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BAPTA Induces Frequency Shifts in vivo of Spontaneous Otoacoustic Emissions of the Bobtail Lizard

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Key Words
Spontaneous otoacoustic emissions · Australian bobtail lizard · Cochlear amplifier

Abstract
Spontaneous otoacoustic emissions (SOAEs) are indicators of active processes in the inner ear and are found in all classes of land vertebrates. In the Australian bobtail lizard, earlier work showed that otoacoustic emissions are generated by an active motility process in the hair-cell bundle. This is likely to be driven by calcium-sensitive mechanisms implicated in other non-mammalian hair cell systems. If so, it should be fundamentally influenced by the extracellular calcium concentration. In in vitro studies, the rate of force generation in hair cell stereovilli is linked to the extracellular calcium concentration. In such preparations, low-calcium solutions, buffered by the calcium chelator BAPTA, were reported to change the frequency of hair cell bundle oscillations. In the present study, BAPTA was iontophoresed into the endolymph of the bobtail skink in vivo, and SOAEs were monitored. Application of BAPTA resulted in a prolonged downward shift in the frequency of individual SOAE spectral peaks. Recovery took more than 1 h, consistent with a slow clearance of BAPTA from endolymph. SOAE peak amplitudes were most often enhanced, suggesting there was no functional disruption of tip links. The direction and degree of frequency shifts were consistent with in vitro and in vivo data showing the effects of changing calcium concentrations in the endolymph directly.

Introduction

In all terrestrial vertebrates including humans, hearing at low sound levels is supported by a fast, mechanical amplifying system integral to the sensory hair cells [Hudspeth, 1997]. Numerous phenomena have been attributed to this 'cochlear amplifier', including the ear's high sensitivity and frequency selectivity, but also faint sounds in the ear canal (otoacoustic emissions) generated spontaneously, or in response to stimulation, by hair cells in the inner ear [Manley, 2001]. The frequency selectivity of tonal suppression of spontaneous otoacoustic emissions (SOAEs) in lizards clearly links these emissions to localized hair-cell activity [Köppl and Manley, 1994]. SOAEs and other phenomena believed to reflect amplification by hair cells have been described for all tetrapods examined so far, suggesting the underlying mechanisms are very old [Manley and Köppl, 1998].

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In mammalian cochleae, active mechanical force can be generated by the uniquely specialized outer hair cells (OHCs). OHCs in vitro are capable of rapid somatic motility driven by changes in the trans-membrane voltage [Brownell et al., 1985; Ashmore, 1987; Santos-Sacchi and Dilger, 1988; Dallos and Evans, 1995]. At low sound levels, the mechanical force set into effect by OHCs apparently provides most of the stimulus to the inner hair cells, which are the true sensory receptors.

The ears of non-mammalian vertebrates lack specialized OHCs and also lack their putative membrane motor molecules and membrane-based motility [He et al., 2003; Köppl and Manley, 2003]. Thus, alternative candidates for the amplifier mechanism and for the source of otoacoustic emissions in non-mammals were sought and found in the hair-cell bundle [Hudspeth, 1997; Martin and Hudspeth, 1999, 2001; Hudspeth et al., 2000; Fettiplace et al., 2001; Manley, 2001; Manley et al., 2001]. In the Australian bobtail lizard *(Tiliqua rugosa)*, the active motility source that generates otoacoustic emissions was demonstrated in vivo to be located in the hair cell stereocilia [Manley et al., 2001; Manley and Kirk, 2002], and is likely to be similar in nature to the transduction channel motor studied in experiments on single hair cells from other non-mammals [Benser et al., 1996; Martin and Hudspeth, 1999, 2001; Fettiplace et al., 2001]. Interestingly, new evidence demonstrates that rapid adaptation is also seen in mammalian hair cells [Kennedy et al., 2003]. These movements may also be involved in cochlear amplifier mechanisms in mammals, making the investigation of active bundle mechanisms of more general interest with regard to hearing in vertebrates.

A transduction channel motor generating SOAEs should demonstrate a physiological sensitivity to changes in extracellular calcium levels, since a likely driving force of the active mechanism is the rapid re-closure of channels on calcium entry. Elevated calcium affects adaptation rates and extends and increases the channels' tendency to close [Eatock et al., 1987; Assad et al., 1989; Crawford et al., 1989]. Such processes have been shown theoretically to be capable of inducing both active amplification and spontaneous oscillation [Choe et al., 1998; Vilfan and Duke, 2003]. Experimental observations in vitro predict that the frequencies of SOAEs produced by a channel motor should be calcium sensitive [Martin and Hudspeth, 1999]. In hair bundles of cells in excised preparations from the bullfrog sacculus, both the frequency of spontaneous oscillations [Martin and Hudspeth, 1999] and the speed of active force transients [Jaramillo et al., 1990; Benser et al., 1996] were faster at higher extracellular calcium levels. Recent studies of the pore of the transduction channel of hair cells proposes that the rate of entry of monovalent ions is affected by calcium binding to a site inside the pore and by additional calcium physically blocking the channel [Farris et al., 2004].

