Block by amiloride and its derivatives of mechano-electrical transduction in outer hair cells of mouse cochlear cultures

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1. The effects of amiloride and amiloride derivatives on mechano-electrical transducer currents in outer hair cells of the cultured neonatal mouse cochlea were examined under whole-cell voltage clamp.

2. At −84 mV transducer currents were reversibly blocked by the extracellular application of the pyrazinecarboxamides amiloride, benzamil, dimethylamiloride, hexamethyleneimino-amiloride, phenamil and methoxynitroiodobenzamil with half-blocking concentrations of 53, 5·5, 40, 4·3, 12 and 1·8 μM, respectively. Hill coefficients were determined for all but the last of these compounds and were 1·7, 1·6, 1·0, 2·2 and 1·6, respectively, suggesting that two drug molecules co-operatively block the transducer channel.

3. Both the structure–activity sequence for amiloride and its derivatives and the mechanism of the block of the transducer channel appear to be different from those reported for the high-affinity amiloride-sensitive epithelial Na^+ channels but similar to those of stretch-activated channels in *Xenopus* oocytes.

4. The block by all pyrazinecarboxamides was voltage dependent with positive membrane potentials releasing the block. The form of the voltage dependence is consistent with a voltage-independent binding of the drug to a site that is accessible at hyperpolarized but not at depolarized potentials, suggesting that the transducer channel undergoes a voltage-dependent conformational change. The channel was not blocked by 1 mM amiloride from the intracellular side at either negative or positive membrane potentials.

5. The kinetics of the block were studied using force steps or voltage jumps. The results suggest that the drug binding site is only accessible when the transducer channel is open (open-channel block) and that the channel cannot close when the drug molecules are bound.

6. The time dependence and voltage dependence of the block together reveal that the transducer channel has at least two open conformational states, the transition between which is voltage dependent.

In hair cells, the receptor cells of the inner ear, mechanical stimuli change the open probability of mechano-sensitive ion channels through which the transducer current flows. These channels are probably located near the tips of the stereocilia (Jaramillo & Hudspeth, 1991) on the apical surface of the cell, although their exact location is unknown.

Two recent observations suggest that the hair cell's transducer channel might be related to amiloride-sensitive ion channels present in other tissues. Firstly, in chick hair cells the transducer channel is reversibly blocked by amiloride with a half-blocking concentration (K_D) of 50 μM (Jørgenson & Ohmori, 1988). Secondly, Hackney, Furness, Benos, Woodley & Barratt (1992) have described immuno-staining near the tips of stereocilia in guinea-pig hair cells with an antibody raised against amiloride-sensitive epithelial Na^+ channels purified from bovine kidneys.

The diuretic drug amiloride is the best known representative of a group of more than 1000 related compounds, collectively called pyrazinecarboxamides. Amiloride characteristically blocks with high affinity ($K_D < 1$ μM), Na^+-selective ion channels of Na^+-absorbing epithelia, like the distal and collecting tubules of the kidney or the skin and bladder of amphibians (reviewed by Smith & Benos, 1991). These voltage-independent Na^+ channels are restricted to the apical membrane of these epithelial cells to allow for a passive diffusion of Na^+ into the cell.

At higher concentrations, amiloride also blocks Na^+-selective transport proteins such as the Na^+–Ca^{2+} and

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Na^+–H^+ exchangers (reviewed by Kleyman & Cragoe, 1988), T- and L-type Ca^{2+} channels (Tang, Presser & Morad, 1988; Garcia *et al.* 1990) and stretch-activated cation channels in *Xenopus* oocyte membranes (Lane, McBride & Hamill, 1991, 1992). For many of these systems structure–activity sequences for amiloride and its derivatives are known, as are details of the molecular blocking mechanisms.

The aims of this study were to establish a structure–activity sequence for the hair cell's transducer channel and to assess the channel's relationship to other amiloride-sensitive ion channels. Furthermore, the amiloride compounds were used to probe for details of the channel's gating mechanism. Some preliminary results have been published in abstract form (Rüsch, Kros, Richardson & Russell, 1991).

METHODS

The preparation

Experiments were done on outer hair cells of organotypic cochlear cultures made from one- or two-day-old mice. Some experimental details and the method to produce the cultures have already been described elsewhere (Russell & Richardson, 1987; Kros, Rüsch & Richardson, 1992). In short, mouse pups were killed by cervical dislocation and the cochleas removed. The isolated organ of Corti with the attached spiral ganglion was explanted onto a collagen-coated glass coverslip and maintained at 37 °C for 1–3 days before being transferred to an experimental chamber that was constantly perfused by a perilymph-like extracellular solution at a flow rate of 10 ml h^{-1}. The extracellular solution contained (mM): 144 NaCl, 0·7 NaH_2PO_4, 5·8 KCl, 1·3 $CaCl_2$, 0·9 $MgCl_2$, 5·6 D-glucose, 10 Hepes–NaOH, pH 7·4. Vitamins and amino acids for Eagle's minimal essential medium were added from concentrates (Gibco, Paisley, Strathclyde). The osmolality was near 320 mosmol kg^{-1}. In some experiments 111 mM *N*-methyl-D-glucamine (NMDG), a large cation, replaced an equimolar amount of sodium. The culture was viewed with an ACM top-focusing microscope (Zeiss, Oberkochen, Germany) with Nomarski differential interference contrast optics, using a ×40 water immersion objective lens.

Electrical stimulation and recording

Electrical recordings were made in the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), using a List EPC-7 amplifier (List Electronic, Darmstadt, Germany). Patch pipettes were drawn from soda-glass capillaries (Clark Electromedical Instruments, Reading, Berkshire), coated with Sylgard resin and heat-polished to tip diameters of about 1 μm. In later experiments it was found that more stable seals were obtained when the pipettes were coated with ski wax (Swix yellow, Astra-Gruppen, Skarer, Norway) and the heat-polishing step was omitted. Patch pipettes had electrical resistances of 2–3 MΩ. They were filled with a solution containing (mM): 135 CsCl, 0·1 $CaCl_2$, 5 EGTA–NaOH, 3·5 $MgCl_2$, 2·5 Na_2ATP, 5 Hepes–NaOH, pH 7·3, osmolality about 280 mosmol kg^{-1}. This caesium-rich solution was used to minimize voltage-dependent potassium currents. In some experiments 105 mM NMDG replaced an equimolar amount of caesium. Gigaohm seals were formed on the basolateral membranes of the hair cells in any of the three rows of outer hair cells after a small tear had been made in the epithelium (see Kros *et al.* 1992). We did not record from inner hair cells in this study. The 128 outer hair cells used for this project had a membrane capacitance of 5·3 (± 0·6) pF (mean (± S.D.); parentheses are used in the rest of this paper to indicate S.D.). The residual series resistance, after electronic compensation, was between 2·4 and 9·0 MΩ, and the time constants of the voltage clamp ranged from 13 to 60 μs. No correction was made for the voltage drop across the series resistance (≤ 5 mV). At −84 mV the linear leak conductance ranged from 0·5 to 2·1 nS. Membrane potentials were corrected for the liquid junction potentials measured between normal intra- and extracellular solutions (−4 mV) and NMDG intra- and NMDG extracellular solutions (−2 mV). All experiments were done at room temperature (21–25 °C). Unless otherwise indicated, the data were usually low-pass filtered (8-pole Bessel) with 3 dB attenuation at 2·5 kHz, digitized at a sampling rate of 5 kHz by a Labmaster 100 kHz DMA data acquisition board (Scientific Solutions, Solon, OH, USA) supported by an IBM-compatible 386 personal computer, and stored on hard or optical disks. The software package ASYST (ASYST Inc., Rochester, NY, USA) was used to generate protocols for mechanical and electrical stimuli and for data analysis. Non-linear regression fits to the data were made with the programme Fig. P (version 6·0, Biosoft, Cambridge) using a Levenberg–Marquardt curve-fitting algorithm.

