Calcium Modulates the Frequency and Amplitude of Spontaneous Otoacoustic Emissions in the Bobtail Skink

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Manley, Geoffrey A., Ulrike Sienknecht, and Christine Köppl. Calcium modulates the frequency and amplitude of spontaneous otoacoustic emissions in the bobtail skink. J Neurophysiol 92: 2685–2693, 2004. Active processes in the inner ear of lizards can be monitored using spontaneous otoacoustic emissions (SOAE) measured outside the eardrum. In the Australian bobtail lizard, SOAE are generated by an active motility process in the hair-cell bundle. This mechanism has been shown to be sensitive to the calcium-chelating agent 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid and is presumed to be related to the calcium-sensitive transduction-channel motor implicated in other nonmammalian hair cell systems. In studies of frog saccular and turtle auditory papillary hair cells in vitro, the frequency and amplitude of bundle oscillations depend on the concentration of calcium in the bathing solutions. In the present study, the calcium concentration in the endolymph was changed in vivo in the Australian bobtail lizard Tiliqua rugosa, and SOAE were monitored. Glass pipettes with large tips and containing different calcium concentrations in their fluids were introduced into scala media, and their contents were allowed to passively flow into the endolymph. Low calcium concentrations resulted in a downward shift in the frequency of SOAE spectral peaks and generally an increase in their amplitudes. Calcium concentrations >2 mM resulted in increases in frequency of SOAE peaks and generally a loss in amplitude. These frequency shifts were consistent with in vitro data on the frequencies and amplitudes of spontaneous oscillation of hair cell bundles and thus also implicate calcium ions in the generation of active motility in nonmammalian hair cells. The data also suggest that in this lizard species, the ionic calcium concentration in the cochlear endolymph is ≈1 mM.

INTRODUCTION

It has been known for many years that the calcium ions in the milieu surrounding vertebrate hair cells are important for normal sensory transduction. Because of their accessibility, most of the early studies of hair-cell transduction mechanisms were carried out using lateral-line hair cells (e.g., Russell and Sellick 1976; Sand 1975). Using frog saccular hair cells, Corey and Hudspeth (1979) showed that hair-cell transducer channels are nonspecific for positive ions, suggesting that the current carried by the different ions would be determined by their relative concentrations and valences. Because the hair cells of the sacculus and the lateral line are embedded in otolithic membrane or cupular material, however, it was (and still is) difficult to measure the concentrations of the different ionic species in the fluid surrounding the transduction channels. In addition, the hair-cell stereovillar membrane is able to extrude calcium ions at a high rate and significantly alter the concentration around the bundle (Yamoah et al. 1998). Attempts to assess the true ionic concentrations in such hair-cell systems have thus mostly been indirect, looking at the effects of different concentrations on hair-cell responses in vitro.

Normal calcium concentration in endolymph

The literature is relatively inconsistent regarding the concentration of calcium in mammalian cochlear endolymph. Whereas, e.g., Bosher and Warren (1978) first measured the total and ionized fraction concentrations to be between 23 and 30 μM, and Ikeda et al. (1987) reported the concentration of ionized calcium in the guinea pig endolymph to be 22 μM, other data (summarized by Ferrary et al. 1988) indicated a range of 23–560 μM total calcium. Recent measurements confirmed that in normal, pigmented guinea pigs, the ionized fraction of calcium in cochlear endolymph lies in the range from 26 μM (in the basal turn) to 38 μM (in the apical turn) (Gill and Salt 1997). In lizards, the only report (Petersen et al. 1978) gives a total calcium concentration of ~2 mM in endolymph (and perilymph) of the alligator lizard. Interestingly, these authors also report the highest value for total calcium concentration in mammalian endolymph, 560 μM from the cat cochlea. The analysis and data of Ferry et al. (1988) indicate that in mammalian cochlear endolymph, the total calcium concentration is ~20 times higher than the ionized fraction. If this was also true in lizards, the ionized fraction in lizard endolymph could be estimated from the data of Petersen et al. (1978) to be ~100 μM. However, nonmammalian cochlear endolymph is likely to be more similar to that of vestibular endolymph because the cochlear duct also contains the otolithic lagener macula. Vestibular calcium concentrations—also in a fluid surrounding otolithic organs—in mammals are higher than those of cochlear endolymph by an order of magnitude with an ionized fraction of ~0.26 mM and a total fraction of 3.0 mM (reviewed by Sterkers et al. 1988).