In the bobtail lizard, lowering endolymphatic calcium levels in the endolymph caused the frequencies of SOAE to fall, and raising the calcium concentration caused them to rise [Manley et al., 2004]. In the turtle auditory papilla and in response to mechanical step stimuli, appropriate calcium conditions lead to damped bundle oscillations in the animal's hearing range [Ricci et al., 1998, 2002; Fettiplace et al., 2001], and lowering calcium levels slowed the time constants in both turtle and mammalian hair cells [Kennedy et al., 2003; Ricci et al., 2003]. The calcium chelator BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′,-tetraacetic acid), causes loss of mechano-sensitivity, elimination of tip links, and loss of voltage-dependent bundle motility [Assad et al., 1991; Marquis and Hudspeth, 1997]. In view of these data, it appeared opportune to study the effects of manipulating calcium levels using the chelator BAPTA on one manifestation of an active mechanism, the SOAE, in vivo.

This report describes the effects of BAPTA on SOAEs from the intact ears of anaesthetized bobtail lizards. No evidence of tip link disruption was found when BAPTA was applied by iontophoresis to scala media. However, SOAE frequencies shifted strongly downwards, consistent with the expectation [e.g. Martin and Hudspeth, 1999] that low extracellular calcium should reduce the frequency of spontaneous oscillations.

Methods

Data were obtained from successful experiments on four Australian bobtail lizards *Tiliqua rugosa* from a group of 11 animals captured in the wild under license in Western Australia. The final sample size was limited by the difficulty of access to scala media in larger animals. The lizards, weighing between 105 and 434 g, were anaesthetized with pentobarbital sodium and diazepam (initial doses averaged 43 $mg \cdot kg^{-1}$ and 1.8 $mg \cdot kg^{-1}$, respectively, typically followed by one-third doses of pentobarbital every 1.5 h and a full diazepam dose every 3 h). Oesophageal temperature was kept as constant as possible near 30°C and the animals were artificially ventilated using air at a volume (~4 ml) and rate (10/min) approximating that of a resting animal. Some lizards, that were used on two sequential days, were cooled to about 10°C overnight – while the anaesthesia was maintained – and slowly rewarmed on the next day. At the end of the experiment, the animals were killed with an overdose of pentobarbital sodium.

The ventro-lateral 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 an OD of ~3 μm) was advanced through scala vestibuli and through the thin vestibular membrane into scala media. The pipettes were filled with either 200 m*M* KCl or with a solution of 150 or 200 m*M* BAPTA (tetra potassium salt, Sigma). During electrode advance, the DC potential was monitored and entry into scala media was indicated by a potential jump to an endocochlear potential of about +6 mV [Manley et al., 2001; Manley and Kirk, 2002]. To ensure that the electrode stayed in scala media, it was advanced a further 0.2–0.4 mm with no further change in potential. An Ag/AgCl wire placed under the skin of the lower jaw served as the reference electrode. Due to subtle changes in electrode properties as a result of BAPTA injections, it was not possible to reliably follow any changes in the magnitude of the EP.

A closed, calibrated microphone system utilizing a Brüel and Kjaer 4166 microphone coupled to a Brüel and Kjaer 2660 low-noise preamplifier was sealed with Vaseline® into the ear canal. Averaged SOAE spectra were displayed on a spectrum analyser (Stanford SR760) and stored to diskette, as required, 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.

BAPTA was injected into scala media by iontophoresis (–2 to –4 μA DC). We assume that the negative current would not have moved potassium in the BAPTA salt into scala media and thus not have affected the potassium concentration in the endolymph. Since negative direct current by itself has a systematic influence on SOAE [Manley and Kirk, 2002], control injections were carried out in which the iontophoresis currents were applied through KCl-filled pipettes. The size of the access hole and the necessary angles of approach were such that only one pipette at a time could be placed in scala media. Thus, control and BAPTA iontophoresis trials were made in successive penetrations with the appropriate pipette inserted. Repeated control trials verified that the differences between control and BAPTA injections as described in the Results were not due to order effects.

