Amiloride causes changes in the mechanical properties of hair cell bundles in the fish lateral line similar to those induced by dihydrostreptomycin

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Amiloride is a known blocker of the mechano-electrical transduction current in sensory hair cells. Measurements of cupular motion in the lateral line organ of fish now show that amiloride concurrently changes the micromechanical properties of the hair cell bundles. The effects of amiloride on the mechanics and receptor potentials of the hair cells resemble those previously observed for the aminoglycoside drug dihydrostreptomycin (DHSM) and are similarly antagonized by Ca^{2+} .

We hypothesize that amiloride and DHSM act on hair cells in two correlated ways, which manifest themselves in both the electrical and mechanical properties of the transduction process. One action is the reduction of the transduction current with a concurrent increase in hair bundle stiffness. The other action is a shift of the hair cell's operating point on a current–displacement curve, with a concomitant shift along the associated hair bundle stiffness–displacement curve. The latter action has the opposite effect to that of the first and thus may lead, at relatively low blocker concentrations, to both an increase of transduction current and a decrease in hair bundle stiffness.

Keywords: hair cell; hair bundle mechanics; gating spring; lateral line; amiloride; aminoglycosides

1. INTRODUCTION

The vertebrate sense of hearing is based on mechanical signals received and processed by sensory hair cells. These cells also perform the primary mechanotransduction in the vestibular system and the lateral line organs of fish, and possess a mechanoreceptive organelle, the hair bundle. Deflections of the hair bundle as small as atomic dimensions can be detected (e.g. Khanna & Leonard 1982).

In contrast to photo- and olfactory reception, the molecular basis of mechanoreception is poorly understood. The engaging machinery of the transduction channel, its molecular identity, and its precise location in the cell's membrane are still unknown. Most likely the channels reside somewhere near the tips of the stereocilia (Hudspeth 1982; Hackney *et al.* 1992; Denk *et al.* 1995). Structural candidates for the engaging machinery in that region are the tip links, tiny strands connecting the individual stereociliar tips (Pickles *et al.* 1984), or membrane patches in the contact region between individual stereocilia (Hackney *et al.* 1992; Furness *et al.* 1997).

The most comprehensive model of mechano-electrical transduction to date assumes that the deflection of a hair bundle is transferred into a direct force on the transduction channel's gate via an elastic element, termed the gating spring (Corey & Hudspeth 1983b; Howard &

Hudspeth 1988). Increased tension on the gating spring enhances the free energy of the channel favouring its opening. Although this 'gating spring model' is usually associated with the tip links, a similar functional description may possibly be based on shearing motion of elastic membrane elements (Hackney *et al.* 1992; Furness *et al.* 1997).

Pharmacological agents binding to channel proteins are widely used to probe the molecular properties of ion channels. The current through the mechano-electrical transduction channel of hair cells is blocked by both aminoglycosides (Kroese *et al.* 1989) and amiloride (Jørgensen 1985; Jørgensen & Ohmori 1988; Rüsch *et al.* 1994). Both blockers possibly have a common binding site somewhere near the contact region of adjacent stereocilia rather than at sites near the tip links (Furness *et al.* 1996). It is therefore interesting to investigate how these blockers interfere with the mechanical properties of the transduction channel. Here we describe such blocker-induced mechanical changes of the hair bundles in the fish lateral line *in vivo*.

Hair cells in the supraorbital lateral line organ of the ruffe (*Acerina cernua* L.) are grouped together in neuromasts, located in sub-epidermal canals on the head (Jakubowski 1963). The hair cell bundles project into overlying accessory structures, called cupulae (Flock 1967). Neuromasts are relatively easily accessible, and it is possible to make steady recordings for several hours of nanometer displacement responses of the cupula while

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monitoring the transduction current via extracellular receptor potentials.

Previous studies on cupular mechanics have shown that the elastic coupling of the cupula with the underlying epithelium can be attributed to the stiffness of the hair cell bundles protruding into the cupula (van Netten & Kroese 1987). Therefore, measurement of the displacement responses of the cupula provides information about the mechanical properties of the hair bundles and, owing to their mechanical coupling to the transduction channels, about the channels' gating mechanism (e.g. van Netten & Khanna 1994). More specifically, cupular displacement in response to low-frequency (< 50 Hz) canal fluid flow is proportional to the inverse of the mechanical hair bundle impedance. Therefore, in the present study, cupular displacement is used as a monitor of hair bundle stiffness and damping.