Calcium modulates transduction currents

Lumpkin et al. (1997) demonstrated that the hair-cell transduction channel in the frog saccus is selective for calcium over monovalent ions and that calcium carries a substantial current even at low concentrations. The calcium transduction current was largest at modest concentrations of monovalent ions. In turtle auditory hair cells, Ricci and Fettiplace (1998)
showed that in the presence of ionic concentrations similar to those assumed to be present in turtle endolymph (they assumed 50 µM calcium), calcium carried ~10% of the total transduction current.

In a study of the effects of calcium concentration on the transducer channels in turtle auditory hair cells, Crawford et al. (1991) showed that when the concentration was lowered from 2.9 to 0.5 mM (while also lowering the magnesium concentration from 2.2 to 0 mM), the transducer current was doubled. On lowering the calcium concentration even further to 50 µM, there was a threefold increase in current but also a loss of adaptation and a shift in the current-displacement relationship. Under these conditions, more than half the maximal current was activated at rest, compared with 10% in the 2.9 mM control solution. In a solution with only 20 µM calcium, the cells displayed a steady current but were nonresponsive. These data suggest that at progressively lower concentrations, the open probability of the transduction channels increases until at 20 µM, all channels are steadily open. This confirms Russell and Sellick’s (1976) suggestion that calcium controls the permeability of the transducer channels. In our discussion, we will assume the basic elements of the tip link version of the gating spring model (see Gillespie and Walker 2001 for a recent review).

When raising the calcium concentration above hair-cell bundles of the frog’s sacculus, Martin et al. (2003) report that the bundle displayed a net movement in the negative direction, suggesting closure of bundle channels. Assad et al. (1989), Eatock et al. (1987) and Hacohen et al. (1989) measured the effects of different ionic concentrations on the adaptation rates of saccular hair cells of the bullfrog. Raising calcium levels increased the relaxation rate of the bundles through the potentiating effect of calcium on the slipping rate of the myosins of the adaptational plaques attached to the tip links. This relaxed the tension on the tip links and allowed channels to close. At rest in low calcium concentrations, more channels were open. Marquis and Hudspeth (1997) showed that the maximum stiffness of intact hair-cell bundles in the frog saccus was attained at a concentration of 250 µM, which they suggest to be the concentration found in frog endolymph.

Recently, Ricci et al. (2003) demonstrated that in hair cells of the turtle auditory papilla, the conductance of individual transducer channels increased systematically from the low- to the high-frequency end of the papilla and that in all locations, the channel conductance and the time course of channel activation and adaptation were substantially slowed by lowering calcium levels, in this case from 2.8 to 0.05 mM (Ricci et al. 2003).

Rapid movements of the stereovillar bundle and their calcium dependence in vitro

With advances in measurement techniques, it became possible to make much more subtle measurements of tension in the gating springs and movement, and this resulted in the discovery of an active bundle motion that was influenced by the calcium concentration. Active bundle motion was first reported by Crawford and Fettiplace (1985), who measured spontaneous displacements of the bundles of turtle auditory hair cells, with amplitudes ≤20 nm, in a fluid containing 2.8 mM CaCl₂. Howard and Hudspeth (1987) observed mechanical resonances in frog saccular hair cells in a solution containing 4 mM CaCl₂ and proposed that these originated from an active force generator. In these early studies, the force generation was attributed either to myosins in the hair cells or to the presence of a (presumably active) kinocilium. Jaramillo et al. (1990) described oscillatory motions of frog saccular hair-cell bundles that occurred both spontaneously and following applied mechanical stimulation. Some rapid movements of the bundles were described as being due to the rebound closure of transduction channels—the bundle, as it were, kicks back. These movements were faster in higher calcium concentrations but only detectable at concentrations >250 µM. Active bundle motion was also attributed to the transduction process itself by Benser et al. (1996), who describe rapid and spontaneous bundle twitches of saccular hair cells in solutions containing 4 mM and 250 µM calcium chloride. These authors suggested that is possible that such rapid movements of hair-cell bundles are responsible for producing the sound energy underlying spontaneous otoacoustic emissions.