Rates of accumulation of BAPTA in scala media during iontophoresis and its subsequent clearance were estimated using the 'Washington University Cochlear Fluids Simulator' (version 1.5) developed by Dr. Alec Salt (see http://oto.wustl.edu/cochlea/). In the simulation, [BAPTA] was estimated 0.4 mm from the injection site, since this was presumed to be the minimal distance between the intended position of the electrode tip and the hair cells producing the SOAEs. Since BAPTA is an anion, it must clear very slowly from scala media. For the simulation, a clearance half time of 58 min was assumed. This was based on the clearance rate constant of $AsF6^-$ in guinea pig [Salt and DeMott, 1994] adjusted for the low (<10 mV) endocochlear potential in lizards. This simulation can only be a rough approximation, however, as the empirical data on which the model is based were derived from the mammalian and not the lizard cochlea and, in addition, the lizard cochlea contains calcareous otoliths in the lagena macula that may strongly buffer the BAPTA effect (see below).

Animals were collected under 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

Consistent with our earlier report [Manley and Kirk, 2002], negative direct current injected into scala media through KCl-filled pipettes systematically changed SOAE spectra, inducing a downward shift in the frequency of individual SOAE peaks. In these control experiments, peak frequencies generally returned fully to baseline values within 15–20 min after the current was turned off. The effect of an injection of BAPTA was qualitatively similar, but the shifts in frequency were larger, and recovery to the baseline frequencies was considerably slower.

Examples of shifts in SOAE peak frequencies can be seen in the series of spectral traces in figure 1. Here, the iontophoresis current (–2.5 μA) was applied for 16.5 min and the ear canal sound field was sampled at intervals of from 2 to 12 min. The lightly-shaded spectral traces were recorded during the passage of current, which began at 0 min. The SOAEs were monitored in this case for 39 min after the onset of the control current (a), and for 70 min after the onset of BAPTA iontophoresis (b), the latter was not quite adequate for full recovery in this case.

Changes, over time, in the frequencies of the three largest SOAE peaks from figure 1 are plotted in figure 2. These peaks were initially found at frequencies of 1766, 2078 and 2438 Hz, but the frequencies of one of them had shifted very slightly (by one 15-Hz frequency bin, as indicated) prior to the iontophoresis of BAPTA. Initially, the downward frequency shift was similar during both control and BAPTA injections. However, after approximately 5 min, the BAPTA-induced shift progressed more rapidly, with peak frequencies falling by more than 40% compared with about 30% in the control condition. A more striking difference, however, was evident in the return to baseline frequencies. Recovery occurred over about 15 min in the control data, but the frequency shifts induced by passing current through the BAPTA electrode, though having an initially similar rate of recovery over the first 5 min or so, recovered much more slowly over the ensuing 45 min.

The assumption was made that the changes measured after iontophoresis of BAPTA were a linear sum of the effects of the iontophoresis current per se and of the presence of BAPTA in endolymph. The assumption is based on the observation that in the first few minutes of a BAPTA injection, i.e. presumably before the BAPTA reached the hair cells, the current effect is identical to that when using a potassium chloride-filled electrode (e.g. fig. 2). Accordingly, an estimate of a 'net' BAPTA effect was obtained by simple subtraction. The results of this