2. MATERIALS AND METHODS

(a) *Preparation*

Twenty-five ruffe (*Acerina cernua* L.) with body lengths ranging from 10 to 14 cm were used for this study. Fish were anaesthetized with an intraperitoneal injection of Saffan (Pitman Moore, ca. 50 mg kg⁻¹ body weight) and placed in a fish tank with tap water, where they were artificially respired and held rigidly in place with head and body clamps.

Supraorbital canal neuromast no. 3 (see Jakubowski 1963) was exposed by gently removing the overlying skin and bony bridge. The condition of the cupula and hair cells was visually monitored with an incident light polarizing microscope (ILPM; Kroese & van Netten 1987).

(b) *Mechanical stimulation*

Neuromasts were stimulated with canal fluid motion produced by a small glass sphere (diameter 0.8 mm) placed inside the canal about 4 mm rostral of the cupula. The sphere was connected to a piezoelectric bimorph moving sinusoidally in the direction of the longitudinal axis of the canal at frequencies of 30, 46 or 250 Hz. The amplitude of the displacement of the sphere was 3 µm, which evoked extracellular receptor potentials well below saturation. At low frequencies, resulting variations of the membrane potential are estimated not to exceed 15 mV (e.g. Kroese & van Netten 1989).

(c) *Measurement of cupular motion*

Cupular motion was measured with a differential heterodyne laser interferometer coupled to the ILPM (e.g. van Netten 1988). Two laser beams, derived from a He-Ne laser (Spectra Physics, 127), were shifted in frequency using two acousto-optic cells ($f_1 = 40.0$ MHz, Polytec; $f_2 = 40.4$ MHz, Isomet, 1201E) and focused on a natural irregularity in the cupula. Backscattered laser light consisted of a carrier ($f_2 - f_1 = 400$ kHz), which was phase modulated in proportion to the displacement of the cupula. Backscattered laser light was detected with a photomultiplier (Hamamatsu, R1477) and demodulated with a modified frequency demodulator (Polytec, OFV 3000). The output signal of the demodulator was proportional to the velocity of the cupula within the range of 10^{-1} – 10^3 µm s⁻¹.

Velocity signals were low-pass filtered with an eight-pole Bessel filter (Frequency Devices) of which the cut-off frequency was set at eight times the stimulus frequency, and amplified before they were digitized with a 16-bit A/D converter (Ariel,

DSP-16) at a sampling frequency of 64 times the stimulus frequency. Responses to stimuli consisting of 16 periods were averaged 20 times. Amplitude and phase of the component of cupular displacement at the stimulus frequency (principal component) were calculated using a fast Fourier transform (FFT) of the averaged velocity response. In figure 1a, examples of amplitude spectra of cupular displacement to a 46 Hz stimulus are depicted, which show the principal components to dominate the responses. Signal-to-noise ratios of the principal components were typically 20 dB.

(d) *Measurement of extracellular receptor potentials*

As a monitor of the AC receptor current of the hair cells, extracellular receptor potentials were measured (e.g. Corey & Hudspeth 1983a) using a silver wire electrode (diameter 0.03 mm), insulated except for the tip, which was placed in the canal near the cupula (ca. 0.6 mm) at its caudal side. An AgCl-coated reference electrode was placed in the trunk of the fish. The signal was AC amplified 10 000 times (PARC 113) and subsequently low-pass filtered with a 16-pole Elliptic filter (Difa) with a cut-off frequency at 16 times the stimulus frequency. The extracellular receptor potential was measured simultaneously with the mechanical response and digitized similarly. Amplitude and phase of the principal component were extracted using an FFT. The principal component of the extracellular receptor potentials occurs at twice the stimulus frequency, owing to the activity of two oppositely orientated populations of hair cells (e.g. Flock 1971). Figure 1b shows examples of amplitude spectra of extracellular receptor potentials to a 46 Hz stimulus. Signal-to-noise ratios of the principal component were typically 30 dB.