Mechanical stimulation

Single hair bundles were stimulated by hydrodynamic coupling to a fluid jet driven by a piezoelectric sound generator (7BB-27-4, Murata, Kyoto, Japan; 27 mm diameter; resonance frequency 4·5 kHz) and directed towards the hair bundle by a glass micropipette (tip diameter 7–15 μm) placed within a distance of 10–15 μm from the hair bundle and approaching the cell from the modiolus of the organ culture. Some details of this stimulator have already been published in Kros *et al.* (1992).

The piezodisc is a two-element assembly of a piezoceramic disc glued to a thin brass disc. It was clamped around its edge in a Perspex housing carefully filled with extracellular solution to remove all air bubbles, and in contact with the bath through the glass micropipette directing the fluid jet onto the hair bundle. The brass disc faced the bath solution from which it was electrically insulated by a thin layer of Sylgard resin and was grounded to provide an electrical shield. Positive driver voltages forced the two-element assembly to bulge towards the bath, exert positive pressure and induce an efflux of fluid from the stimulus pipette which deflected the stereocilia towards the kinocilium. Negative voltages caused underpressure in the stimulator and suction of fluid into the pipette which induced deflections of the hair bundle away from the kinocilium. The command signal was generated by computer and low-pass filtered at 1 kHz (8-pole Bessel) to avoid exciting resonances of the piezodisc before being fed into a high-voltage driver (maximally ± 50 V output). This produced a 10–90 % rise time of the command voltage of 460 μs when recorded through a 2·5 kHz 8-pole Bessel filter. The calibration of the stimulator with carbon or glass fibres is described in Kros *et al.* (1992). The force exerted by the stimulator was linearly related to the driving voltage. The stimulator was equally effective in producing forces in both directions. Force steps had rise times similar to that of the piezo-driver voltage and were uniform over 1 s, the longest time tested. Hair bundles were either stimulated by 119 or 40 Hz

sinusoids or by force steps of up to 130 ms duration. In the latter case positive and negative steps were alternated to avoid build-up of over- or underpressure in the stimulator. With either stimulus, the amplitude was increased until the currents saturated, and was kept constant throughout the experiment.

A fluid-filled tube served as a second exit from the stimulator housing. After every change of the superfusing solution, it was used to fill the stimulator pipette with the new solution by suction through its tip before stimulation was resumed. This was essential in superfusion experiments because otherwise drug-free solution from inside the stimulator would have diluted the superfusate around the hair bundle during positive stimuli.

In the early stages of this project, hair cells were stimulated with sine waves and the cell's response was measured at the maxima and minima of the driver voltage as little phase difference was observed between the driver voltage and the cell's response (see Fig. 1, inset, and Kros *et al.* 1992). Later observations indicated that the drug binds to the open channel only. As the time constants (τ) of drug binding (see Fig. 5) and the duration of a half-cycle of a 119 Hz sinusoid (4·2 ms) are comparable, measurements taken at the peak of the sine wave do not represent the steady state of the block. Assuming a well-saturating mechanical stimulus, at the peaks of the 119 Hz sinusoid the block would be only 88% complete in 60 μM amiloride (mean $\tau = 1{\cdot}0$ ms) and 48% in 10 μM benzamil (mean $\tau = 3{\cdot}2$ ms). Therefore, the cell's response to positive sinusoidal deflections was measured 1/8 of a cycle after the peak of the stimulus to allow more time for the drug to bind while still ensuring a reasonable signal-to-noise ratio in the current. At this point of the stimulus the block would reach 96% of the steady-state value for 60 μM amiloride and 63% for 10 μM benzamil. Responses to negative stimuli were always measured at the peak of the stimulus. When hair cells were stimulated by force steps the currents were measured under steady-state conditions of the block. As a result of the incomplete block mentioned above, the K_D values derived from 119 Hz sinusoidal stimuli are expected to be about 3 μM larger (both for amiloride and benzamil) than those obtained from steps. Since in practice no difference between these two data sets (closed and open symbols in Fig. 1) was found, they were pooled.

Drug solutions

The following drugs were used: amiloride hydrochloride (Sigma, Poole, Dorset, and Research Biochemicals Inc. (RBI), Natick, MA, USA), benzamil hydrochloride (RBI and as a gift from T.R. Kleyman, University of Pennsylvania, Philadelphia, PA, USA) dimethylamiloride (DMA), hexamethyleneiminoamiloride (HMA), 2′-methoxy-5′-nitroiodobenzamil (I-NMBA) and phenamil (gifts from T.R. Kleyman); 5-(*N*-propyl-*N*-butyl)-dichlorobenzamil (PBDCB); a gift from Merck Sharp & Dohme, Hoddesdon, Hertfordshire). A mass spectroscopical analysis of the samples of benzamil and phenamil obtained from T.R. Kleyman confirmed their purity. Drugs obtained from different sources had the same blocking efficacy. Drugs were dissolved in extracellular solution, in some cases by heating to 45 °C for several hours, to give the following stock solutions (mM): 1 amiloride, 1 benzamil, 1 DMA, 0·05 HMA, 0·1 phenamil, 10 I-NMBA and 1 PBDCB. The last two appeared to be very hydrophobic and had to be dissolved initially in dimethylsulphoxide (DMSO). The PBDCB solution tested on hair cells contained 1% DMSO and the I-NMBA solutions 0·05 and 0·1% DMSO. Equal amounts of DMSO were added to the control solutions of corresponding experiments.

Amiloride, benzamil, DMA, HMA and phenamil are weak bases with pK values of 8·7, 8·1, 8·8, 8·5, and 7·8, respectively, and a probability of being protonated at pH 7·4 of 0·95, 0·83, 0·96, 0·93, and 0·72, following the Henderson–Hasselbach equation. Only the protonated form interacts with the epithelial Na^+ channel (Kleyman & Cragoe, 1988). As our results provide no indication whether the charge of the drug is relevant for the block of the transducer channel no corrections for the deprotonation at pH 7·4 were made.

Driven by gravity, drug solutions were applied locally through a glass micropipette the tip of which (diameter 50 μm) was placed about 100 μm from the cell. Polystyrene latex beads (Sigma) of 1·1 μm diameter were added to the superfusate at a concentration of about 4×10^6 ml^{-1} to visualize its flow. As a control, drug-free extracellular solution was superfused between two drug tests. Up to four concentrations of one drug were tested on any one cell. Benzamil was extensively tested to investigate the blocking mechanism. In concentrations larger than 10 μM, however, its effect was irreversible. Each culture and the contaminated pipettes were therefore abandoned after every superfusion with benzamil.