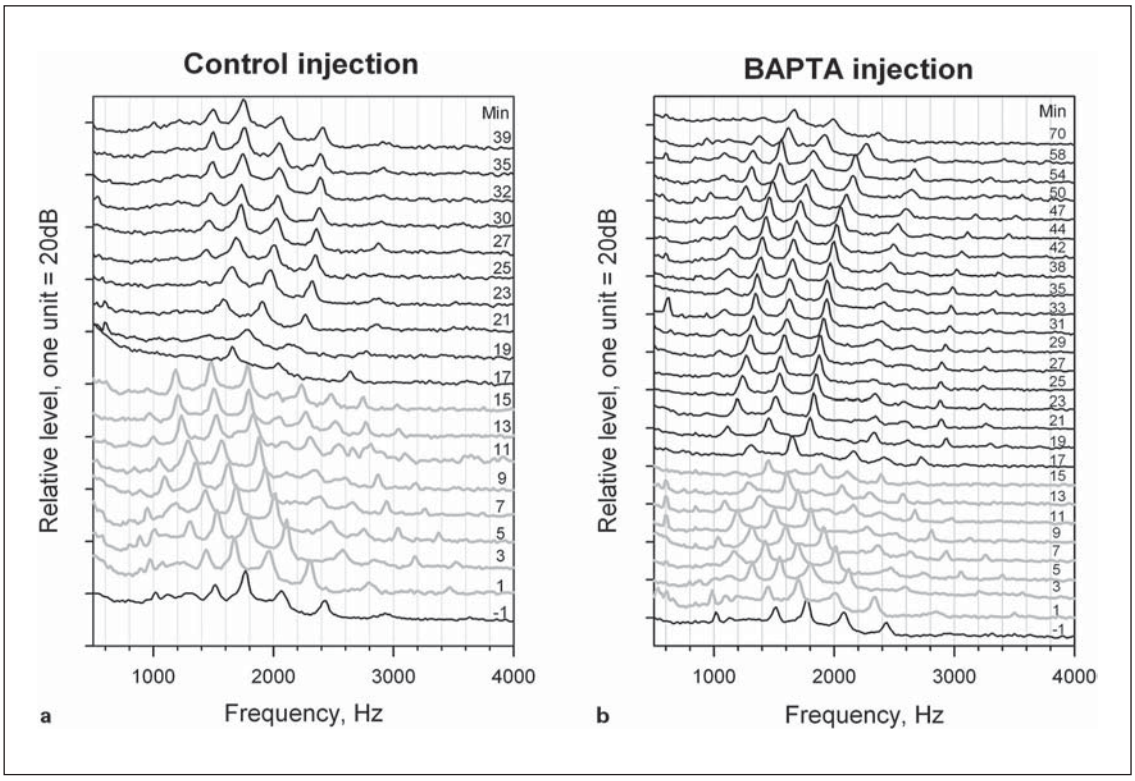

Fig. 1. Waterfall display of SOAE spectra from one ear measured successively from bottom to top at time intervals as indicated in minutes to the right of each spectral trace. Iontophoresis current (–2.5 μA DC) began at 0 min and was applied for 16.5 min. Grey-shaded spectral traces were recorded during the period of iontophoresis. Current was passed first through a pipette containing 200 m*M* KCl (**a**; control) and then through a pipette filled with 150 m*M* BAPTA (**b**). The trace shift at lower frequencies in the control at 17 min was due to a loud external noise. Note that during current injection, amplitudes increased, and small SOAE peaks appeared at frequencies where they were not seen in the resting case. In both **a** and **b**, one tick on the ordinate represents a relative separation of 20 dB.

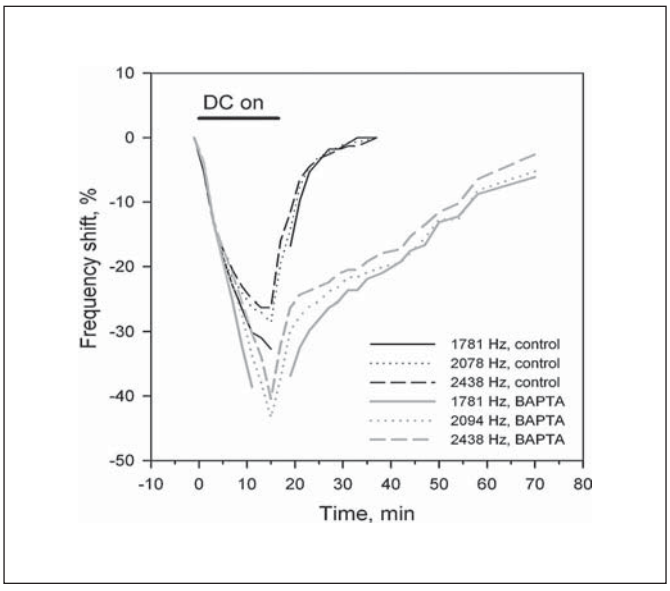

Fig. 2. Development and recovery over time of the shifts in frequency induced by control current and BAPTA iontophoresis for the three largest SOAE peaks in figure 1. The frequencies of one of the peaks had shifted slightly prior to iontophoresis of BAPTA. Shift in frequency is expressed on the vertical axis in percentage units relative to the initial baseline frequencies, which are indicated in the figure legend. The horizontal bar indicates the current-injection or the iontophoresis interval. A break in the trace indicates that the SOAE peak was temporarily not measurable due to loss of amplitude.