(e) *Application of amiloride and dihydrostreptomycin*

The fish tank (volume 0.7 l) was part of a closed water circuit (volume 3.5 l), which was initially filled with tap water. Amiloride hydrochloride (RBI) was applied to produce concentrations of 10, 25, 50, 100, 200 or 300 µM. Control experiments using ink showed that the concentration in the fish tank reached 90% of its final level within 6 min after application. The washout time was similar. Dihydrostreptomycin (DHSM) (Sigma) was applied using the same procedure, to obtain concentrations of 18 or 34 µM.

(f) *Application of Ca²⁺ and EGTA*

The concentration of Ca²⁺ in normal tap water was approximately 1.5 mM. The Ca²⁺ concentration was increased to 5 mM (one experiment) or 10 mM (two experiments) by adding CaCl₂ (Merck). The influence of adding CaCl₂ on the pH (7.2) and the conductance of the fluid appeared to be negligible. The Ca²⁺ concentration was decreased by adding EGTA (Aldrich) (0.75 mM in two experiments and 1.0 mM in one experiment). In the experiments with EGTA, 5 mM HEPES and 3.4 mM NaOH were added in order to maintain a constant pH of 7.2. Because we were interested in the direction of the change due to a rise or a drop in the extracellular Ca²⁺ concentration, no attempts were made to measure the resulting exact concentrations of Ca²⁺.

3. RESULTS

Hydrodynamic stimuli at low frequencies (e.g. 30 and 46 Hz) evoke cupular displacements that are inversely

Figure 1. Amplitude spectra of (a) cupular displacement and (b) extracellular receptor potential responses to a 46 Hz stimulus, before ($t=0$ min, control, open squares=mech., open diamonds=elec.) and after ($t=13$ min, amiloride, filled squares=mech., filled diamonds=elec.) application of 100 μM amiloride. The principal response components of the spectra (cupular displacement at $f_{\text{stim}}=46$ Hz and extracellular receptor potential at $2f_{\text{stim}}=92$ Hz) show a significant increase after application of amiloride (ca. 15 nm and 1.8 μV , whereas the noise levels are ca. 2 nm and 0.1 μV , respectively). (c, d) Amplitude and phase of principal response components of cupular displacement (filled circles, left y-axes) and extracellular receptor potentials (open circles, right y-axes) at (c) 46 Hz and (d) 250 Hz, as a function of time ($t=0$ min, application of 100 μM amiloride; $t=40$ min, washout of amiloride). The vertical arrow in (c) indicates a washout transient.

proportional to the collective impedance of the hair cell bundles protruding into the cupular base (van Netten 1991). Measurement of cupular motion at these frequencies thus provides information on induced changes in hair bundle mechanics. Cupular motion, induced by hydrodynamic stimuli at 250 Hz, is not related to hair bundle mechanics. Thus, if amiloride were to change hair bundle mechanics, cupular motion should only be affected at low frequencies.

(a) Effects of amiloride on cupular motion

At low frequencies, application of amiloride in concentrations of 25–300 μM clearly influences the motion of the cupula ($N=16$).

As an example, effects of 100 μM amiloride on cupular motion at 46 Hz are shown in figure 1c (filled circles). Amiloride induces a transient rise in the amplitude of cupular displacement, peaking at about 13 minutes after application. The increase is accompanied by a phase lag, which continues after the transient amplitude rise. As expected, when amiloride affects hair bundle mechanics, no changes in cupular displacement are observed at 250 Hz (figure 1d, filled circles).

The effects on the amplitude and phase of cupular displacement depend on the concentration of amiloride. At lower amiloride concentrations (25 and 50 μM), the effects on cupular motion are comparable, but much slower (maximum amplitude after about 40 minutes, not shown).