In turtle auditory hair cells (Ricci et al. 1998), the frequency of the damped oscillation of the transduction current (58–230 Hz) seen in response to a step stimulus depended on the external Ca²⁺ concentration. Martin and Hudspeth (1999) and Martin et al. (2003) studied active bundle movements in frog saccular hair cells using response measurements in a fluid containing 250 µM calcium. The observed oscillation frequencies (5–130 and 5–50 Hz, respectively) were lower than the range known from afferent-fiber recording from the intact saccus, and thus they suggested that the in vivo calcium concentration may in fact be higher. Martin et al. (2003) also reported in this preparation that increasing the calcium concentration above their standard of 250 µM rendered oscillations faster. The frequency could be doubled at the most, and the amplitudes fell until the oscillations were suppressed. Lowering the calcium concentration <250 µM moderately with chelators had the opposite effect. For a given hair bundle, the slow oscillations were often the largest. No oscillations were observed when the calcium concentration exceeded 1 mM or fell <100 µM.

Spontaneous bundle movements produce spontaneous otoacoustic emissions

All of the preceding measurements were made using extirpated organs studied in vitro. The only study that used in vivo conditions is that of Manley and Kirk (2002b) using spontaneous otoacoustic emissions (SOAE) from the basilar papilla of the Australian bobtail skink *Tiliqua rugosa*. In that study, the introduction of bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetatic acid (BAPTA), a powerful calcium chelating agent, into scala media above the basilar papilla resulted in a drop in the frequency of SOAE that was often accompanied by a rise in amplitudes. Taken together with the in vitro data described in the preceding text and evidence that SOAE are the result of hair-cell bundle oscillations (e.g., Manley 2001; Manley et al. 2001), these data suggested that in this preparation, the oscillation frequencies of hair-cell bundles were reduced and their amplitudes raised due to the presence of lowered calcium concentrations. These changes in frequency and amplitude are fully compatible to the in vitro data described in the preceding text for other hair-cell systems. Because it is not possible to
know the calcium concentration that results from BAPTA iontophoresis into scala media, however, we decided to measure the effects on SOAE when solutions of different calcium concentrations are introduced in vivo to modulate the calcium levels near the hair cells.

METHODS

Data were obtained from experiments on 12 ears of eight Australian bobtail lizards (Tiliqua rugosa) captured in the wild under license in Western Australia. The lizards, weighing between 103 and 339 g (median: 107.5 g), were anesthetized with pentobarbital sodium and Diazepam as described previously (Manley et al. 2001). Esophageal temperature was between 26.9 and 29.8°C, but within an experimental time series was kept constant ±0.2°C, and the animals were artificially ventilated with room air.

The ventrolateral surface of the otic capsule was exposed through the skin and muscle layers of the lower jaw. A small hole (<0.5 mm) was made in the bone covering scala vestibuli and a glass micropipette electrode broken to between 8 and 28 µm OD (mean: 18.3 µm) was advanced through scala vestibuli and the thin vestibular membrane into scala media. Previous studies had shown that to enable bulk flow out of such electrodes (unaided by electrophoresis), an electrode diameter of ≥6 µm is required. The pipettes were filled with mixed solutions of KCl and CaCl₂, with the following compositions: (KCl concentration + CaCl₂ concentration in mM) 200 + 0, 197.5 + 0.625, 195 + 1.25, 190 + 2.5, 180 + 5, 180 + 10, 180 + 20, 180 + 50, and 100 + 100. During electrode advance, the DC potential was monitored using a WPI Cyto 721 electrometer, and entry into scala media was indicated by a jump to an endocochlear potential of about +6 mV (5.4 ± 1.56, mean ± SD, n = 58) (Manley and Kirk 2002a; Manley et al. 2001). To ensure that the electrode stayed in scala media, it was advanced a further 0.2–0.5 mm with no further change in potential. An Ag/AgCl wire placed under the skin of the lower jaw served as the reference electrode.