RESULTS

Steady-state block of the transducer channel at hyperpolarized potentials

Block by amiloride

Neonatal mouse cochlear hair cells responded with large inward transducer currents at a holding potential of −84 mV when the fluid jet deflected the hair bundle towards the kinocilium, and with a reduction of the holding current when the deflection was in the opposite direction (inset, Fig. 1; see also Kros *et al.* 1992). The transducer currents were reduced when the hair cell was superfused by 100 μM amiloride. The effect was established without noticeable delay when the drug solution reached the hair cell, as judged by the flow of latex beads included in the drug solution. On discontinuing the superfusion the transducer currents recovered within minutes. The dose–response curve of amiloride shown in Fig. 1*A* was the best fit to the data according to the logistic curve:

$$\frac{I}{I_{\text{control}}} = \frac{1}{1 + ([\text{D}]/K_{\text{D}})^{n_{\text{H}}}}, \quad (1)$$

where I and I_{control} are the transducer currents in the presence and absence of the drug D, respectively, K_D is the dissociation constant and n_H the Hill coefficient. The form of eqn (1) is based on the assumption that a co-operative interaction between multiple binding sites is necessary to block the channel. The K_D for amiloride was 53 [± 3] μM and the Hill coefficient was 1·7 [± 0·3] ($n = 8$ cells; here and in the rest of this paper values in square brackets are the 95% confidence limits).

Position 5 and 2-carbonylguanidino substituents have higher binding affinities

To investigate how the addition of side chains to the core structure (see Table 1) changes the binding affinity of amiloride, three different types of derivatives were tested, with modifications at the 5-position of the pyrazine ring (DMA and HMA), at the guanidium group (phenamil, benzamil, and I-NMBA), and at both (PBDCB). The best fits to eqn (1) gave the following half-blocking concentrations (μM): 40 [± 17] DMA ($n = 3$ cells); 4·3 [± 2·3] HMA ($n = 2$ cells); 12 [± 6] phenamil ($n = 3$ cells); 5·5 [± 0·7] benzamil ($n = 15$ cells); and 1·8 I-NMBA ($n = 2$ cells, assuming $n_H = 1·6$). Compared with amiloride, the binding affinities of all compounds with additional side chains on either side of the molecule were increased by up to an order of magnitude, with the more lipophilic compounds (Table 1) binding more strongly. The lipophilicity coefficient for the strongest blocker tested, I-NMBA, is not known, but its extremely low solubility in aqueous solutions suggests this substance is more lipophilic than the other single-position substituents tested.

As an example of an even more lipophilic compound (Kleyman & Cragoe, 1988) with hydrophobic side chains on both sides of the core structure, we tried to block transduction with PBDCB, which is only soluble at 10 μM in 1% DMSO. This seemed to make the cell membrane leaky and the recordings unstable, and therefore this compound was only tested at this single concentration in two cells. The estimated K_D was > 10 μM, which demonstrates that simply increasing the lipophilicity does not necessarily increase the efficacy of the block, perhaps because the molecule becomes too bulky to bind effectively.

The Hill coefficients were: DMA, 1·0 [± 0·3]; HMA, 2·2 [± 0·9]; phenamil, 1·6 [± 0·5]; and benzamil, 1·6 [± 0·2]. Hill coefficients were not calculated for I-NMBA or PBDCB, because insufficient data were collected. Because the Hill coefficients were somewhat smaller than 2 for most compounds tested, it seems reasonable to assume that the drug molecules bind to two binding sites with incomplete co-operativity.

Voltage dependence of the block

Current–voltage curves

Figure 2*A* shows that the mechanically induced transducer currents reversed when the membrane potential was stepped from negative to positive values. The currents reversed close to 0 mV and the current–voltage curves measured in normal extracellular solutions (Fig. 3) showed the non-linearity that has previously been observed and tentatively explained by a voltage-dependent block of the transduction channel by divalent cations (Kros *et al.* 1992).

At hyperpolarized potentials transducer currents were reduced by all of the seven compounds tested in this study in a time-dependent manner. For example, in Fig. 2*B* it can be seen that in the presence of benzamil after an initial peak the currents relaxed exponentially to a steady state, somewhat resembling the current relaxations caused by adaptation (Fig. 4*A* in Kros *et al.* 1992). We considered the possibility that drug binding might simply increase the stiffness of the hair bundle (although at present there is no evidence for such a suggestion), and make the force stimulus

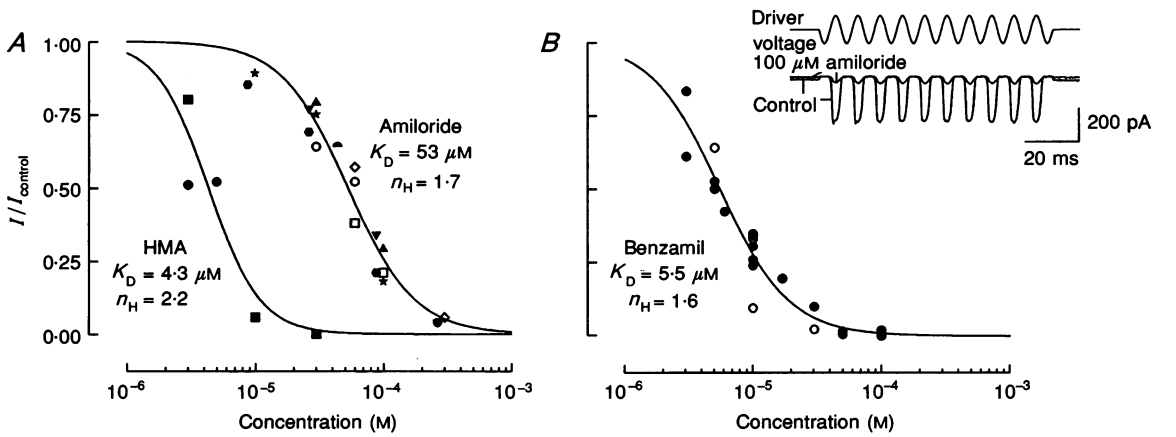


Figure 1. Dose–response curves for block of transducer current at −84 mV for (*A*) amiloride and hexamethyleneiminoamiloride (HMA) and (*B*) benzamil

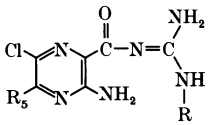
Hair cells were mechanically stimulated near saturation with step stimuli (open symbols) or 119 Hz sinusoids (filled symbols). Each symbol type represents a different cell (8 cells for amiloride, 2 for HMA), except in the case of benzamil, where data for step stimuli and sinusoids were derived from 1 and 14 cells, respectively. Continuous lines were calculated according to a co-operative binding mechanism (eqn (1)). The fitting parameters are indicated in the figure. Inset, block of transducer currents by amiloride. The hair cell was voltage clamped at −84 mV and stimulated with bursts of 119 Hz sinusoids. Top, voltage across the piezodisc driving the saline column in the stimulating glass micropipette (amplitude, 8 V). Bottom, two successive current traces (each averaged from 16 presentations) recorded before and during superfusion with 100 μM amiloride. Series resistance, 2·8 MΩ.

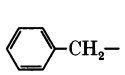
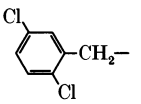
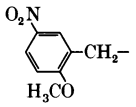
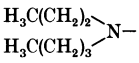
less effective in deflecting the bundle. The transducer currents might then fail to saturate, thus showing more prominent and rapid adaptation than with saturating stimuli (Crawford, Evans & Fettiplace, 1989; Kros *et al.* 1992). Figure 2*C* demonstrates that adaptation was, however, not responsible for the time course of the currents in the presence of the drug: when stimulated in control solution with non-saturating mechanical steps at a constant potential of −84 mV, very little sign of adaptation was present in this cell, and what little adaptation there was had a much slower time course than the drug-induced current relaxations. It will be shown later that the decline of the currents in the presence of the drug occurs instead because the drug molecules only bind to open channels.