(b) Effects of amiloride on the extracellular receptor potential

The effects of 100 μM amiloride on the extracellular receptor potential are shown in figure 1c,d (open circles). At 250 Hz, the amplitude of the extracellular receptor potential decreases strongly and there is a small change in its phase (figure 1d). Because no concurrent changes in hair bundle motion are induced, these reductions can be attributed to the electrical effect of blockage by amiloride. A dose-response (extracellular receptor potential) curve, using results from 15 experiments covering a concentration range of 10–300 μM , was derived at this stimulus frequency (250 Hz). Fitting the Hill equation (e.g. Aidley & Stanfield 1996; see also § 4d) to these results, using a χ^2 minimization method, yielded a K_D ($\pm$ s.e.) of 46 ± 3 μM ($N=15$) and a Hill coefficient of 1.8 ± 0.2 ($N=15$).

The initial effect of the channel blocker amiloride on the extracellular receptor potential at low frequencies (46 Hz, figure 1c) is not a decrease but rather an increase in its amplitude. The initial changes in amplitude and phase closely follow cupular motion. The increase in electrical response, however, is not likely to be solely attributable to the increased mechanical input. The maximum amplitude of the extracellular receptor potential at 46 Hz ($t=13$ min) is found when about half of the number of transduction channels are blocked, as estimated from the reduction of the extracellular receptor potential with a factor of about two at 250 Hz (figure 1d) at $t=13$ min. The increase in the electrical responses at 46 Hz, after correction for blockage, usually exceeded the increase expected from the input-output relationship between cupular motion and extracellular receptor potential. An additional cause of the increase of the extracellular receptor potential will be discussed (§ 4b).

(c) Effects of Ca^{2+}

An increase of the extracellular Ca^{2+} concentration inhibits the effects of amiloride (figure 2, 10 mM Ca^{2+} was applied at $t=-10$ min). The increased extracellular Ca^{2+} concentration reduces the amplitude of the extracellular receptor potentials (e.g. Corey & Hudspeth 1983b). After the additional application of 100 μM of amiloride at $t=0$ min, no changes in the amplitude and phase of both the cupular displacement and the extracellular receptor potential due to amiloride were observed such as were found after application of 100 μM amiloride only (figure 1c).

It was found that the Ca^{2+} chelator EGTA enhances the effects of amiloride. The effects of amiloride (50 and 100 μM) in combination with EGTA on the mechanics and extracellular receptor potential were comparable to the effects of a 2–4 times higher amiloride concentration in normal tap water (not shown).

(d) Comparable effects of dihydrostreptomycin

The effects of DHSM are similar to those found for amiloride. Figure 3 shows the effects of DHSM on both cupular motion (filled circles) and the extracellular receptor potential (open circles) at a stimulus frequency of 30 Hz. In this experiment, 18 μM DHSM was applied at $t=0$ min and, after washout of this dose, 34 μM DHSM was applied at $t=135$ min. The difference in the behaviour of the response at the two drug concentrations is clearly visible. During the first concentration, which was

Figure 2. The almost abolished effect of 100 μM amiloride during an increased Ca^{2+} level (10 mM) on the amplitude and phase of cupular displacement (filled circles, left y-axes) and the extracellular receptor potential (open circles, right y-axes) in response to a stimulus frequency of 30 Hz ($t=-10$ min, application of Ca^{2+} ; $t=0$ min, application of 100 μM amiloride).

close to two times K_D ($=8$ μM ; Kroese *et al.* 1989), an almost stable increased amplitude in both cupular displacement and extracellular receptor potential is found, whereas at the higher drug concentration, the rise in amplitudes is followed by a sharp decrease.

4. DISCUSSION

The results of this study unequivocally demonstrate that amiloride changes the motion of the cupula *in vivo* in response to canal fluid flow at low frequencies. It can thus be concluded that amiloride changes the mechanical properties of the hair bundles, as the motion of the cupula at these frequencies is controlled by the mechanics of the hair bundles of the underlying hair cells (van Netten & Kroese 1987; van Netten & Khanna 1994). Further, the effects of amiloride on hair bundle mechanics are similar to those induced by the aminoglycoside drug DHSM.

