cd$^{2+}$ was more effective than Ni$^{2+}$ in depressing the inward current. When these ions were tested at 5-μM concentration, the decrease in peak current magni-
The organic Ca$^{2+}$ antagonist amiloride (Figure 3D) exerted a weak depressant effect on Ba$^{2+}$ current even when applied at high concentrations. In 6 experiments performed with 500 μM amiloride, the decrease in peak current was only 14% ± 5%.

Ba$^{2+}$ currents were highly sensitive to the dihydropyridine derivative Bay K 8644. In 8 cells, 5 μM Bay K 8644 rapidly increased the magnitude of the inward current by 122% ± 19% at a membrane potential of −40 mV (Figure 4A). As shown in the I/V relationships in Figure 4C, the effect of Bay K 8644 was particularly prominent at low depolarizations and became progressively smaller with increasing depolarizing pulses. The inward current activation was shifted toward more negative potentials, and the maximal current was attained at −40 mV, with an average shift of 20 mV on the voltage axis with respect to control conditions. Unlike other observations made in excitable cells (19,20), however, Bay K 8644 did not cause any significant decay of the Ca$^{2+}$ current during 150-ms test pulses, even at positive potentials (Figure 4B). The action of Bay K 8644 was completely reversible after 20 to 30 seconds of washing.

**Discussion**

The present results suggest that hair cells from the frog semicircular canals are endowed with a homogeneous population of voltage-dependent channels selective for Ca$^{2+}$ ions. In fact, these cells invariably produced a fast and noninactivating inward current that was blocked more effectively by Cd$^{2+}$ than by Ni$^{2+}$ and clearly increased in magnitude when Ba$^{2+}$ was substituted for Ca$^{2+}$. These results are in agreement with those obtained in previous investigations in vestibular hair cells (6–9) and failed to demonstrate the presence of a transient Ca$^{2+}$ current reported recently in both type I and type II hair cells of guinea pig crista ampullaris (21). Whatever the reason for these discrepancies, our experiments highlight additional properties of the sustained Ca$^{2+}$ current in vestibular hair cells: the inward current was...
reversibly suppressed at all potentials by low concentrations of the Ca\(^{2+}\) blocker \(\omega\)-conotoxin (20) and was markedly increased in a voltage-dependent manner by the Ca\(^{2+}\) agonist Bay K 8644 (19,20). Conversely, the organic Ca\(^{2+}\) blocker amiloride (22) exerted a negligible depressant effect, even when applied at high concentrations.

It is currently accepted that in neurons there are two main types of Ca\(^{2+}\) channels, called the L and the T channels (23) or the HVA and the LVA channels (24). A third type of Ca\(^{2+}\) channel, called the N channel, has been found in chick dorsal root ganglia (23, 25), in frog sympathetic neurons (26), and in mammalian peripheral neurons (27). These three types of channels have been classified on the basis of their kinetic properties. T-type channels, which are activated at negative membrane potentials and rapidly inactivated, provide transient currents at small depolarization from the resting level. L channels, which open at more positive potentials and show very little inactivation, produce long-lasting currents at large depolarization values. N-type channels show intermediate kinetic properties between L and T channels. Moreover, these three types of channels can be differentiated on the basis of their sensitivity to several pharmacological agents, such as Cd\(^{2+}\), dihydropyridines, and \(\omega\)-conotoxin.

Our findings indicate that the Ca\(^{2+}\) channels sustaining the inward current in canal hair cells exhibit several properties typical for neuronal L channels, in agreement with a recent investigation in cochlear hair cells (28). In fact, this current did not show the inactivation typical for T and N currents (23), was very sensitive to Bay K 8644 and \(\omega\)-conotoxin, which are substances highly specific for L channels in different cells (19,20,26), and insensitive to amiloride, a specific T-channel blocker (22). However the Ca\(^{2+}\) current in frog canal hair cells differed from classical neuronal L current (20) for its more negative potential of activation (−60 vs −20 mV), reversibility of the block exerted by \(\omega\)-conotoxin, and lack of decay during long-lasting test pulses in the presence of Bay K 8644. The possibility that these differences may reflect variations in the structure of the subunits constituting Ca\(^{2+}\) channels in hair cells and neuronal L channels (20) cannot be excluded.

Although Ca\(^{2+}\) entry into hair cells can occur through leakage as well as through transduction channels (5,29), the fact that Ca\(^{2+}\) channels in canal hair cells could be activated at potentials close to the resting level
suggests that these channels may be involved in the control of both resting and evoked transmitter release from the basal pole of the receptor cell. This suggestion is supported by recent findings by our group (30) and by Rossi and Martini (31), indicating that ionic manipulations expected to affect \( \text{Ca}^{2+} \) currents remarkably modify afferent transmitter discharge was manner by inorganic \( \text{Ca}^{2+} \) blockers; 3) a consistent increase of both EPSPs and spike frequency in the afferent discharge was observed when \( \text{Ca}^{2+} \) was replaced with \( \text{Ba}^{2+} \).

The magnitude of \( \text{Ca}^{2+} \) inward current in canal hair cells is very small compared to the voltage-dependent outward currents, indicating that the number of \( \text{Ca}^{2+} \) channels in the cell membrane, even taking into account their low elementary conductance, must be relatively low. Based on noise analysis of \( \text{Ca}^{2+} \) currents in 5 mM \( \text{Ca}^{2+} \), a mean elementary current value of 0.09 pA at \(-12 \text{ mV}\) has been recorded from chromaffin cells (17). A similar value (0.12 pA) has been reported under similar conditions for rat sensory neurons (32).

If we assume that all \( \text{Ca}^{2+} \) channels in hair cells were open when the maximal \( \text{Ca}^{2+} \) current (160 pA) was recorded at the test potential of \(-20 \text{ mV}\), each hair cell should possess about 1600 \( \text{Ca}^{2+} \) channels. Since the mean surface area of frog canal hair cells has been estimated to be 839 \( \mu \text{m}^2 \) (10), the maximal density of \( \text{Ca}^{2+} \) channels in these cells would be about 2 channels/\( \mu \text{m}^2 \). This value is relatively low when compared to the 45 channels/\( \mu \text{m}^2 \) and 30 to 60 channels/\( \mu \text{m}^2 \) estimated for mammalian sympathetic neurons (33) and snail neurons (34), respectively, or to the 8 to 10 channels/\( \mu \text{m}^2 \) estimated for bovine chromaffin cells (17). It should be pointed out, however, that the estimate of a mean density value per unit of cell surface may be misleading. In fact, a recent investigation in frog saccule hair cells demonstrated directly that the \( \text{Ca}^{2+} \) channels that mediate the control of transmitter release are clustered in the basolateral membrane close to the sites of extrusion of the transmitter (33).

Two features of \( \text{Ca}^{2+} \) currents in frog canal hair cells that might have significant functional implications are their fast rate of activation and the lack of inactivation. In theory, these properties should allow the release of afferent transmitter without appreciable distortions in response to both rapid and long-lasting changes in the membrane potential produced by the receptor current. If it is accepted that the primary role of \( \text{Ca}^{2+} \) channels is to couple mechanical transduction to transmitter release, then the individual transfer functions linking the time course of natural stimuli to the frequency of the afferent discharge must be determined elsewhere: the mechanical coupling systems and the voltage-and ion-dependent channels of the basolateral membrane are the most probable candidates.

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