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Rapid Communication

Aminoglycoside Antibiotics Impair Calcium Entry but Not Viability and Motility in Isolated Cochlear Outer Hair Cells

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Cochlear outer hair cells have been well established as primary targets of the ototoxic actions of aminoglycoside antibiotics. These cells, isolated from the guinea pig cochlea and maintained in short-term culture, were used as a model for evaluating the acute effects of gentamicin on cell viability, depolarization-induced transmembrane calcium flux, and depolarization-induced motile responses.

On the basis of morphology and fluorochromasia, the presence of extracellular gentamicin as high as 5 mM did not affect the viability of the cells for up to 6 hr, the longest time tested. Viable cells showed binding of fluorescently tagged gentamicin to their base but excluded the drug from their cytoplasm. In response to $[K^+]$-depolarization, intracellular calcium levels (monitored with the fluorescent calcium-sensitive dye fluo-3) increased from a resting value of 218 ± 102 nM to 2,018 ± 1,077 nM concomitant with a cell shortening of 0.7% ± 1.3%. The depolarization-induced calcium increase was apparently caused by calcium entry into the cell as it was inhibited by the calcium-channel blocker methoxyverapamil and prevented in the absence of extracellular calcium. Both gentamicin and neomycin blocked the $[K^+]$-induced calcium increase at an IC_{50} of 50 μM. Despite the inhibition of calcium entry the ability of the outer hair cells to shorten under $[K^+]$-depolarization was not impaired; in fact, cell shortening was even more pronounced in the absence of calcium influx (2.6% ± 1.4%). This argues effectively against the existence of a calcium-dependent actomyosin-mediated component in $[K^+]$-induced shape changes.

The results suggest the existence of voltage-gated calcium channels in outer hair cells and that calcium influx through these channels is impaired by the aminoglycoside antibiotics neomycin and gentamicin. This action may be part of the acute ototoxic mechanism of these molecules. Furthermore, the results not only confirm the calcium independence of the depolarization-induced motility but also suggest that calcium influx into outer hair cells opposes cell shortening.

Key words: fluo-3, $[K^+]$-depolarization, calcium-channels, gentamicin, neomycin

INTRODUCTION

Isolated outer hair cells maintained in short-term culture have recently been established as models for the study of hair cell physiology (Brownell et al., 1985; Ashmore and Meech, 1986) and pathology (Dulon et al., 1987; Williams et al., 1987). Since the cochleotoxicity of aminoglycoside antibiotics preferentially affects this sensory cell type (Hawkins, 1976) this preparation should be a particularly good model to study the toxic mechanisms of these drugs. Aminoglycosides indeed bind to isolated hair cells and displace membranous calcium (Williams et al., 1987). Such actions at the level of the outer hair cells were in good agreement with previous demonstrations of aminoglycoside/calcium antagonism in the inner ear. Neomycin had been shown to compete with calcium binding in the organ of Corti and lateral wall tissues of the guinea pig in vitro (Orsulakova et al., 1976); and the suppression of the cochlear microphonic potential (which is postulated to originate mostly in the outer hair cells) during cochlear perfusion of gentamicin

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was prevented by calcium (Takada and Schacht, 1982). The aminoglycoside antibiotics also interfere with calcium actions in other tissues: neuromuscular transmission is competitively blocked (Pittinger and Adamson, 1972, for review) as are contractions of smooth (Adams and Goodman, 1975; Corrado et al., 1975; Del Pozo and Baeyens, 1986) and cardiac muscles (Adams, 1975; Hino et al., 1982). More recently, these drugs were shown to impair transmembrane Ca^{2+} flux in isolated nerve terminals, presumably by blocking Ca^{2+}-channels (Atchison et al., 1988), and to reduce binding of ω-conotoxin GVIA, a putative inhibitor of "N-type" Ca^{2+} channels, in cerebral cortex membranes (Knaus et al., 1987). Furthermore, transduction channels in bullfrog hair cells are sensitive to aminoglycosides (Hudspeth and Kroese, 1983; Hudspeth, 1985).

The aim of this study was to determine how acute exposure to the aminoglycoside antibiotics neomycin and gentamicin affected viability and calcium flux in outer hair cells. Calcium entry was induced by depolarizing the cells with KCl and monitored with the fluorescent calcium indicator, fluo-3 (Minta et al., 1987). $[K^+]$-depolarization also induces shape changes in outer hair cells; and, consequently, we studied the effects of the aminoglycosides on these motile properties and the link between calcium influx and $[K^+]$-induced motility.