The changes in the mechanics of hair cell bundles are synchronized with the electrical effects of amiloride on the transduction channels (46 Hz, figure 1c). In the present study, the electrical effect of blockage is most clearly evidenced by the reduction of the extracellular receptor potential at high frequencies (250 Hz), at which the mechanical input to the hair bundles is not changed by amiloride (figure 1d), thus enabling the separation of mechanical and electrical effects. The dose-response (extracellular receptor potential) curve of blocking by amiloride, uncorrected for binding time constants (e.g. Rüscher *et al.* 1994) and fitted by the Hill equation, yields $K_D=46 \pm 3$ (s.e.) μM and $n=1.8 \pm 0.2$ (s.e.). This closely resembles the result of Rüscher *et al.* (1994): $K_D=53 \pm 3$

Figure 3. The effect of 18 μM and 34 μM DHSM on the amplitude and phase of cupular displacement (filled circles, left y -axes) and the extracellular receptor potential (open circles, right y -axes) in response to a stimulus frequency of 30 Hz ($t=0$ min, application of 18 μM DHSM; $t=70$ min, washout of DHSM; $t=135$ min, application of 34 μM DHSM; $t=195$ min, washout of DHSM). The vertical arrow indicates washout transients.

(95% confidence limits) μM and $n=1.7 \pm 0.3$ (95% confidence limits), and to some extent also agrees with the finding of Jørgensen & Ohmori (1988): $K_D=50 \mu\text{M}$ and $n=1$ (see § 4d).

Owing to the external application and the high diffusion barriers of the sensory epithelium, amiloride seems most likely to be exerting its mechanical and electrical action on the hair cells directly via the transduction channels and not, for instance, via the efferent system or other actions on the animal. This is confirmed by the general agreement of the present *in vivo* electrical blocking results with those of the *in vitro* patch-clamp studies by Rüsch *et al.* (1994) and Jørgensen & Ohmori (1988). Furthermore, other physical properties affecting cupular motion, like fluid viscosity and fluid density, are very unlikely to have been changed by the amiloride concentrations used. Also, cupular shape and volume, as visualized with the ILPM, were found not to change.

(a) Model explaining an increase in mechanical responses

Under normal conditions, cupular responses to low frequencies (e.g. 30 or 46 Hz) of fluid flow past the cupula are predominantly impeded by the reactive component (stiffness) of hair bundle impedance (van Netten & Kroese 1987, 1989).

It has been proposed previously that the action of DHSM on hair bundle mechanics at low frequencies was to decrease the impedance resulting in increased motion of the cupula (van Netten & Kroese 1989). This proposal was based on a specific micromechanical model of the hair bundle (figure 4), accounting for mechanically mediated

Figure 4. Mechanical model of the hair bundle, consisting of a resistive element, R , placed in series with the stiffness of the gating spring, S_{gs} , and parallel to the pivotal stiffness of the hair bundle, S_{piv} . A reduction of R only explains the observed mechanical changes at intermediate drug concentrations (ca. K_D). X_{gs} , gating spring extension; X_{hb} , hair bundle displacement.

adaptation of the transduction current (Howard & Hudspeth 1987). This model consists of a spring, S_{piv} , associated with the elastic pivoting of the bundle around its rootlet, in parallel with a damping element, R , in series with the gating spring, S_{gs} , that is thought to engage the transduction channels. Increased cupular response and reduction of the phase lead at low frequencies, as induced at intermediate concentrations of DHSM, could be explained by a fourfold reduction of the damping of element R , while S_{piv} and S_{gs} remained constant.

The experimental results of the present study show a remarkable similarity between the effects induced by amiloride and those found after DHSM application. Indeed, using the same mechanical model, a fourfold reduction in R explains the measured changes in both amplitude and phase of cupular motion as induced at intermediate amiloride concentrations (50 μM). However, a reduction of R also reduces the tension on S_{gs} . In the model, a reduced tension decreases the transduction current (Howard & Hudspeth 1987). Thus, reducing R cannot explain the measured increase in the extracellular receptor potential (46 Hz). Another complication is that the transient increase in both cupular motion and extracellular receptor potential cannot readily be explained by a simple monotonic reduction of R .

To describe transient increases in both mechanical and electrical responses, we consider in the next section an alternative hypothesis, which involves a reduction in hair bundle impedance via a change in hair bundle stiffness component, S_{gs} .