A closed, calibrated microphone system using a Brüel & Kjaer 4166 microphone and a Brüel & Kjaer 2660 low-noise preamplifier was sealed with petroleum jelly (Vaseline) into the ear canal. Averaged SOAE spectra were captured using a personal computer running Labview software and deriving the input from the microphone system and an interface card (NI PCI-6024E). Signals were sampled at 10 kHz and high-pass filtered (500 Hz, 3-pole Butterworth) and averaged spectra (100 Hanning-windowed RMS averages) were stored to disk for further analysis. SOAE spectra were monitored before surgery and during and after electrode penetration to ensure that no damage to the inner ear ensued (manifested in a drop in SOAE amplitudes). If doubt existed as to the location of the electrode tip, the injection of negative current through the electrode (2–5 µA for 2–3 min) was used to cause a downward shift of SOAE frequencies (Manley and Kirk 2002a). Failure to elicit SOAE frequency shifts indicated that the electrode was no longer within scala media. The collection of SOAE spectra was begun as soon as feasible after electrode penetration, and this was routinely possible within 30 s, often within 15–20 s. This time resolution was necessary when using higher calcium concentrations, which caused rapid frequency shifts after very short delays. Because larger increases in frequency were accompanied by falls in SOAE peak amplitudes, it was often necessary to rapidly withdraw the electrode within minutes of its penetration, to permit recovery of hair-cell activity within a useful time period.

Between one and nine penetrations (mean: 3.6) were carried out per ear with no attempt to use a particular sequence of calcium concentrations. The SOAE peak frequency was defined as the frequency bin with the highest amplitude. Subsequent penetrations were attempted after the peak frequencies had returned to near their original values and had been stable over a period of ≥5 min. To quantify the rates of frequency shift, linear regressions were fitted to the data points beginning at the time where a consistent shift in frequency of >1% was induced, and covering a subsequent time interval of a maximum of 2 min. In a minority of extreme cases, where the SOAE changed very rapidly, this included only two or three data points. Rates of SOAE amplitude change were derived in the same way and over the same time intervals, provided the change in amplitude exceeded 1 dB.

Exceptions were only made in cases where a reversal of the amplitude change occurred; here, the time interval of analysis was shortened accordingly to include only the initial effect.

Animals were collected under a license from the Western Australian Department of Conservation and Land Management, and experiments were carried out with the approval of the Animal Experimentation Ethics Committee of the University of Western Australia.

RESULTS

We report the results of 31 electrode penetrations in 12 ears, for which the frequency changes in 80 SOAE peaks were quantified as a function of the calcium concentration in the electrode. The calcium concentrations used were 0, 0.625, 1.25, 2.5, 5, 10, 20, 50, and 100 mM. The highest SOAE peak frequency examined before shifts were induced was 3,257 Hz, the lowest 1,014 Hz. Between one and five SOAE peaks were quantified per ear, using only the larger peaks in each spectrum to minimize the effect of small-amplitude fluctuations on the measurement accuracy and increase the time range of measurements in cases where amplitudes fell.

At the two extremes of calcium concentration, the SOAE peaks were strongly affected, and the behavior of the peaks under the two conditions was diametrically opposite. When using very high or very low calcium concentrations, the electrode was usually withdrawn within a few minutes, to avoid extreme effects with very long recovery times. When electrodes containing only 200 mM KCl (presumably with a finite but extremely small calcium content) penetrated scala media, they initiated a rapid drift of peak SOAE frequencies downward. This was usually accompanied by an increase in peak amplitudes (Fig. 1). In contrast, high concentrations of calcium in the electrode initiated a rapid shift of SOAE peak frequencies upward, accompanied by a loss of amplitudes to below the noise floor and a long recovery time (Fig. 2). Data obtained using very high concentration values were very difficult to quantify, however, because the SOAE amplitudes sometimes fell to the noise in < 1 min. Slower, smaller shifts were obtained for intermediate concentrations (Fig. 3). The largest frequency shifts observed for each SOAE peak and for all calcium concentrations used are shown in Fig. 4, irrespective of whether the electrode was withdrawn during the shift period. Thus the peak values in this figure do not necessarily represent the maximum possible shift. Because often the frequency shifts were large and because amplitudes tend to decrease dramatically when peak frequencies fall <1 kHz (Manley and Kirk 2001), the electrode was usually withdrawn before stable conditions were found.