MATERIALS AND METHODS

Cell Preparation

Pigmented guinea pigs (280–340 g) were decapitated and the temporal bones quickly removed. The bulla and the bony walls of the cochlea were immediately opened, and the three upper turns of the organ of Corti dissected. These were kept at room temperature (20–22°C) in Hanks' balanced salt solution (HBSS) buffered to pH 7.4 with 5 mM sodium HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (Dulon et al., 1987; Zajic and Schacht, 1987). The outer hair cells were mechanically isolated by gentle flux and efflux of the organ of Corti through a micropipette (Brownell et al., 1985). The osmolalities of all solutions in the study were adjusted to 300 ± 2 mOsm and measured before and after the experiments with a micro-osmometer (Roebling, Berlin, FRG). If the osmolality exceeded these limits at the end of an experiment (e.g., due to evaporation), results were considered invalid. This precaution was taken because length and shape of outer hair cells is highly sensitive to osmotic variations in the surrounding medium (Dulon et al., 1987).

Cell Viability: Staining With Fluorescein Diacetate and Propidium Iodide

Simultaneous double-staining by fluorescein diacetate (FDA) and propidium iodide (PI) was used for the determination of cell viability (Jones and Senft, 1985). Fluorescein diacetate (FDA), a non-polar and non-fluorescent ester, passes through cell membranes and is hydrolyzed by intracellular esterases to produce fluorescein. Fluorescein accumulates inside viable cells since, as a polar compound, it is released only slowly through an intact plasma membrane. The cells therefore exhibit green fluorescence (520–560 nm) when excited by blue light (450–490 nm). While FDA exclusively stains viable cells, PI is excluded by intact cell membranes and only stains non-viable cells by intercalating with their DNA and RNA to form a bright red fluorescent complex in the nucleus when excited by green light (515–560 nm). Stock solutions were prepared by separately dissolving 5 mg FDA (Sigma, St. Louis, MO) in 1 ml acetone and 350 μg of PI (Sigma) in 1 ml of HBSS. Mixed FDA and PI working solutions were always freshly prepared in HBSS at concentrations of 10 μg FDA and 5 μg PI/ml, and the cells were stained for 3 min and then rinsed with HBSS. The percentage of viable cells at different times was determined by counting approximately 100 cells under fluorescence microscopy with a hemocytometer.

Application of Aminoglycosides to Hair Cells

For viability studies, cells were incubated for 6 hr at room temperature in HBSS containing the aminoglycosides gentamicin sulfate (Schering Corporation, Bloomfield, NJ) or neomycin sulfate (Pharma-Tek Inc, Humington, NY) at concentrations of up to 5 mM. Calcium flux and motility were studied in response to $[K^+]$-depolarization after a 30 min incubation with the aminoglycosides, and only in cells no older than 2 hr after the dissociation procedure.

Preparation and Uptake of Fluorescein-Labelled Gentamicin

Fluoresceinthiocarbamyl gentamicin (FTC-gentamicin) was synthesized by reacting 230 mg of gentamicin (gentamicin sulfate) with 250 mg of fluorescein isothiocyanate (FITC) isomer I from Sigma in 50 ml of sodium carbonate-bicarbonate buffer (50 mM; pH 9.0) for 2 hr at room temperature (Watson et al., 1976). FTC-gentamicin was purified by chromatography over Sephadex G-15 and concentrated on a Dowex-50 column. Column fractions were monitored for gentamicin content by enzyme-immunoassay (Emit, Syva-Biomerieux, France) and for fluorescein content by conventional fluorometry. The ratio of fluorescein:gentamicin was 1:1.4, and no unreacted FITC could be detected by TLC (Watson et al., 1976).

Cell Imaging

Hair cells were incubated for 30 min at room temperature (22–25°C) with the permeant acetoxy methyl

ester derivative of fluo-3, fluo-3/AM (Molecular Probes Inc., Junction City, OR) at a final concentration of 2 μM. They were maintained in a saturated humid chamber in a 50 μl droplet of HBSS on glass cover slips (thickness, 0.13 mm) and subsequently carefully rinsed with HBSS.

Cells loaded with fluo-3 were observed with an inverted microscope (Nikon, DIAPHO-TMD) fitted with an epifluorescence system (light source HBO 100, AC; a 450–490 nm band pass exciter filter, 510 nm dichroic mirror, and a 520 nm barrier filter for excitation and emission, respectively) and a Leitz 160/- NPL FLUOTAR 50/1.00 oil immersion objective. In order to prevent photobleaching, the excitation irradiance was reduced by a 97.5% neutral density filter to 0.14×10^5 W/m^2. Under these conditions, no significant photobleaching was noted with continuous illumination of 2 min (maximum exposure time in this study).