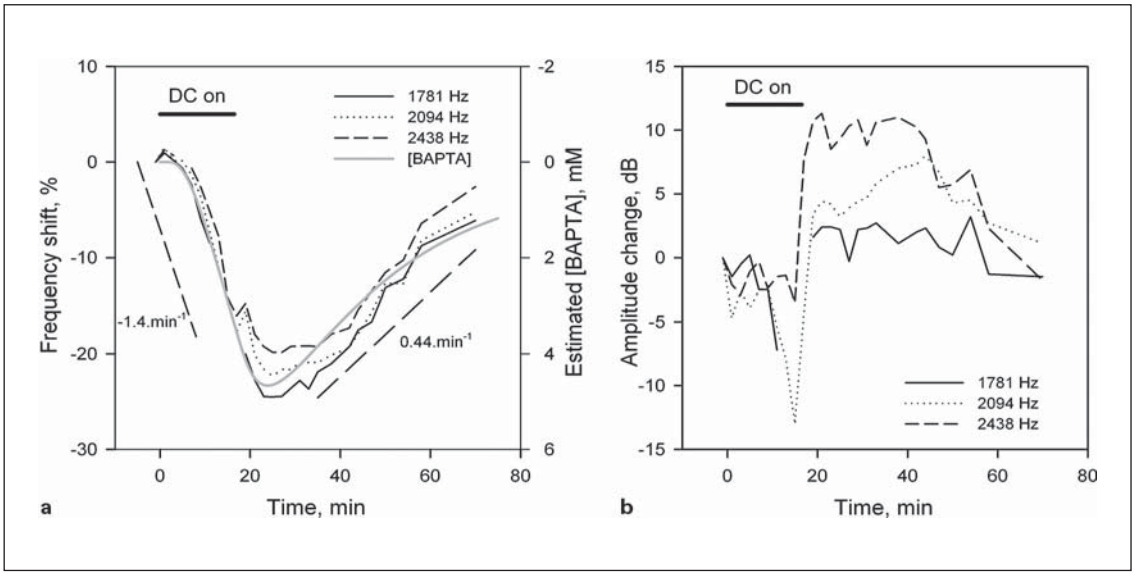

Fig. 3. a 'Net' effect of BAPTA on SOAE frequency (i.e. the difference between the effect of the control current and the BAPTA injection), derived from the data of figure 2. Frequency shifts induced by the control current were subtracted from those produced when current was passed through the BAPTA pipette. Linear estimates of onset and recovery rates (dashed lines) were fitted to the means of the three peaks between 7 and 25 min (onset) and between 31 and 70 min (recovery). The lightly shaded, solid line is an estimate of the accumulation of BAPTA near the hair cells and its subsequent clearance (note the inverted ordinate concentration scale). **b** 'Net' effect of BAPTA on the amplitudes of the three SOAE peaks as a function of time, derived by subtracting the amplitude shifts induced by the control current from those produced when current was passed through the BAPTA pipette. In both **a** and **b**, the horizontal bar indicates the current-injection interval.

procedure applied to the data of figure 2 are shown in figure 3a. Only the amplitudes and frequencies of the three largest SOAE peaks were analyzed. The shift in frequency (fig. 3a) had an onset rate of –1.4% of frequency per min and a recovery rate of 0.44% per min (average of the three peaks). Changes in amplitude (fig. 3b) were quite variable across peak frequencies, but the dominant trend after 10–15 min of BAPTA application was a reversible increase. The lowest-frequency SOAE peak showed the smallest increase in amplitude (2–3 dB) and the highest-frequency SOAE peak showed the largest increase in amplitude (9–10 dB). That this was not always the case can be seen in figure 4, which shows the results of a BAPTA injection in a different ear.

The lightly shaded curve in figure 3a is an estimate, calculated using the Washington University Cochlear Fluids Simulator (see Methods), of the concentration of BAPTA in endolymph in the vicinity of the hair cells. The scales on the two vertical axes were set arbitrarily so that the slopes of the curves, representing the change in [BAPTA] (on an inverted scale) and the downward frequency shift, are approximately the same during the passage of iontophoresis current. The relationship is a 10% shift in frequency per 2 m*M* increase in [BAPTA]; the approximate maximum [BAPTA] reached was 5 m*M*.

The results from three more animals consistently show frequency shifts with a relatively fast onset rate (from –0.46 to –1.84% per min) and a slower rate of recovery (0.04–0.19% per min). Another example is shown in figure 4a. Amplitude changes were again variable, but, in two animals, an increase in amplitude during the slow recovery of the SOAE peak frequency was the dominant effect (fig. 4b). The estimates of [BAPTA] using the scaling relationship of 10% frequency shift per 2 m*M* increase in [BAPTA] consistently provided a reasonably good fit to the data during iontophoresis.

The accumulation of BAPTA in endolymph would have been proportional to the level of the iontophoresis current, and this was reflected in the shifts in SOAE frequencies. The fastest rate of frequency shift in the different animals was induced by the highest current level (4 μA) and the slowest rate by the lowest current level (2 μA) and the two intermediate current levels used gave intermediate rates of shift (Pearson $r = -0.88358$, $p < 0.01$). This correlation is reflected in consistent fits between the rate of frequency shift and the estimated accu-