Depolarization gradually removed the block (Figs 2*B*, 3 and 4) and at large positive potentials outward currents were significantly reduced only when the drug concentration was well above its half-blocking concentration at hyperpolarized levels. For example, superfusing the cell of Fig. 3*A* with 60 μM amiloride reduced the chord conductance at −104 mV from 2·0 to 1·2 nS, but did not change it at +106 mV (2·7 nS in control solution); 300 μM amiloride almost completely blocked the channel at −104 mV (conductance 3·5 % of control) and reduced the chord conductance at +106 mV to 63 % of its control value. In another cell, at −104 mV 10 μM benzamil reduced the conductance from 6·1 nS measured in control solution to 1·2 nS (Fig. 3*B*). At increasingly positive membrane potentials the blocking effect was gradually reduced until, at +121 mV, almost the full control conductance (5·4 nS) was measured in the presence of benzamil (5·1 nS).

The shape of the voltage dependence of the block is most clearly appreciated by plotting the transducer current as a fraction of the control current *versus* the membrane potential. Figure 4 shows for amiloride and benzamil that this current ratio is a sigmoidal function of the membrane

Table 1. Structure–activity sequences for different amiloride-sensitive channels and ion exchangers



	Amiloride	DMA	Phenamil	Benzamil	HMA	PBDCB	I-NMBA	
R	H–	H–			H–			
R_5	H_2N–	$(CH_3)_2N$–	H_2N–	H_2N–			H_2N–	
Relative lipophilicity	1 (5)	1·2	20	11	19	—	—	Kleyman & Cragoe, 1988
				Blocking efficacy relative to amiloride				
Transducer channel, mouse	1 (53)	1·3	4·4	9·6	12·3	< 5*	29·4	This study
Stretch-activated channel, *Xenopus*	1 (500)	1·4	—	5·3	[14·7] [BrHMA]	—	—	Lane *et al.* 1992
Na^+ channel, low affinity	1 (10)	2·2	2·9	[3·8] [DCB]	—	—	—	Asher *et al.* 1987
Na^+ channel, low affinity	1 (4)	[2] [EIPA]	100	20	—	—	—	Barbry *et al.* 1987
Na^+ channel, high affinity	1 (0·34)	0·04	17	9	< 0·04	—	[7] [NMBA]	Kleyman & Cragoe, 1988
Ca^{2+} channel, L-type	1 (100)	3	—	4	—	400	[77]	Garcia *et al.* 1990
Na^+–Ca^{2+} exchanger	1 (1100)	2	5·5	11	11	300	[28] [NMBA]	Kleyman & Cragoe, 1988
Na^+–H^+ exchanger	1 (84)	20	< 0·01	< 0·08	524	—	—	Kleyman & Cragoe, 1988

Top left, core structure of the pyrazinecarboxamides; a modified pyrazine ring carries a guanidium group at the 2-position. First two rows of table, molecular structures of side chains of the tested pyrazinecarboxamides and the lipophilicity coefficients of the tested drugs relative to that of amiloride. Bottom, the blocking efficacy of drugs are given relative to that of amiloride. For amiloride the absolute half-blocking concentrations (in μM) and the absolute lipophilicity coefficient (percentage partitioning in the chloroform phase when equal volumes of the solvent and an aqueous phosphate buffer (pH 7·4) containing the drug are mixed) are stated in parentheses in the second column. Values of structurally similar drugs (in square brackets) were used where data of the test drugs were not available. EIPA: *N*-5-ethylisopropylamiloride; DCB, dichlorobenzamil; *estimate based on measurements in two cells with 10 μM PBDCB. The iodide in I-NMBA replaces the chloride in the core structure.