Frequency shifts of up to ~30.6 and +10.4% were observed (Fig. 4A). At 5 and 10 mM calcium concentration, there was a significant correlation between the maximal change in frequency observed and the center frequency of the SOAE with the larger shifts shown by lower-frequency SOAE peaks (Fig. 5). This was not the case for other concentrations, and this is probably mainly due to the fact that at the highest concentra-
tions, the electrode was withdrawn very quickly to attempt to prevent total loss of SOAE amplitudes, and thus the data do not reflect what would have happened at the true final concentrations. Thus the largest positive frequency shifts (+9.9 and +10.4%) were not limited to the highest concentrations but were observed at very different calcium concentrations (2.5 and 50 mM). At high calcium concentrations, this upward frequency shift could happen so quickly (<1 min) that it was not possible to structure the time resolution (minimum time between measurements 15 s) so as to catch the highest frequency that would have been readable. The observed changes in amplitude accompanying downward frequency shifts were up to +8 dB and those accompanying upward frequency shifts were maximally −11 dB (Fig. 4B).

Figure 4 includes all data collected, and thus usually several data points per electrode penetration (1 for each SOAE) as well as some repeated measurements from individual ears for the same calcium concentration. The same picture emerges, however, if to avoid mixing dependent and independent data, repeated measurements are eliminated and only one data point per penetration (from the largest SOAE) is entered (Fig. 6, A and B and Fig. 7). Although there was a wide scatter for 10 mM data, these figures also indicate that the polarity of this calcium effect reverses at a concentration near 1.25 mM. All frequency shifts were zero or negative for concentrations ≤1.25 mM, and all frequency shifts were zero or positive for concentrations of ≥2.5 mM (Figs. 4A and 6A). Interestingly, in both cases when using the concentration 1.25 mM, the frequency initially...
shifted down a little then recovered and shifted up a little with the electrode still within scala media (Fig. 3). Similarly, peak SOAE amplitudes generally increased when using calcium concentrations of <1.25 mM, and peak amplitudes generally fell for concentrations of 2.5 mM or more (Figs. 4B and 6B).

SOAE peak frequencies and amplitudes showed recovery toward their initial values after electrode withdrawal. In cases in which high calcium concentrations were used, however, recovery times were often so long (e.g., 19 h), that in many cases continuous monitoring of the recovery process was not attempted.

**DISCUSSION**

SOAE in the bobtail skink originate in the high-frequency area of the papilla (Köppl and Manley 1993), which neurophysiological data have shown covers the frequency range from ~1 to 4.8 kHz (Köppl and Manley 1990). The hair cells of this area are micromechanically tuned, and groups of hair cells that are linked by individual tectorial sallets oscillate spontaneously at their best frequency, generating SOAE (Köppl and Manley 1993, 1994; Manley 1997). Because the SOAE peaks studied in the present analysis ranged in center frequency from 1,014 to 3,257 Hz, it can be assumed that effects for hair cells over most of the high-frequency papillar area were sampled.

The goal of this research was to use this in vivo preparation to try to establish the endolymphatic calcium concentration at which no SOAE frequency shifts occurred on the assumption that this would represent the concentration that normally prevails above the hair cells. Because at the concentrations near the reversal concentration the electrode could be left in scala media (Fig. 3). An example of a waterfall spectral display showing the effect of introducing 1.25 mM calcium solution (plus 190 mM KCl) into scala media of the bobtail skink. The lowest spectrum was taken 4 min before the electrode penetrated scala media, the other spectra (from bottom to top) at the times in minutes as shown on the right of each spectrum. Only the times for every 2nd spectrum are shown. In this case, the electrode remained in scala media. To avoid overlap, each spectrum is shifted upward by 6 dB in relation to the previous one. The vertical dashed dark gray lines are for orientation on the initial frequencies of the 2 largest SOAE spectral peaks. During the last 2 spectra, here shown in light gray, negative current (3 μA) was injected through the electrode to verify that its tip remained within scala media. This caused a downward shift of the spectral peaks.