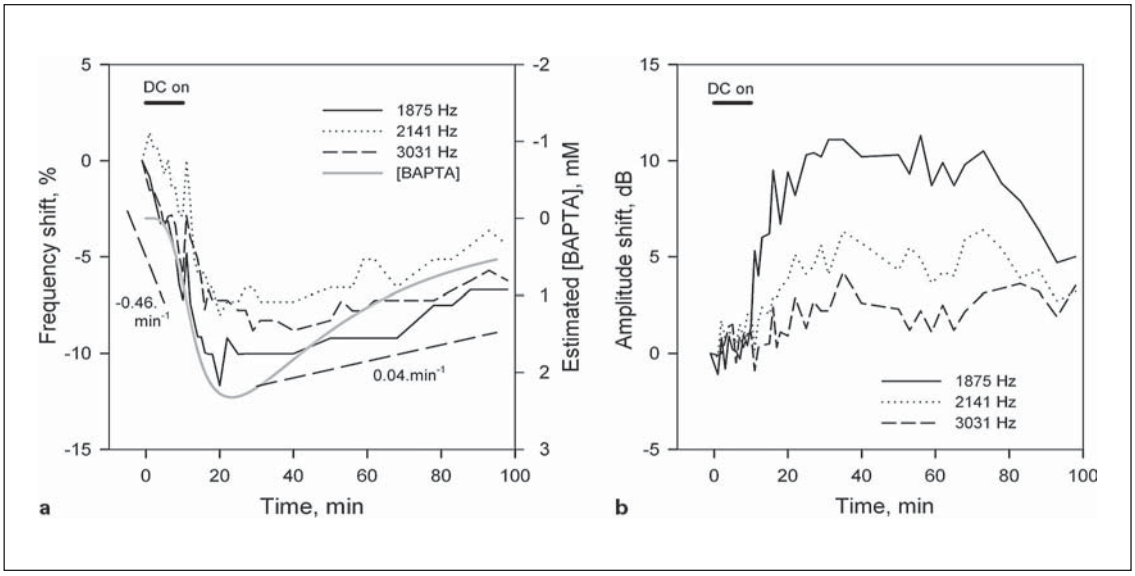

Fig. 4. Net BAPTA effects (see caption to fig. 3) on frequency (**a**) and amplitude (**b**) of three SOAEs in a different ear. In this case, BAPTA was injected iontophoretically using a smaller direct current (–2 μA) for 10 min, a shorter time than in figures 1–3. Both the onset rate and recovery rate are slower than in figures 1–3 and the fit to the estimated [BAPTA] is poorer. As an example, the values and parameter settings used in the estimation of [BAPTA] in this case were as shown in table 1.

Table 1. Values and parameter settings used in the calculation of the [BAPTA] curve in figure 4a

Diffusion coefficient, $\times 10^{-5}$ cm^2/s	0.64329
Iontophoresis current, nA	2000
Iontophoresis transport number	0.197
Iontophoresis onset half time for max. transport number, min	1
Iontophoresis location in endolymph	1.45
Location of detector 1 in endolymph	0.2
Clearance half-time endolymph to blood, min	35
Endolymph half-time for endolymph–ST exchange, min	70
ST half-time for ST–SV exchange, min	25
Apical communication enabled, area of helicotrema, mm^2	0.03
Vestibule area, mm^2	0.961
Vestibule length, mm	4
Distance along ST where cochlear aqueduct enters, mm	1

Radial ST–SV communication half-time normalized to 0.1 mm^2 CSA. Diffusion from base of SV to vestibule enabled.

mulation of BAPTA in endolymph during iontophoresis. A close relationship between changes in [BAPTA] and the rate of frequency shift was not, however, consistently present following iontophoresis. In two instances, the final measurements of peak frequency and [BAPTA] are close, but there are pronounced discrepancies between the exponential decay of [BAPTA] and the time course of the frequency shifts during the recovery phase (fig. 4a). The variability in the recovery patterns is in contrast with what appears to be a fairly straightforward relationship between the downward frequency shift and the estimated increase in [BAPTA] during iontophoresis.

Discussion

A transduction channel motor should depend for its effectiveness on bundle integrity, including that of the filamentous 'tip links' [Pickles et al., 1984] that connect adjacent stereovilli [Assad et al., 1991; Hudspeth, 1997]. High BAPTA concentrations eliminate tip links of hair cells in vitro [Assad et al., 1991; Zhao et al., 1996; Marquis and Hudspeth, 1997; Meyer et al., 1998]. In bullfrog saccular cells, direct in vitro BAPTA application to the hair bundle for a few hundred milliseconds was sufficient

to abolish the transduction current. Voltage-dependent bundle motility and the tip links themselves were eliminated after brief direct application of BAPTA to the hair-cell bundle [Assad et al., 1991].

On the basis of Salt's simulation program (see Methods), [BAPTA] in endolymph near the hair cells was estimated to have exceeded 5 m*M* for several minutes in two animals, yet SOAE amplitudes were either increased (fig. 3b, 4b) or essentially unaltered. If intact tip links were critical for the generation of SOAEs, then the preservation and even enhancement of SOAE amplitude would indicate that the tip links were not broken. It is of course possible, but highly unlikely, that the tip links were broken, but lizard SOAEs do not depend on tip links transmitting force from a transduction channel motor [Hudspeth, 1997]. Lizard hair cells are probably incapable of generating force through somatic motility since they lack the specialized membrane structures of mammalian OHCs [Köppl et al., 2004]. More directly, earlier reports presented strong evidence that electrically evoked otoacoustic emissions from bobtail lizard ears were generated by a stereovilli-based motility source [Manley et al., 2001] and that SOAEs arose from this source [Manley and Kirk, 2002].