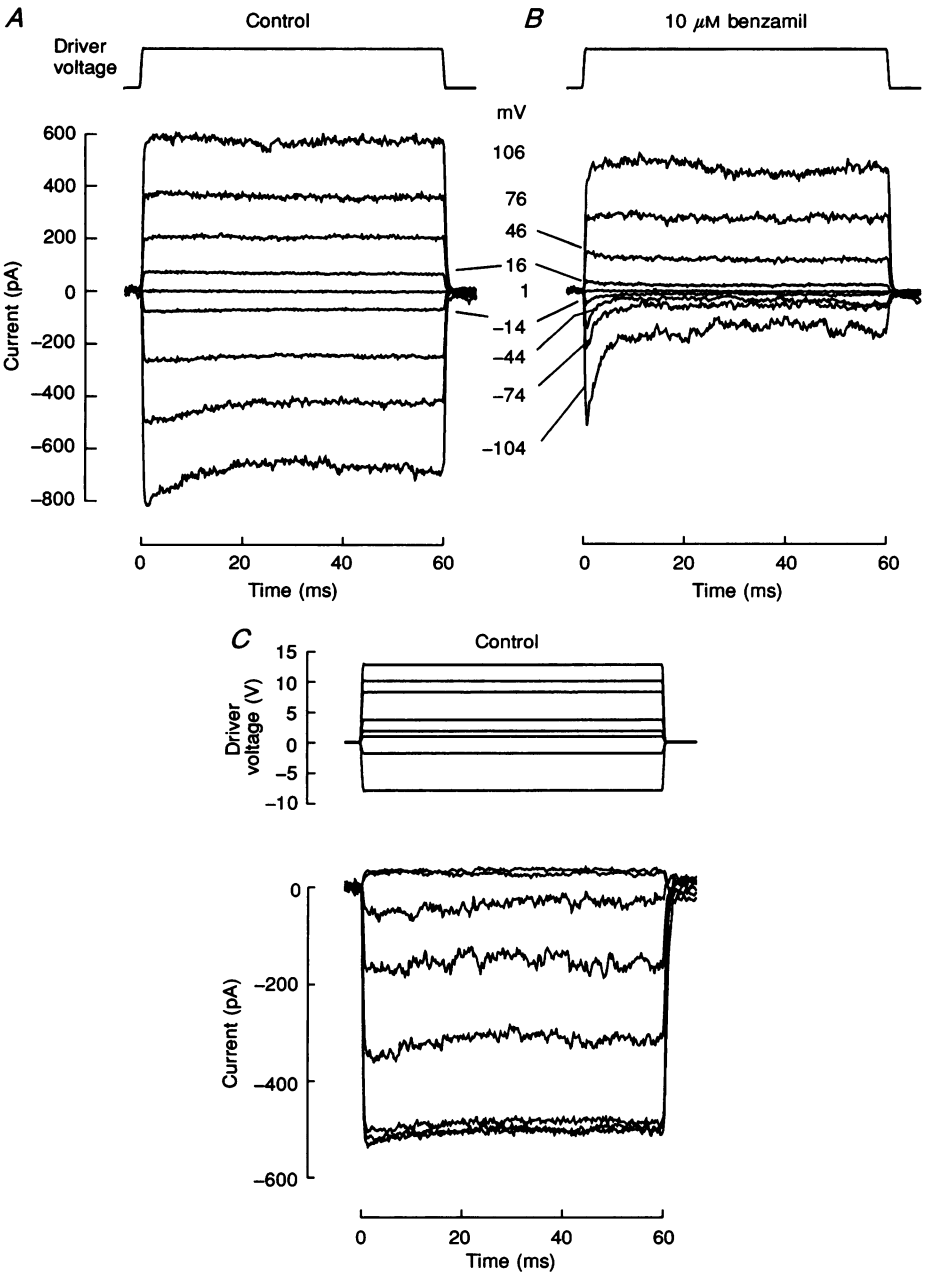


Figure 2. Transducer currents before and during superfusion with 10 μM benzamil
All responses are single traces. For every potential, the membrane current in the absence of a mechanical stimulus was set to zero. *A* and *B*, the cell was mechanically stimulated by the fluid jet at different membrane potentials to give near-saturating responses with little sign of adaptation. The membrane potential was changed from −84 mV to the values shown next to the current traces 50 ms before the onset, at $t = 0$ ms, of the mechanical force step, indicated by the driver voltage (10 V) to the piezodisc shown above. Series resistance, 7·1 MΩ. *A*, records in control solution. *B*, responses of the same cell when subjected to the same protocol in the presence of 10 μM benzamil. Note the time dependence of the block at negative potentials. *C*, transducer currents before superfusion with benzamil, in the same cell at a constant potential of −84 mV but at different driver voltages (shown above the current traces). Positive driver voltage deflected the stereocilia towards the kinocilium and caused an increase in inward transducer current; negative driver voltage moved the stereocilia away from the kinocilium and eliminated the transducer's contribution to the holding current. Note that almost no adaptation was shown by this cell, even for non-saturating force steps.

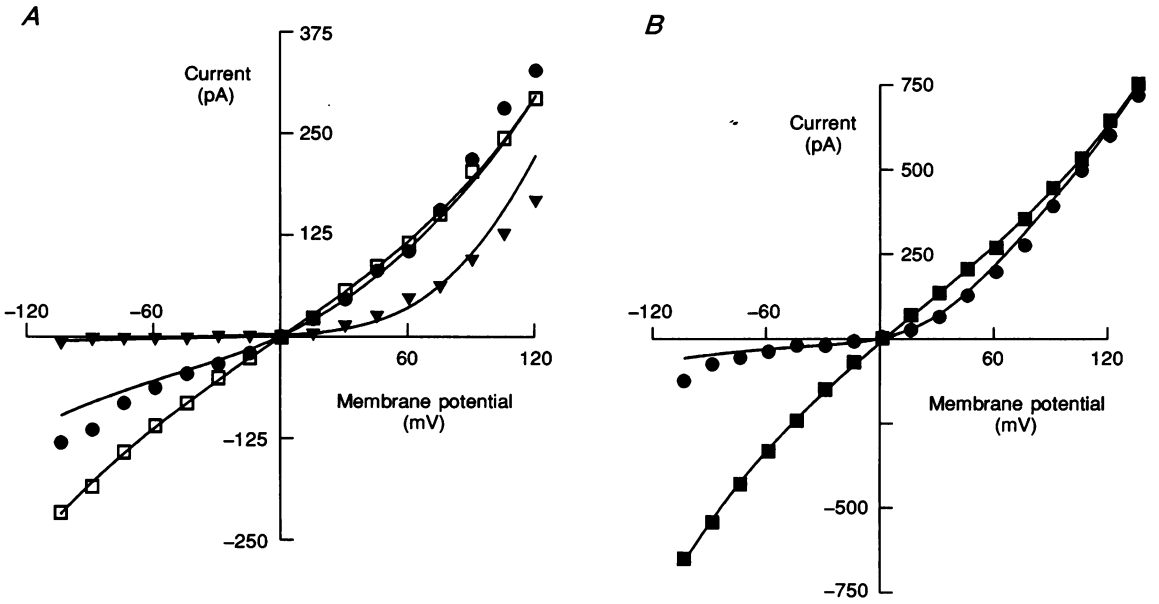


Figure 3. Current–voltage curves in amiloride (*A*) and benzamil (*B*)
Transducer currents in response to sinusoidal (*A*) and step stimuli (*B*) were measured as explained in Methods. The continuous lines fitted to the control data have been calculated according to a scheme used to describe the non-linearity of $I-V$ curves of transducer channels (Kros *et al.* 1992). This mechanism assumes that divalent cations block the transducer conductance in a voltage-dependent manner according to a single energy barrier model:

$$I(V) = k(\exp((1-\gamma)(V-V_r)/V_s) - \exp(-\gamma(V-V_r)/V_s)),$$

with V, the membrane potential; V_r, the reversal potential; V_s, a measure for the steepness of the rectification; γ, fractional distance of the energy barrier from the outside of the membrane's electric field and k, a proportionality constant. Fits to $I-V$ curves measured in the presence of the drug were made according to the conformation model (eqn (4)) with $I_{control}$ given by the above equation. *A*, $I-V$ curves of one cell exposed to 60 μM amiloride, then control and finally 300 μM amiloride solutions. Series resistance, 4·0 MΩ. Parameters of fits are as follows. Control: $\gamma = 0{\cdot}47$, $V_r = 1{\cdot}5$ mV, $V_s = 46{\cdot}7$ mV, $k = 83{\cdot}7$ pA. 60 μM and 300 μM amiloride (eqn (4) combined with equation above): γ, V_r, V_s and k as in control, $K_D = 53$ μM, $n_H = 2{\cdot}0$, equivalent valency (z) = 0·85, equilibrium constant at 0 mV (E_{D0}) = 0·6. *B*, $I-V$ curve of another cell (the same as that in Fig. 2) in control and 10 μM benzamil. Series resistance: 7·1 MΩ. Control: $\gamma = 0{\cdot}54$, $V_r = 3{\cdot}3$ mV, $V_s = 49{\cdot}9$ mV, $k = 236$ pA. 10 μM benzamil: γ, V_r, V_s and k as in control, $K_D = 3{\cdot}2$ μM, $n_H = 2{\cdot}0$, $z = 1{\cdot}11$, $E_{D0} = 0{\cdot}4$. *A*: □, control; ●, 60 μM amiloride; ▼, 300 μM amiloride. *B*: ■, control; ●, 10 μM benzamil.

potential. At depolarized potentials all curves for different concentrations and different drugs merge and asymptotically approach 1, indicating the complete release from block.

At hyperpolarized potentials the situation is different as a complete block of the channel is never reached. Instead, the data asymptotically tend towards different values between 0 and 1 depending on the binding affinity of the drug and its concentration, as described by the dose–response curves at hyperpolarized potentials (Fig. 1). Below −50 mV the block is practically independent of the membrane potential. Fits to all four data sets of Fig. 4 had the same limiting voltage dependence of an e-fold change in $I/I_{control}$ per 23 mV at negative potentials.

This type of voltage-dependent block could not be explained by a simple Woodhull model, where a charged blocker molecule binds to a site inside the transmembrane electric field (Woodhull, 1973). Such a mechanism should lead to either a complete block or a complete release of the block when the membrane potential is at extreme potentials. Instead, two alternative models were considered to explain the data, referred to as the competition model and the conformation model (see Discussion). To choose between these models it was necessary to examine whether the voltage-dependent release of the block was less prominent when the cation flux through the channel was reduced.