(b) *Shift of operating point of transduction by amiloride and DHSM*

The enhanced cupular motion and AC receptor current at low frequencies after amiloride and also DHSM application can be explained qualitatively by a shift in the operating point of transduction. The operating point shifts along the X_{gs} -axis of the receptor current–displacement ($I_{\text{tr}}-X_{\text{gs}}$) and stiffness–displacement ($S_{\text{hb}}-X_{\text{gs}}$) curves into the positive direction (u $\rightarrow$ b in figure 5). To properly account for the physical blocking action of the drugs, both curves also have to be rescaled (dashed curves), as outlined below.

The ($I_{\text{tr}}-X_{\text{gs}}$) and ($S_{\text{hb}}-X_{\text{gs}}$) curves specifically relate the extension of the gating spring, X_{gs} , to the evoked transduction current, I_{tr} , and the effective hair bundle stiffness, S_{hb} . The deviation from constant normalized stiffness, termed gating compliance, $\Delta(X_{\text{gs}})$, results from the gating springs and the transduction channels engaged by them. Such curves have been found experimentally, and their (basic) shapes can be described by a two-state model of the transduction channel (Corey & Hudspeth 1983*b*; Howard & Hudspeth 1988; Russell *et al.* 1992; van Netten & Khanna 1994).

As can be seen in figure 5*b*, a shift in the hair cell's operating point to the right (u $\rightarrow$ b), in combination with rescaling the ($I_{\text{tr}}-X_{\text{gs}}$) curve (dashed curve), results in an enhanced sensitivity of the AC receptor current in response to AC changes in gating spring extension. In figure 5*a*, a concomitant shift along the ($S_{\text{hb}}-X_{\text{gs}}$) curve and rescaling (dashed curve) decreases the effective stiffness of the hair bundle. The decreased hair bundle stiffness results in an enlarged cupular vibration and thus hair cell input. This enlarged hair cell input, together with the enhanced current sensitivity, increases the AC receptor current to a small sinusoidal stimulus of low-frequency fluid flow.

For the electrical as well as the mechanical response, the enhancing effects of the shift in the hair cell's operating point are counteracted by the physical blocking action of the drug, which is indicated by the rescaled dashed curves in figure 5. This is obvious for the $I_{\text{tr}}-X_{\text{gs}}$ curve, where rescaling simply implies multiplication with the fraction of

Figure 5. Hypothetical action of amiloride and DHSM on hair cell transduction. (a) Normalized hair bundle stiffness, S_{hb} , and (b) transduction current, I_{tr} , are plotted as a function of the extension of the gating spring, X_{gs} , as described by a two-state model of the transduction channel (Corey & Hudspeth 1983*b*; Howard & Hudspeth 1988). The solid lines represent the control input–output curves and the dashed lines the situation in which part of the channels are blocked (drug concentration $\approx K_D$). Blockage of transduction channels not only rescales the transduction current and the gating compliance, $\Delta(X_{\text{gs}})$ (dashed curves), as resulting from the physical block, it also shifts the hair cell's operating point from point 'u' to point 'b' (see §4). At point b, therefore, the effective hair bundle stiffness is decreased, but also the hair cell's electrical sensitivity to (small) changes in gating spring extension is increased. The combination of rescaling and shifting thus results in an enlarged mechanical (AC) response and electrical (AC) response to a vibrational hydrodynamic stimulus. At drug concentrations substantially larger than K_D (not shown in this figure), the gating compliance decreases further, resulting in a decreased mechanical response. The electrical response then decreases, due to the decreased mechanical input as well as the increased electrical blockage of the transduction channels.

unblocked channels. A related mechanical effect is that the gating compliance, $\Delta(X_{\text{gs}})$, is multiplied with the same fraction (figure 5*a*). Such a reduction of $\Delta(X_{\text{gs}})$ is to be expected if the transduction channels that are blocked no longer change their conformational state in response to changes in the gating spring tension, and as a consequence do not contribute any more to the gating compliance. This assumption is in accordance with Howard & Hudspeth's finding (1988) that the gating compliance disappears at fairly high concentrations ($\gg K_D$) of the aminoglycoside gentamicin. It is also in line with evidence that DHSM and amiloride only bind to the transduction channel when it is open (Kroese *et al.* 1989; Denk *et al.* 1992; Rüscher *et al.* 1994).