We consider it more likely that the injections of BAPTA did not eliminate the tip links and thus Salt's model is not entirely appropriate for the present preparation. It is possible that the recovery time course could be more related to Ca homeostasis mechanisms (without any reduction of endolymphatic BAPTA) rather than a definite decline in BAPTA concentration; however, it is likely that calcium homeostasis would have longer time courses than those shown for the initial phase of the changes we observed in SOAE [cf. e.g., Ferrary et al., 1988; Payan et al., 2002]. Endogenous [Ca^{2+}] regulatory systems [Lumpkin and Hudspeth, 1998; Yamoah et al., 1998] may have additionally compensated, and it is likely that the concentrations of BAPTA in endolymph near the hair cells were, in any case, overestimated, for the following two reasons. First, the distance between the electrode tip and the hair cells (see Methods), and hence the estimated [BAPTA] near the hair bundles is subject to uncertainty. The electrode penetrated near the low-to-mid-frequency papillar area and the high-frequency end could be at least 1 mm further away. Penetration of scala media is difficult in this species, and the angle of approach is governed by the bone and muscle structure of the lower jaw, which varies considerably across individuals of different ages. We cannot be certain that the electrodes were always at the intended distance from the hair cells. Second, and more significantly, the lagenar macula, an organ that in lizards is within the cochlear duct and thus physically continuous with the fluid in scala media [Wever, 1978, p 77], has many calcareous otoliths that presumably would have buffered endolymphatic [Ca^{2+}] [Assad et al., 1991; Payan et al., 2002]. The lagena would have served as a potent BAPTA 'sink', substantially limiting accumulation near the hair cells and, depending on its accessibility, affecting the recovery process (see below).

The possibility exists that in our preparation, changes in ionic homeostasis might explain some of the effects seen. However, if lizard endolymph is supplied with calcium in a similar way to that of bony fish [Payan et al., 2002], then calcium in the endolymph can be replenished from the blood system at a very rapid rate. In the trout, the entire endolymphatic calcium content can in theory be resupplied within about 25 min [Payan et al., 2002]. In addition, in the trout, the continuous growth of the otolith incorporates the equivalent of 8 endolymphatic calcium pools every day. Even if BAPTA were to interfere with the calcium pumps partly responsible for calcium homeostasis, in these animals calcium can also be rapidly replenished through scavenging from the otoliths. Since the effects observed here so strongly resemble those seen in vitro (where there is no EP and there are no EP-generating or ion-homeostatic structures that can be disturbed by adding BAPTA), we assume that to a first approximation, what we see was due to direct effects at the hair-cell level.

It is of course possible that changes in calcium concentrations affect the tectorial membrane, since this non-cellular structure has been shown in mammals to be sensitive to the ionic milieu. Shah et al. [1995], for example, showed that the mouse tectorial membrane shrinks up to 14% in very-low-calcium solutions. Any change in the mass of the tectorial membrane will affect micro-mechanical resonances in the cochlea. In lizards such as the bobtail skink, the mass of local tectorial units is an important component determining the resonant frequency [Manley et al., 1988]. However, the tectorial membrane differs in its make-up between the different animal groups, and in the alligator lizard the tectorial membrane is very insensitive to changes in the calcium concentration (thickness changes of less than 2%) [Freeman and Weiss, 1995]. It therefore seems very unlikely that the present calcium effects can be the result of reversible changes in tectorial masses.

Doubts as to the precise concentrations of BAPTA aside, the downward shifts in SOAE peak frequencies are significant in view of evidence linking [Ca^{2+}] and the

speed with which a transduction-channel motor develops its force in several species of non-mammals [Jaramillo et al., 1990; Benser et al., 1996; Ricci and Fettiplace, 1998; Martin and Hudspeth, 1999; Manley et al., 2004]. The control currents used here also produced downward frequency shifts, albeit of lesser magnitude and much shorter duration. This again is consistent with a calcium dependency. Negative direct current in scala media would depolarize the hair cell's apical membrane and reduce the electrochemical gradient for calcium entry [Manley and Kirk, 2002]. It has been proposed that calcium binds to a site inside the pore of the hair-cell transduction channel, reducing the pore's inner diameter and the rate of entry of monovalent ions [Farris et al., 2004]. At higher calcium concentrations, these authors also propose that calcium can physically block the channel. This proposal is compatible with the present data, if it is assumed that the rate of entry of monovalent ions controls the spontaneous oscillation frequency of the hair-cell bundle.