Channel block is independent of cation flow

To reduce the cation flux through the transducer channel we replaced about 70 % of the intra- and extracellular monovalent cations by NMDG, which does not contribute to the current through the channel (Kros *et al.* 1992). As it is technically difficult to perfuse cells intracellularly, we

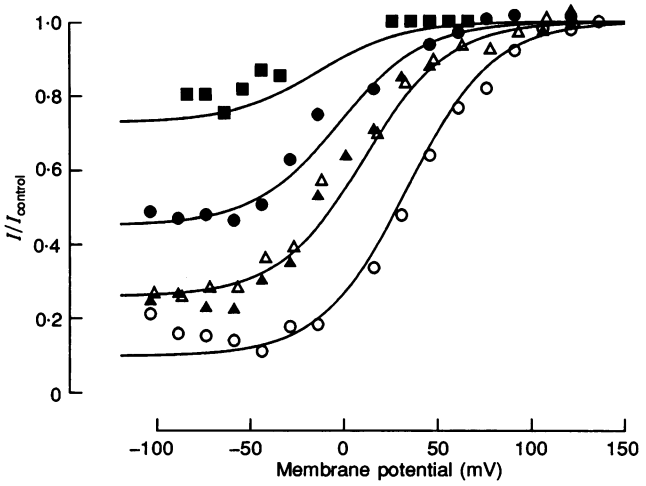


Figure 4. Voltage-dependent block in amiloride and benzamil
Hair cells were stimulated by force steps (except 30 μM–119 Hz sinusoid). The ordinate shows the transducer current as a fraction of the current in drug-free control solution. Data for benzamil are derived from Fig. 3*B*; data for amiloride are from 4 different cells. ■, 30 μM amiloride; ●, 60 μM amiloride; ▲, 100 μM amiloride; △, 100 μM amiloride with 70 % of the monovalent cations replaced by NMDG; ○, 10 μM benzamil. Continuous lines are fits according to the conformation model (eqn (4)). All four data sets have a limiting voltage dependence at negative potentials of 23 mV per e-fold change of conductance (i.e. equivalent valency $z = 1{\cdot}11$). Other fit parameters are as follows. Amiloride (parameters were kept constant for all concentrations, and do not necessarily represent the best fit for each drug concentration): $K_D = 53$ μM, $n_H = 1{\cdot}7$, $E_{D0} = 0{\cdot}4$. Benzamil: $K_D = 3{\cdot}2$ μM, $n_H = 2{\cdot}0$, $E_{D0} = 0{\cdot}4$.

preferred to evaluate the channel block by 100 μM amiloride in normal and NMDG solutions in different cells. In cells studied in the presence of intra- and extracellular NMDG, the total transducer current at any potential before superfusion with amiloride was only about 25 % of the current in the normal cells. The voltage dependence of the block, however, overlapped completely, without a shift of the current–voltage curve in the NMDG solutions (Fig. 4, 100 μM amiloride). The voltage at which 50 % of the voltage-dependent block is released ($V_{½}$) was similar in the control and the NMDG experiments. For 100 μM amiloride, two cells in control solutions had a $V_{½}$ of the conductance–voltage curves of $-2{\cdot}8$ ($\pm$ 7·3) mV (mean ($\pm$ S.D.)). Three cells in NMDG solutions had a $V_{½}$ of $+3{\cdot}3$ ($\pm$ 6·5) mV, which was not significantly different from the controls.

The binding site is inaccessible from the intracellular solution

To investigate whether amiloride has any effect on the transducer channel when applied from the intracellular

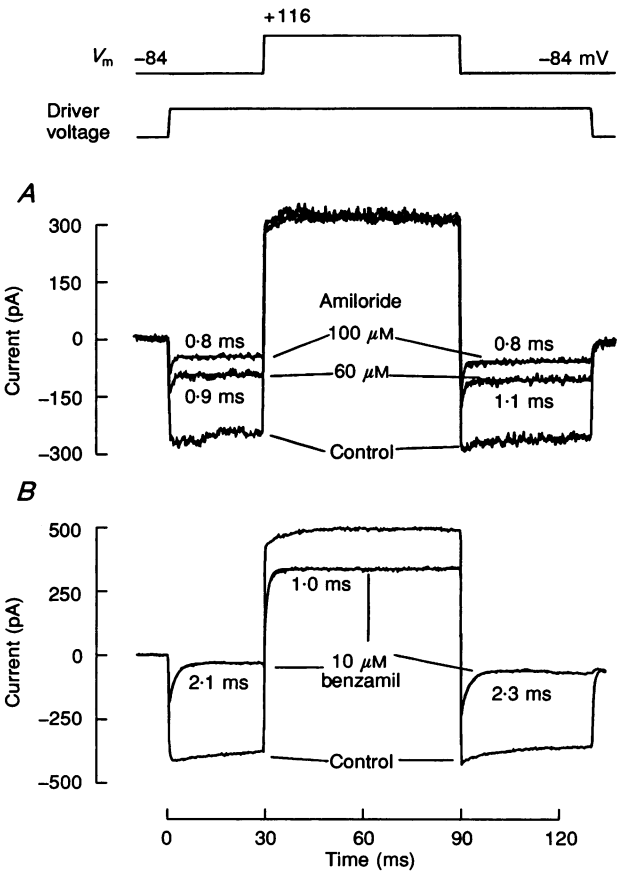


Figure 5. Kinetics of the channel block for force steps and voltage jumps
Cells were first superfused with normal extracellular solution and subsequently by drug solutions as indicated in the figure. Pure electrical stimuli and combinations of mechanical and electrical stimuli were alternated, and responses to electrical stimulation were subtracted from the responses to combination stimuli to eliminate linear leak and voltage-activated currents. Fitted continuous lines are single exponentials with their time constants printed next to them. Top, monitor of the membrane potential V_m and the driver voltage to the piezodisc of the mechanical stimulator. *A*, mechanical and electrical step stimulation in 60 and 100 μM amiloride. Current traces are averages of 7 presentations. Series resistance, 4·8 MΩ. *B*, step experiments in 10 μM benzamil. Averages of 19 presentations. Series resistance, 6·0 MΩ.

side, 1 mM amiloride was included in the patch pipette and the cell's responses to mechanical step stimuli were measured at different membrane potentials.

In three cells we found saturating chord conductances at −84 mV of between 3·2 and 5·1 nS. The mean conductance of 3·9 (± 1·0) nS was not significantly different from the maximum conductance in control solutions we reported recently, 5·3 (± 3·4) nS ($n = 11$) (Kros *et al.* 1992). Current–voltage curves were indistinguishable from control curves such as those shown in Fig. 3. Transfer functions of the transducer current (Kros *et al.* 1992) also had the same shape as in controls (data not shown).

Drug binding kinetics

Open-channel block

An important question that could give information about the nature of the blocking mechanism is whether the drug binds to the closed channel or whether the channel must first open before block can occur. Figure 5 shows saturating step responses of cells which were first superfused with normal extracellular solution and subsequently by 60 μM and 100 μM amiloride, or 10 μM benzamil (see also Fig. 2*A* and *B* for another cell). In control solutions the currents developed quickly, rising in less than 1 ms to the tonic current plateau which persisted throughout the duration of the force step. At −84 mV in the presence of the drugs, a mechanical stimulus initially activated inward currents, although these current transients were never as large as the control currents at the onset of the force step. This observation suggests that the closed transducer channel is not blocked by the drug. The seemingly curtailed amplitudes of the current transients could be fully explained by the limited rise time of the stimulus, as not all transducer channels are opened before the first ones become blocked by the drug.

After the transient peak, currents relaxed approximately according to a single exponential to a new steady-state level. This time course of the current flow can be explained by an open-channel block where the channel has to be opened first before the drug molecules can bind to the channel and cause the block. The time constant of the exponential and the degree of suppression of the steady-state current depended on the concentration and dissociation constant of the superfused drug. At concentrations blocking about 80 % of the control transducer current, benzamil (10 μM), with the higher binding affinity, had a slower relaxation time constant of 3·2 (± 0·8) ms (5 cells), than amiloride (100 μM), where τ was 0·81 ms (1 cell). In 5 μM benzamil τ increased to 4·0 (± 0·4) ms (3 cells); in 60 μM amiloride it was 0·96 (± 0·04) ms (3 cells).