Cell fluorescence was monitored via a Silicon Intensified Target video camera (SIT 66, Dage-MTI Inc., USA) and recorded for analysis on a video cassette recorder (Mitsubishi SuperVHS model HS-423UR). The recorded images were analyzed with a Quantex QX-7-210 image processing system (Quantex Corp., Sunnyvale, CA). The response of the camera and digitization system was linear under the experimental conditions as tested with a series of neutral density filters.

Sixteen to 32 sequential frames from the video cassette recorder or the camera were averaged and the radiances of the cell images were measured by averaging the pixel radiance value in a defined cell area. The cell area considered was essentially an elongated rectangle covering the cell from the cuticular plate to the nucleus, excluding the nucleus and the edges of the cell.

The concentrations of intracellular calcium were estimated according to the equation described for quin2 (Tsien et al., 1982): $[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F)$, where K_d is the reported dissociation constant (400 nM) for the fluo-3/calcium complex (Minta et al., 1987); F is the observed fluorescence in arbitrary units of intact fluo-3 loaded cells; F_{max} (fluorescence maximum) was obtained by using ionomycin (10 μM) to equilibrate the cell with external calcium; F_{min} (fluorescence minimum) was calculated from the equation: $F_{min} = (F_{max} - F_{auto})/40 + F_{auto}$, where 1/40 is the fluorescence ratio of the metal-free fluo-3 to the Ca^{2+} complex and F_{auto} (cell autofluorescence) was obtained after the addition of $MnCl_2$ (2 mM) and lysis of the cell.

Stimulation and Hair Cell Motility

Cell length was simultaneously monitored with the fluorescence or separately on bright field images and analyzed with the Quantex-QX-7-210. The system provided measurements with an standard error of ± 0.2 μm as tested with fluorescent microspheres of known diameter.

For [K^+]-depolarization (70 mM KCl fianl concentration), 50 μl of a 140 mM KCl-HBSS solution (Na^+ replaced by KCl and osmolality adjusted to 300 ± 2 mOsm) was gently added with a pipette to the 50 μl of normal HBSS-containing the cells. For depolarization in calcium-free conditions, KCl-HBSS solution was prepared without calcium and supplemented with 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) (osmolality 300 ± 2 mOsm).

As evaluated on 10 cells, the addition of normal HBSS (5 mM KCl) under the same conditions did not lead to any significant changes in the fluorescent signal of fluo-3 or of cell length.

Hair cells bathed in calcium-HBSS which did not respond with an increase in fluorescence after [K^+]-depolarization and the application of the calcium ionophore were excluded from the study.

RESULTS

Viability of Outer Hair Cells In Vitro

The viability of outer hair cells at various times after isolation was monitored by fluorochromasia, an objective test for viable cells (Rotman et al., 1966). In the presence of FDA and PI, viable cells exhibited green fluorescence when excited by blue light (Fig. 1a) and no red fluorescence in their nucleus when excited by green light. On the contrary, cells with non-intact membranes presented weak or no fluorescence in blue light while their nucleus was brightly fluorescent in green light (Fig. 1b).

After isolation, 72% ± 5% of the outer hair cells were viable. They did not show signs of deterioration for up to two hours in vitro (Fig. 2a). Subsequently, however, the percentage of viable cells slowly decreased until at 6 hr less than 30% of the cells appeared intact. The damaged cells showed the characteristic features of deterioration previously described by Zajic and Schacht (1987): loss of birefringence, numerous granulations and Brownian activity within the cytoplasm, and rounding and swelling. In HBSS containing gentamicin (5 mM) the survival time of the isolated outer hair cells, as judged by both morphology and fluorochromasia, did not differ from the control incubations over a period of 6 hr (Fig. 2b).

Uptake of Gentamicin-FTC

Binding of gentamicin to the cells was measured after various times of incubation (up to 4 hr) with FTC-gentamicin (100–200 μM). Intact cells did not exhibit

TABLE I. Effect of Neomycin on Calcium Entry and Cell Shortening†

Addition to medium	$[Ca]_i$ (nM)		Length (% change)
	Resting	Depolarized	
None (control)	218 ± 102	2,108 ± 1077	−0.75 ± 1.3
1 mM neomycin	200 ± 76	375*·** ± 157	−2.0*** ± 1.1

†The intracellular calcium concentrations were calculated as described in Materials and Methods before and after 60 sec depolarization with 70 mM $[K^+]$ in the absence (n = 11) and the presence of 1 mM neomycin (n = 7).
*Different from resting value, $P < 0.01$ (paired t-test).
**Different from control, $P < 0.01$ (unpaired t-test).
***Different from control, $P < 0.05$ (unpaired t-test).

intra-cytoplasmic fluorescence, and binding was essentially restricted to the synaptic ending of the cells and to a lesser degree to the area around the cuticular plate. This bound FTC-gentamicin appeared to be localized extracellularly (Fig. 3a,b), but it was impossible at the light microscopic level to determine whether it was bound to the cell plasma membrane or to the residues of nerve endings and supporting cells frequently observed in this area. On the other hand, intracellular fluorescence was detected in cells with morphological features of damaged cells, suggesting that FTC-gentamicin penetrated only through damaged cell membranes (Fig. 3c,d).