It is somewhat inconsistent with some of the described in vitro behaviour that there was either little change or an increase in the amplitude of SOAE peaks associated with the BAPTA-induced frequency shifts. Low calcium diminished the magnitude of hair bundle oscillations in saccular preparations excised from the bullfrog [Jaramillo et al., 1990]. However, Martin et al. [2003] report that in individual hair bundles of the frog sacculus, the highest oscillation amplitudes were those measured at the lower frequencies and in response to reduced calcium levels. The reason for these discrepancies is not clear, although the amplitude change will presumably depend on the absolute calcium concentrations used in each case. Data from experiments manipulating the endolymphatic calcium concentration directly in vivo in the bobtail lizard were consistent with the present data, in that lowered calcium levels generally increased SOAE amplitudes [Manley et al., 2004].

In the present data, there was consistently a close correspondence between the rate of net frequency shift and the estimated accumulation of BAPTA during iontophoresis (fig. 3). After iontophoresis, however, the recovery of SOAE frequencies did not always follow the estimated clearance of BAPTA from endolymph, and in some instances there was a pronounced deviation. The reasons for this are unclear. SOAE frequencies in unoperated ears of this lizard species are stable over repeated measurements for up to 4 months [Köppl and Manley, 1993] and, in the present study, they were relatively invariant for up to 30 min or more (data not shown) in the absence of iontophoresis current. After passage of control currents, recovery was rapid and baselines were quite stable to within one or two frequency bins (about 16 Hz). This is consistent with an extensive study of the effects of current on SOAE of this lizard species, in which it was shown that short-duration negative and positive current have opposite and reversible effects [Manley and Kirk, 2001]. The irregular changes seen during the recovery phase from BAPTA injections, that did not follow the simulated clearance of BAPTA and thus the presumed recovery of calcium levels, may indicate some unknown change to a complex system, affected either directly or indirectly by the presence of BAPTA in endolymph. It is likely that the effective concentration of BAPTA during both the accumulation and recovery phases were influenced by calcium in the lagenar otoliths. In amniote vertebrates, the otoliths are single-crystal calcite structures that vary greatly in size even within one organ [Fermin et al., 1998]. Any effect of interactions between BAPTA and the otoconia would have been larger during recovery, as the time available for such interactions during the recovery phase was much longer. Such contingencies could, however, also not be taken into account in the model simulation of the rise and fall of BAPTA concentration.

The ubiquity of calcium's involvement in cellular biochemical processes makes it difficult to pin down the site(s) at which changes in calcium concentration act in the cell [Fettiplace, 1992]. In the present data, however, the concentration was changed in the endolymph above the hair cell's stereovillar bundle, and previous data indicate that calcium entering the transduction channels is rapidly sequestered by cellular buffers such as calmodulin and parvalbumin [Oberholtzer et al., 1988; Jaramillo, 1995; Hall et al., 1997; Lumpkin and Hudspeth, 1998; Heller et al., 2002] or pumped out of the cell by plasma membrane Ca^{2+} ATPase [Yamoah et al., 1998]. The endogenous buffer of the hair bundle of turtle hair cells is equivalent to 0.1 (in low-frequency hair cells) and to 0.4 m*M* BAPTA (in high-frequency hair cells) [Ricci et al., 1998]. For the reasons given above, the diffusion of calcium down the stereovilli is strictly limited [Lumpkin and Hudspeth, 1998]. These data strongly suggest that the effects of changing the calcium concentration in scala media would be limited to events that occur in the stereovillar bundle and not in the cell soma.

The stereovillar bundle of hair cells has a thick glycocalyx [Santi and Anderson, 1987], and the stereovilli are interconnected in a number of ways [Osborne et al., 1984; Furness and Hackney, 1985; Hackney and Furness, 1995]. There are both links at the tips and along the sides of stereovilli. Even if the tip links are broken, the bundle

does not lose its integrity [Assad et al., 1991]. Thus the effects of moderately varying calcium levels on bundle stiffness is likely to be essentially due to changes in the properties of the tip links and their associated transduction channels [Marquis and Hudspeth, 1997]. Since these data are also fully consistent with the effects of directly manipulating the fluid calcium levels in this species [Manley et al., 2004], it can be concluded that the BAPTA effect described here are due to effects on the transduction channels or structures closely associated with them.

Although it can be difficult to relate the integrated behaviour of the intact hearing organ to the known physiology of individual hair cells [Kirk, 2001], the capability of a transduction channel motor to contribute active gain with the known properties of cochlear amplification and otoacoustic emissions has been demonstrated [Martin and Hudspeth, 2001]. Our data are the first in vivo study of the effects of BAPTA on spontaneous emissions, they are entirely consistent with the effects of directly manipulating calcium levels by the infusion of fluids into the bobtail's scala media [Manley et al., 2004], and show strong similarity to data previously reported on hair-cell bundle movements using in vitro systems.

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