As the block of the transducer channel is abolished by large depolarizations, the hypothesis of an open-channel block could be tested in a voltage-jump experiment. If the drug binds only to the open channel, the current is expected to relax with the same time course at the end of a depolarizing voltage jump, when the channels are open, and when the channels are initially opened mechanically by a step stimulus.

Panels *A* and *B* in Fig. 5 show such voltage-jump experiments in 60 and 100 μM amiloride, and 10 μM benzamil. The cells' transducer channels were first opened by a saturating mechanical step stimulus and blocked by either amiloride or benzamil. The drug was then released by clamping the membrane potential to +116 mV. In amiloride solutions (60 and 100 μM) the release of the drug at the onset of the depolarization and the corresponding reactivation of the transducer current was too fast to be distinguished from the control current. In 10 μM benzamil the time course of the reversed current could be fitted by a single exponential with $\tau = 0{\cdot}99$ (± 0·03) ms (2 cells). On stepping the membrane potential back to −84 mV the transducer current became inward again and rose to its transient maximum within 200 μs (10–90 % limits). Thereafter the current amplitude relaxed exponentially with time constants of 1·1 (± 0·1) ms ($n = 3$ cells) in 60 μM amiloride and 0·76 ms (1 cell) in 100 μM amiloride. In 10 μM benzamil the corresponding time constant was 2·3 (± 0·3) ms (2 cells). These time constants are very similar to those observed at the onset of the mechanical step stimulus. It is therefore likely that they represent the time course of the drug binding to the open channel.

The relaxation time constant of the block was also examined as a function of the membrane potential. In the

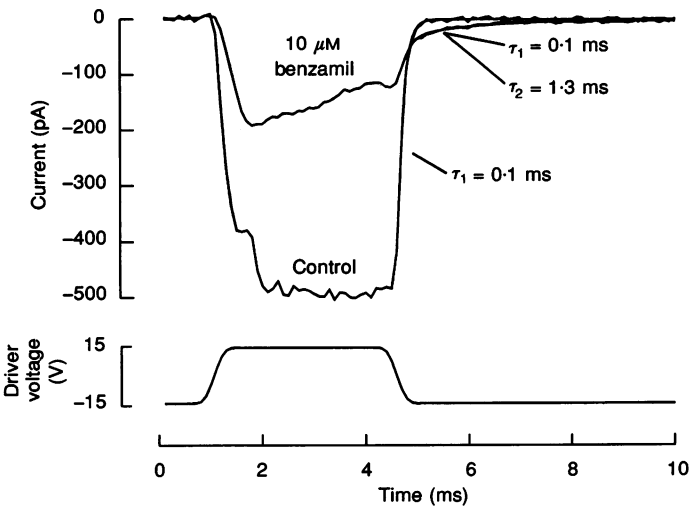


Figure 6. Kinetics of release of the block by benzamil
The record starts with the hair bundle deflected in the inhibitory direction and all transducer channels closed. Voltage clamped to −84 mV, the cell was first superfused by normal extracellular solution and subsequently by 10 μM benzamil. Tail currents at the end of the mechanical step were fitted by a single exponential (control) or a double exponential (10 μM benzamil), superimposed on the data with their time constants printed next to them, simplifying their sigmoidal time course (due to the Bessel filter) by neglecting the very onset. The relative size of the fast and slow component was 3:1. Data filtered at 5 kHz and digitized at 10 kHz. Series resistance, 3·9 MΩ.

presence of 10 μM benzamil, two outer hair cells, including the one shown in Fig. 2, were stepped through a series of different membrane potentials as indicated in Fig. 2. Their transducer channels were opened 50 ms after the onset of this voltage step by a saturating force step. Relaxation time constants evaluated from fits to current transients could be obtained in the range −84 to +46 mV, except about 10 mV below and above the reversal potentials where currents were too small. In these two cells no systematic change of the relaxation time constant with change in membrane potential was observed (cell shown in Fig. 2: 2·2 (± 0·5) ms, fits at $n = 10$ different potentials; other cell: 2·6 (± 0·4) ms, $n = 6$).

Tail currents in the presence of blockers

In control solutions, the time course of the onset and offset of the transducer current was apparently limited by that of the driver voltage put across the piezodisc. As is shown in Fig. 6, tail currents on termination of mechanical steps in control solutions, put through an 8-pole Bessel filter at 10 kHz, had a 10–90 % rise time of 300 μs and, ignoring the very onset of the current trace, were sufficiently well described by single exponentials with a mean time constant of 0·12 (± 0·04) ms ($n = 2$ cells).

In the presence of 10 μM benzamil the tail currents had an additional slow component and double exponentials had to be used to fit the current traces. In three cells, the average fast time constant was unaltered compared to the control (0·13 (± 0·04) ms). The slow component had a mean time constant of 1·6 (± 1·0) ms. In two cells superfused by 30, 60 or 100 μM amiloride the time course of the tail currents was not changed compared to the control. The slow component indicates that the channel can only close after the drug molecules have been released from their binding sites (see Discussion).

DISCUSSION

Steady-state block of transducer currents by amiloride and derivatives

Our results demonstrate that amiloride and its derivatives reversibly block the current flow through the transducer channel of mouse cochlear outer hair cells. The observed half-blocking concentration of amiloride ($K_D = 53$ μM) at hyperpolarized potentials is in good agreement with the value of 50 μM published by Jørgenson & Ohmori (1988) for chick hair cells. Their report of block by a single drug molecule contrasts with our data in favour of a co-operative block by two molecules.

Comparison of structure–activity sequences

The block of hair cell transducer channels by amiloride and the immunostaining of hair cell stereocilia by antibodies directed against epithelial Na^+ channels of bovine kidneys (Hackney *et al.* 1992) raise the question of whether these channels are genetically related. Table 1 shows unambiguously that the hair cell transducer channel and the classic form of the epithelial Na^+ channel, which has a high affinity for amiloride, are different. The structure–activity sequences for the two channels do not match. The Na^+ channel shows a reduction in affinity for drugs modified in the 5-position (DMA and HMA; for review see Kleyman & Cragoe, 1988; Smith & Benos, 1991), whereas the transducer channel shows an increase.

Recently, evidence has been found for the existence of a second class of epithelial Na^+ channels with K_D values > 1 μM for amiloride and structure–activity sequences somewhat similar to that of the transducer channel (Table 1; for review see Smith & Benos, 1991). These channels have a large single channel conductance and a poor cation selectivity (for example, 23 pS and a permeability ratio P_{Na^+}/P_{K^+} of 1·5, Vigne, Champigny, Marsault, Barbry, Frelin & Lazdunski, 1989). Information about the blocking mechanism is not available, except for evidence of negative co-operativity as judged from Hill coefficients smaller than 1 (calculated from published data for amiloride according to eqn (1): $n_H = 0·6$, Fig. 2 in Moran, Asher, Cragoe & Garty, 1988; $n_H = 0·6$, Fig. 6 in Vigne *et al.* 1989). This comparison shows that, despite the existence of different types of epithelial Na^+ channels, none has the same properties as the transducer channel.