**FIG. 4.** A: Maximal shifts in the frequency of SOAE spectral peaks as a percentage of their original frequency that occurred under the influence of different calcium concentrations as compared with their frequency before electrode penetration of scala media. To plot these data on a logarithmic concentration scale, the 0-calcium data were assigned the value 0.01 mM. The dashed, dark gray line represents no change in frequency. Less than 2.5 mM, all frequency shifts were negative, whereas ≥2.5 mM, all frequency shifts were positive. B: maximum changes in the amplitude of SOAE peaks in decibels under the influence of different calcium concentrations as compared with their amplitude before electrode penetration of scala media. Low calcium concentrations generally lead to an increase in amplitude, whereas concentrations >2.5 mM generally lead to a loss of amplitude. The dashed, dark gray line represents no change in amplitude.
media, it can be assumed that in these cases, the calcium concentration near the hair-cell bundles was closer to that in the electrode than where more extreme concentrations were used and the electrode quickly removed. If this assumption is correct, the data indicate that in the endolymph of this lizard species, the calcium concentration is near or greater than 1.25 mM. Because the time course of the SOAE frequencies at 1.25 mM showed an initial frequency fall followed by a recovery with the electrode in place, it is likely that 1.25 mM is indeed the calcium concentration near the hair-cell bundles was closer to that in the electrode than where more extreme concentrations were used and the electrode quickly removed. If this assumption is correct, the data indicate that in the endolymph of this lizard species, the calcium concentration is near or greater than 1.25 mM. Because the time course of the SOAE frequencies at 1.25 mM showed an initial frequency fall followed by a recovery with the electrode in place, it is likely that 1.25 mM is indeed the calcium concentration near the normal value.

An exact definition of the “reversal concentration” for the calcium effect described cannot be obtained using the present data, however, because the precise distance of the electrode from the hair cells was not known for any experiment and in any case would have been different for hair cells at different locations along the papilla. In addition, the times of electrode withdrawal to prevent further change in calcium levels were individually assessed; this led to very different exposure times. Under these conditions, it is not possible to accurately determine the admixture of fluids near a given set of hair cells. Because the dynamics of fluid content control in lizards are not at all known (see, e.g., Manley and Kirk 2002a), and calcium is involved in so many intracellular processes, such an analysis would in any case be of limited usefulness. It might be objected that the changes in the osmolarity of the solutions in the electrodes (due to the changing relative concentrations of mon- and divalent ions) would have had a significant effect on the hair cells. However, in the extreme cases, the electrodes were quickly retracted, and the cells were not exposed for long periods. In the important range of calcium concentrations changes, from 0.625 to 2.5 mM, the variation in osmolarity was only ~2% (calculated to be between 354 and 363 mosM).

Although the exact location of the electrode tip in each case is not known, a simple assessment of the geometry of the cochlea, the placement of the hole in the bone, and the electrode depth suggests that the electrodes would have been nearer the 1-kHz location than the basal end, where frequencies near 4.6 kHz are located. This is the simplest explanation for the larger frequency shifts seen for lower SOAE center frequencies, at least for calcium concentrations of 5 and 10 mM (Fig. 5). At least at intermediate calcium concentrations, the different papillar regions might equilibrate at different concentrations, depending on the distance of the electrode tip.

The large difference in recovery rates between very low calcium concentrations, from which the SOAE recovered relatively quickly, and high calcium concentrations, from which they needed hours to recover, is not easily explained. However, if lizard endolymph is supplied with calcium in a similar way to that of fish (Payan et al. 2002), a possible explanation would lie in the very rapid rate at which calcium can enter the endolymph from the blood system. In the trout, the endolymph calcium pool is turned over 55 times a day, thus the entire endolymph calcium content can in theory be resupplied within ~25 min (Payan et al. 2002). However, in the trout, the otolith grows continuously, and this growth incorporates the equivalent of eight endolymphatic calcium pools per day. Although no information is available on otolith calcium uptake in lizards, it is likely that endolymph calcium can be much more rapidly replenished through homeostatic mechanisms (including dissolution of otoliths) than it can be lost to the blood system or bound within cells.

The size of the changes in SOAE peak levels was roughly the same in upward and downward directions of frequency shift. Larger downward shifts of frequency would certainly have occurred had the electrode not been withdrawn to prevent falls of frequency far <1 kHz with consequent loss of amplitude. Larger upward shifts could not possibly have been observed, however, because in all cases, the SOAE amplitudes fell below the noise floor when frequency shifted by about...
depolarization would reduce calcium influx, which is the equivalent of reducing the calcium concentration in the endolymph. As with the data of the present study, the largest shifts in frequency were seen for equivalent reductions of calcium concentration, and this was accompanied by increases of SOAE amplitudes. In both sets of data also, increases in frequency seem to be limited to a maximum of 8–10%, before amplitudes fall to the noise level. Martin et al. (2003) also report amplitude loss accompanying a rise in bundle frequencies after raising calcium concentration and that in individual hair bundles, the highest amplitudes were those measured at the lower frequencies shown in reduced calcium levels. The effects of BAPTA injections into scala media (Manley and Kirk 2002b), buffering the calcium to very low levels, reflect rather precisely what happened when zero-calcium KCl solution was used in the present experiments. The great similarity of these three data sets also indicate that variations in the osmolarity of the solutions used in the present experiments played no essential role in producing the effects observed.