For the receptor potential and the mechanical response to increase, the enhancing mechanism (shifting) has to predominate the counteracting effect of the physical block (rescaling). This happens, as expected, at low and intermediate drug concentrations ($\leq K_D$). In line with this is the observed transient behaviour of cupular response and receptor potential after application of higher concentrations of the drug ($>K_D$). During the diffusion of amiloride through the cupula, concentrations local to the hair cells become first in the order of K_D , thus giving rise to an increased cupular vibration and AC receptor current. After a few minutes, concentrations rise substantially above K_D , and the initially increased amplitudes decrease, because the effects of the reduction of the gating compliance and the blockage of the transduction current start to dominate the responses (figure 1c). The shape of the response on drug concentration is also clearly evident from the experiment with two different DHSM concentrations (figure 3). It also shows that after washout of the high concentration solely, the responses show an additional transient rise (arrow), as expected. Such washout effects were usually not so clearly observable using amiloride (figure 1c, arrow), presumably because of its poorer reversibility.

Similar concentration- and frequency-dependent behaviour has been previously reported for the action of DHSM on lateral line receptor potentials (Kroese & van den Bercken 1982). This can now similarly be understood.

In figure 5, the hypothetical effect of the drugs on only one hair cell is shown. In the present experiments, cupular motion and the extracellular receptor potentials are affected by two oppositely orientated populations of hair cells (Flock 1971). If this is taken into account, the hypothesized changes in the mechanical responses and the extracellular receptor potentials are in the same direction as those for one hair cell population, and can thus be similarly interpreted.

Many changes induced by the drugs amiloride and DHSM can be interpreted with the present hypothesis. Several parameters still have to be quantified, specifically the extent of the shift of the hair cell's operating point ($X_b(c) - X_u$) along the X_{gs} -axis, as a function of concentration, c . Precise determination of its extent, especially at higher concentrations, is difficult because of the dominating blockage of receptor current and reduction of the gating compliance due to the physical block.

The observed phase drops are not explained by the present hypothesis. These phase drops might be attributed to possible concurrent changes in R (see § 4a). In terms of the micromechanical model of the hair bundle (figure 4), this means that the drugs might cause a reduction of the damping element (R) in addition to the proposed shift of the operating point and the physical block.

(c) Relationship of the shifting mechanism to Ca^{2+} and adaptation

The above functional description of the action of low concentrations of amiloride and DHSM corresponds to an increase in the transduction current under no stimulus conditions (silent current). This agrees with reports on the effects of other aminoglycosides on isolated hair cells, such as an increased silent current (Hacohen *et al.* 1989; Assad & Corey 1992) and changes (delayed relaxation) in resting

position of the hair bundle into the (negative) direction commensurate with an increased silent current (Denk *et al.* 1992).

These observations were related to the influence of the drugs on the influx of Ca^{2+} through the transduction channels, which is thought to control the process of adaptation (Eatock *et al.* 1987). It has been proposed that the influx of Ca^{2+} through the transduction channels results in an increased probability of the transduction channels to close, either via its effect on an adaptation motor (Howard & Hudspeth 1987; Hacohen *et al.* 1989; Assad & Corey 1992), or through a Ca^{2+} -dependent conformational change in the transduction channel (Crawford *et al.* 1989). Less Ca^{2+} entering the transduction channels results in the opposite effect, so that the open probability increases (Hacohen *et al.* 1989; Crawford *et al.* 1991). It may thus be possible that amiloride and DHSM, by preventing Ca^{2+} from entering the cell, cause an increased open probability of the remaining unblocked transduction channels resulting in an enlarged silent current. This could also explain the increased spontaneous afferent nerve activity observed in the lateral line organ after DHSM application (Kroese & van den Bercken 1982).

An interference of amiloride with the Ca^{2+} influx is further supported by the presently demonstrated antagonizing effect of Ca^{2+} on the action of amiloride. A similar antagonizing effect of Ca^{2+} on the action of DHSM was described previously (Kroese & van den Bercken 1982).