Lane *et al.* (1991) recently reported a co-operative block of a stretch-activated conductance in the membrane of *Xenopus* oocytes by amiloride ($K_D = 500$ μM measured in the absence of external Na^+ and Ca^{2+} and in the presence of 1 mM EGTA). This channel has a large single-channel conductance (about 60 pS, calculated from Fig. 3*B* in Lane *et al.* 1991), is non-selective for cations, and its structure–activity sequence (Lane *et al.* 1992) corresponds to the sequence reported here. The channel block has the same form of voltage dependence, although it is less steep (39 mV per e-fold change) than that of the transducer channel (23–30 mV per e-fold change).

The obvious similarities between these two channels suggest a common origin, but it is necessary to verify an open-channel block for the stretch-activated conductance, a point not addressed by Lane *et al.* (1991).

A conformational change of the channel explains the voltage dependence of the block

The peculiar nature of the voltage dependence of the block (Fig. 4), which never becomes complete at hyperpolarized potentials, makes voltage-dependent binding to charged sites inside the channel, in the membrane's electric field, extremely unlikely, although not impossible (see one version of the punch-through model discussed by Lane *et al.* (1991)). Instead, two models in which the drug molecules bind to sites outside the electric field were considered: the competition model and the conformation model.

The competition model is analogous to the mechanism proposed by Armstrong (1971) for the voltage-dependent release of block of K^+ channels by intracellular TEA and derivatives. It assumes that drug molecules bind to a site just outside the electric field (so that the drug binding at hyperpolarized potentials is independent of voltage), but in the ion permeation pathway. Depolarization accelerates

cations from inside the cell through the transmembrane field, and a fraction of these ions gains sufficient energy to displace the drug molecules from their binding sites. The effective concentration near the binding sites of cations (C) with sufficient kinetic energy to displace the drug molecules is given by :

$$[C] = [C_0]\exp\left(\frac{zF}{RT}V\right), \quad (2)$$

$[C_0]$ is the effective concentration at a potential of 0 mV; z is the valency of the cations; F, R and T have their usual meaning, and V is the membrane potential. If z is set to 1, on the assumption that monovalent cations are the most prevalent ones, then reducing the concentration of permeant cations by 70 %, as in the NMDG experiments, would lead one to expect a shift of the current–voltage curves in Fig. 4 of 31 mV to the right, quite independent of the parameters of the drug binding reaction. The absence of such a shift invalidates this model, as does the voltage independence of the time constant of the block: the backward reaction rate constants should become faster at depolarized potentials due to competition with cations (Armstrong, 1971).

Our data are, however, consistent with a binding model, the conformation model, in which the voltage dependence of the block results from an intrinsic property of the channel itself. Similar models were used to explain voltage-dependent block of stretch-sensitive channels in frog oocytes (Lane *et al.* 1991), and to describe the voltage-dependent block of Na^+ channels by guanidium toxins (Moczydlowski, Hall, Garber, Strichartz & Miller, 1984). The simplest version of this model assumes that, when the cell is depolarized, the channel protein changes its conformation to a second open state, obstructing the binding sites somewhere on the extracellular side of the channel outside the membrane's electric field. The dissociation constant (K_D) of the drug binding is voltage independent, but the equilibrium constant (E_D) of the conformational change, defined as the ratio of the blockable to the unblockable open states, is a function of the membrane potential:

$$E_D = E_{D0}\exp\left(-\frac{zF}{RT}V\right), \quad (3)$$

where E_{D0} is the equilibrium constant at 0 mV membrane potential, and z is the equivalent valency associated with the conformational change. The steady-state transducer current is described by:

$$\frac{I}{I_{control}} = \frac{1}{1+\dfrac{[D]^{n_H}}{K_D^{n_H}+(K_D^{n_H}/E_D)}}. \quad (4)$$

This model was used for the fits in Figs 3 and 4. Fitted values for z varied from 0·85 to 1·11, corresponding to a limiting voltage-dependence of the block, at negative potentials, of 30 to 23 mV per e-fold change of $I/I_{control}$. Our values for z are in between the 0·65 of Lane *et al.* (1991) and the 1·67 reported by Jørgensen & Ohmori (1988). The latter used a model in which amiloride binding actually induces the conformational change in the channel. This model does not fit our data. It seems unlikely, however, that transducer channels of mammalian hair cells are more similar in this respect to stretch-sensitive channels in amphibian oocytes than to transducer channels of avian hair cells.

Open-channel block

The mechanical step and voltage jump experiments (Figs 2*B* and 5) clearly support the notion that the channel must open before it can be blocked. This finding is in contrast to the study by Jørgensen & Ohmori (1988) in which the authors state that the blocking effect is apparently independent of mechanical gating. However, using a low-frequency (10 Hz) triangular waveform and the rapidly equilibrating drug amiloride, the effects of open-channel block would have been impossible to detect in their experiments.

The slow component in the tail currents shown in Fig. 6 is consistent with the notion that the channel cannot close with the drug bound, but has to release the drug first and reach a conducting open state before finally changing to the closed state. This observation rules out the possibility that blocker molecules become trapped inside the channel when it closes. The slow component resembles the slow offset kinetics at the end of step displacements in turtle cochlear hair cells described by Crawford *et al.* (1989). In turtle hair cells the offset responses slow down with less Ca^{2+} entering the cell. If the slow component in the tail currents of mouse cochlear outer hair cells were a Ca^{2+}-related effect, this component should develop when the current flow into the cell is limited by a comparable steady-state block by either 100 μM amiloride or 10 μM benzamil. The slow component in the tail currents, however, was seen only in the presence of benzamil, ruling out the possibility that the slow component depends on intracellular Ca^{2+}.

Kinetics of binding and release of the drug

The presence of at least two drug binding sites precludes derivation of the reaction rates from the macroscopic properties of the block. However, some qualitative observations can be made. Time constants for binding and release were consistently faster for amiloride than for benzamil, presumably reflecting larger backward rate constants for the drug with the lower affinity for the binding sites. Both for amiloride and benzamil, the time constants of drug binding decreased with increasing concentration of the drug. This was expected, as each of the forward reaction rates should be proportional to the concentration of the drug.

The time constants of release of the drug, upon large depolarizations as well as upon forcing the channels to close at hyperpolarized potentials, are faster than the time constants of drug binding. A possible explanation is that both a mechanical force that closes the channel and large depolarizations may reduce the affinity of the binding sites for the bound drug molecules. This would lead to a more

complicated variant of the conformation model than the simple version from which eqns (3) and (4) were derived.

The transducer channel has at least two open states

The present results point to two open states of the transducer channel, the first of which prevails at hyperpolarized potentials and exposes extracellular binding sites for amiloride that are not available when the channel is closed. In the second open state, favoured at depolarized potentials, there is again no access to the binding sites.

This finding of a second open state has an interesting parallel in experiments on turtle cochlear hair cells (Crawford *et al.* 1989; Crawford, Evans & Fettiplace, 1991). The authors suggest the existence of a second open state to explain slow offset kinetics under conditions which abolish adaptation. Although it appears that their additional open state is not voltage dependent but controlled by the intracellular Ca^{2+} concentration, the second open state is favoured at depolarized potentials. At present the possibility cannot be excluded that the voltage-dependent conformational change which we have exposed in the transducer channel may in part or completely be caused by the same mechanism that is responsible for the second open state described by Crawford *et al.* (1989, 1991). This raises an analogy to Ca^{2+}-activated K^+ channels (Barrett, Magleby & Pallotta, 1982) where the voltage dependence of channel opening is in part conferred by the voltage-dependent entry of Ca^{2+} into the cell.

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