The question remains as to how the changes in calcium concentrations are causing the frequency and level changes observed. It has to be considered whether the ionic milieu substantially affects, for example, the tectorial membrane, and that this affects the micro-mechanical resonant frequency of the hair-cell-saccular units. A substantial change in tectorial mass would certainly affect the resonant frequency (Manley et al. 1988). However, if the tectorial material of the boxtail skink is similar to that of the alligator lizard, the data of Freeman and Weiss (1995) would suggest that large changes in the calcium concentration would only minimally affect the tectorial membrane (1–2% thickness change in their data). Thus it is necessary to look instead to the hair cells for an explanation.

The changes in SOAE peak frequencies observed in the present experiments are likely to be mainly due to effects of calcium on the mechanical tension exerted on the tip links of the transduction channels and on the timing of the oscillation cycle of the active process. Slower channel closure would lead to an increased cycle time of the bundle oscillation. Lowering calcium would simultaneously increase the tension on the tip links. The increased proportion of open channels, shifting the operating point of the hair cell’s transfer function toward symmetry, should enable greater bundle excursions to occur, consistent with the higher amplitudes generally observed with lower calcium concentrations. The opposite would be true of raised calcium levels. When calcium concentrations are high enough, the proportion of open channels would be insufficient to sustain spontaneous movement, leading to a collapse of the SOAE. This effect would explain the lower amplitudes and—assuming that normally the open probability of the channels is low—the apparent limitation of increases in frequency to a maximum of ~10%.

Recent models of the roles of calcium in hair-bundle adaptation and motility offer explanations for the present data. Hacohen et al. (1989) showed that in saccular hair cells of the bullfrog, increased calcium potentiated the relaxation rate and could be expected to reduce the mechanical tension on the tip links. An increased calcium concentration facilitates detachment of the myosin molecules from the actin core of the stereovilli. This relaxes the tension on the tip links and allows channels to close. When changing the calcium concentration near hair-cell bundles of the frog’s sacculus, Martin et al.

![Graph showing rate of amplitude change vs. rate of frequency shift](http://jn.physiology.org/lookup/suppl/doi:10.1152/jn.00011.2004/-/DC1/fig7.jpg)
(2003) report closure of bundle channels with increased, and opening of channels with decreased, calcium concentrations. Consistent with this, lowering the calcium concentration above turtle auditory hair cells increased the transducer current (Crawford et al. 1991) and increased channel opening probability (Ricci et al. 2003).

Lizard SOAE are the result of an active process in the hair-cell’s stereovillar bundle (Manley et al. 2001). Thus the SOAE amplitude would be dependent on the open probability of transduction channels because the ability of calcium to enter channels and cause their premature closure would be directly proportional to the number of open channels. The fact that amplitudes can be substantially raised by lowering calcium levels suggests that the proportion of channels open under normal conditions is <50%. Thus the operating point of the hair cells would be asymmetrically placed on the transfer function, as also indicated by data from the turtle basilar papilla and frog saccus. Using those data and correcting for higher calcium concentrations (see preceding text) suggests that in the intact hearing organ of the bobtail lizard, the open probability of the transduction channels is 20–25%.

As noted in the introduction, it can be expected that in nonmammals, the endolymph of the cochlear duct is more similar to that of the vestibular system than is the case in mammals. In mammals, the calcium concentration (ionized fraction) in vestibular endolymph is between 0.26 and 0.47 mM (Ferrary et al. 1988). Although it can be assumed that in the present experiments the electrode concentration represented fully ionized calcium, it remains unknown whether all the calcium reached the hair cells in this state especially at low concentrations. The nonionized calcium fraction in endolymph is assumed to have a buffering function (Ferrary et al. 1988; Sterkers et al. 1988) and is presumably able to change the ionized fraction quickly. Little is known, however, about the equilibria between ionized and nonionized calcium fractions in the endolymph on the one hand and these fractions and the calcium of the otoliths on the other hand. In addition, it would not be very surprising if the calcium levels measured in bulk endolymph were lower than those effective near the hair-cell bundle because hair-cell stereovillar membranes contain the calcium ATPase PMCA, and this enzyme can extrude sufficient calcium out of the bundle, raising the concentration outside the transduction channels (Yamoah et al. 1998).