(d) Bias in determination of K_D and Hill coefficient

The use of the Hill equation to describe the electrical effect of blockage of an ion channel as a function of concentration is justified when only an electrical block (i.e. only rescaling in figure 5b) is implied (e.g. Aidley & Stanfield 1996). The shifting mechanism and the rescaling of the gating compliance can mathematically be added to the Hill equation, resulting in the relative (small) AC receptor current as a function of the concentration of the drug, c :

$$\frac{I_{Ac}(c)}{I_{Ac}(0)} = \frac{S_u(X_u)}{S_b(X_b(c))} \times \frac{\left(\frac{dI_b(X_{gs})}{dX_{gs}}\right)_{X_b(c)}}{\left(\frac{dI_u(X_{gs})}{dX_{gs}}\right)_{X_u}} =$$

$$\frac{1 - \Delta(X_u)}{1 - \frac{1}{1 + (c/K_D)^n} \Delta(X_b(c))} \times \frac{\left(\frac{dI_u(X_{gs})}{dX_{gs}}\right)_{X_b(c)}}{\left(\frac{dI_u(X_{gs})}{dX_{gs}}\right)_{X_u}} \times \frac{1}{1 + (c/K_D)^n},$$

where the subscripts u and b refer to the unblocked and blocked situations, S denotes hair bundle stiffness, and where n th order cooperativity is assumed. X_u is the resting gating spring extension under unblocked conditions and $X_b(c)$ under blocked conditions. Note that the last factor after the last equal sign represents the right-hand part of the Hill equation.

The first factor after the last equal sign reflects the change resulting from the change in stiffness (shift and rescaling in figure 5a). Based on the shapes of curves, such as those depicted in figure 5a, this factor tends to

increase the current at low and intermediate concentrations ($\leq K_D$), while slightly decreasing it at concentrations above K_D owing to the decreased gating compliance. This could, under stiffness-dependent conditions, effectively yield an overestimate of the Hill coefficient. Because in our study the Hill coefficient was derived at stimulus frequencies of 250 Hz, and thus under stiffness-independent conditions, the factor related to the stiffness change did not affect our results ($=1$).

The second factor after the last equal sign accounts for the change in sensitivity of the $I_{tr}-X_{gs}$ curve (shift in figure 5b). The extent of the shift is not quantitatively known. Thus, the effect of the second factor is difficult to predict.

It may thus be concluded that fitting the dose-transduction current curves with the Hill equation may be biased depending on the experimental conditions (e.g. imposed or stiffness-controlled hair bundle motion, extracellular Ca^{2+} concentration) and also on the proportion of intact transduction channels.

(e) *Comparison of modes of action of amiloride and DHSM*

As discussed above, results from fitting dose-response curves with the Hill equation may be biased. This especially complicates a proper comparison of the data-sensitive Hill coefficients obtained from various preparations in combination with different techniques. Values found for the dissociation constant (K_D) of amiloride, nonetheless, are quite similar ($K_D \approx 50 \mu M$) and are of the same order as those found for various aminoglycosides (2–95 μM ; Kroese *et al.* 1989). Experimental results on voltage dependence of the block by both drugs also seem to be similar (Rüsch *et al.* 1994; Kroese *et al.* 1989; Kimitsuki & Ohmori 1993). Further, both drugs bind to the transduction channels when they are open and have no effect when applied intracellularly (Kroese *et al.* 1989; Rüsch *et al.* 1994).

Immunolabelling of the contact region of neighbouring stereocilia with an antibody raised against an amiloride-sensitive channel fails if the hair cells are incubated with DHSM, suggesting a common binding site (Furness *et al.* 1996), which is possibly related to the transduction channel (Hackney *et al.* 1992).

Our study also shows that amiloride and DHSM not only have a similar effect on the electrical properties of the transduction channels but also on their mechanical properties. If the proposed shifting mechanism is a direct consequence of reduced Ca^{2+} influx, we would expect any blocker that prevents the channel from changing its conformational state to induce similar mechanical changes.

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