The reversal of the effect of calcium on SOAE frequency near the introduced concentration of 1.25 (Figs. 4 and 6) suggests an in vivo calcium concentration >1 mM in lizard cochlear endolymph. This is higher than suggested in any previous physiological study but close to the total calcium concentration of 2 mM given for the alligator lizard by Peterson et al. (1978). Concentrations in the endolymph of mammals are not truly comparable because in the course of the evolution of the mammalian cochlea, the lagener macula has been lost and there are no otoliths in the cochlear duct. This is correlated with a reduction of the calcium concentration in mammalian cochlear endolymph as compared with vestibular endolymph to extremely low levels (~30 μM).

In other nonmammalian systems, in vitro hair-cell studies have been carried out using higher calcium concentrations than in mammals. However, the results both of the in vitro measurement series in frog saccus and in turtle basilar papilla may be explained if the calcium concentrations used in those experiments were lower than in the intact organ. Instead of 70 μM for the turtle cochllea and 250 μM for the frog saccus, calcium concentrations of between 200 μM and 1 mM would perhaps be more appropriate. In turtles, hair cells in 70 μM calcium displayed damped oscillations with frequencies that were ~65% of those typical of the intact organ. For example, one cell that showed 77 Hz in 70 μM calcium solution, oscillated at 92 Hz (+19%) in 350 μM (Ricci et al. 1998, their Fig. 8B). A halving of the time constant resulted from a 10-fold change in calcium concentration, which means that an increase in frequency from 65% to normal would require a substantial change in calcium levels. Consistent with this, the fraction of transducer current activated at rest in 70 μM calcium was 28%, whereas in the intact organ it was only 10–15%. In sum, these data suggest that the normal calcium endolymph concentration in the turtle cochlear duct is ≃200 μM. Fettiplace (1992) noted that the stimulus-response relations obtained in vitro from isolated turtle cochlear hair cells bathed in 2.8 mM calcium were unexpectedly closer to in vivo relations than were in vitro relations obtained in 70 μM calcium. Fettiplace (1992) suggested that perilymphatic calcium levels must in vivo have more impact on transduction than expected, presumably by setting intracellular calcium levels. However, our data suggest a simpler explanation: that in fact, the 2.8 mM calcium level was closer to the normal in vivo level.

Similarly, measurements of active bundle movements in frog saccular hair cells by Martin and Hudspeth (1999) were made in a fluid containing 250 μM calcium. The observed oscillation frequencies were lower than the range in the intact saccus, and the authors suggested that in view of the calcium dependence of the oscillation frequency, the in vivo calcium concentration may be higher. In the frog saccus, it is possible that calcium concentrations are buffered to a higher concentration than in the turtle cochlea because local buffering conditions must ensure the stability of the crystalline structures of the saccular otoliths. If we assume that endolymph is at pH 7.4, the solubility of the calcium carbonate of the otoliths would produce a concentration of ~2.4 mM (http://www.chem.usu.edu/~sbialkow/Classes/3600/Alpha/alpha3.html). Bensen et al. (1996) were able to observe rapid bundle twitches of saccular hair cells even in solutions containing 4 mM calcium. These and our data strongly suggest that the concentration above auditory hair cells in frogs and reptiles is very much higher than in mammals.

In summary, the present data support indications in the literature that the ionic calcium concentration in the cochlear endolymph of nonmammals is between 250 μM and 1 mM. Future in vitro studies using higher concentrations of calcium than previously used would be essential to finally clarify the role of bundle motility in the generation of SOAE.

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REFERENCES


Lumpkin EA, Marquis RE, and Hudspeth AJ. The selectivity of the hair cell’s mechano-electrical-transduction channel promotes Ca$^{2+}$-flux at low Ca$^{2+}$ concentrations. *Proc Natl Acad Sci USA* 94: 10997–11002, 1997.


Martin P and Hudspeth AJ. Active hair-bundle movements can amplify a hair cell’s response to oscillatory mechanical stimuli. *Proc Natl Acad Sci USA* 96: 14306–14311, 1999.