Hair Cell Motility and Intracellular Ionized Calcium Under $[K^+]$-Depolarization

Hair cells bathed in calcium-HBSS (1.25 mM calcium). In the unstimulated state, the calcium-dependent fluorescence in outer hair cells was highest around the nucleus and under the cuticular plate (figure 4). A similar heterogeneity has been reported in vestibular hair cells of the chick loaded with another calcium-indicator, fura-2 (Ohmori, 1988). This heterogeneity may reflect true intracellular differences in calcium distribution as described for single heart cells (Wier et al., 1987). It should be noted, however, that the fluorescent signal can also be affected by differences in thickness of the cell body or uneven distribution of the dye in these locations. Indeed, the latter explanation appears likely since heterogeneity of fluorescence was still observed after the equilibrium of intracellular and extracellular calcium concentrations by the ionophore ionomycin.

The application of 70 mM KCl induced longitudinal shortening of the outer hair cells (Table I) as previously described (Zenner et al., 1985; Dulon et al., 1988). Simultaneously, a large and rapid increase of the cytoplasmic fluorescence was observed (Fig. 4) which appeared to reach a plateau after 30–60 sec (Fig. 5).

In order to estimate the concentration of intracellular calcium, a maximum and minimum value of fluo-3 fluorescence was determined. The fluorescence maximum of the calcium/dye complex was obtained 30–60 sec after permeabilizing the cells to external calcium by the addition of 10 μM ionomycin (Figs. 4, 5). The subsequent addition of Mn^{2+} (2 mM) and lysis of the cells gave the background signal. According to the equation described in Materials and Methods, the rise in intracellular calcium was estimated to be tenfold after 60 sec of $[K^+]$-depolarization, increasing from a resting value of 218 ± 102 nM to 2,018 ± 1,077 nM (Table I). The addition of the calcium ionophore after $[K^+]$-depolarization led to a consistent 1–2% elongation beyond the original length of the cell (Fig. 4c).

Hair cells bathed in calcium-free HBSS. In the absence of extracellular calcium, i.e., when the cells were placed in HBSS without $CaCl_2$ complemented with 1 or 2 mM EGTA, $[K^+]$-depolarization did not lead to significant changes in cell fluorescence. Cell shortening, however, was not suppressed but, on the contrary, was more pronounced (2.6% ± 1.4%; n = 7). The subsequent addition of ionomycin in the presence of external calcium produced an increase in fluorescence and the cells elongated by 1–2% beyond their original length.

Hair cells bathed in calcium-HBSS containing methoxyverapamil, neomycin, or gentamicin. The $[K^+]$-induced calcium influx was impaired by the addition of the organic Ca^{2+} channel antagonist methoxyverapamil to the external medium. At a concentration of 500 μM, the increase in fluorescence after 60 sec of $[K^+]$-depolarization was reduced to about 15% ± 14% (n = 5) of the value observed in the absence of the antagonist. Although impairing calcium influx, the drug did not block the depolarization-induced shortening of the cells.

Calcium entry in response to $[K^+]$-depolarization was also inhibited by the aminoglycoside antibiotics, gentamicin, and neomycin (Fig. 6). At an external calcium concentration of 1.25 mM, calcium uptake was significantly depressed by 10 μM neomycin, and the highest concentration of neomycin tested, 5 mM, reduced uptake to less than 10% of the control value. The IC_{50} for neomycin was estimated to be 50 μM (Fig. 6). Gentamicin (tested at 1 mM only) also caused reduction of the calcium entry at a similar magnitude to neomycin (Fig. 6). Despite their marked effect on depolarization-induced Ca^{2+} entry the aminoglycosides did not suppress the shortening of the hair cells (Table I). On the contrary, the magnitude of shortening was larger in the presence of the drug (Table I).

The addition of the calcium ionophore after $[K^+]$-depolarization of the cells treated with methoxyverapamil, gentamicin, or neomycin, also led to a 1–2% cell elongation beyond the original cell length.

DISCUSSION

The rise in intracellular calcium concentration under $[K^+]$-depolarization suggests the presence of volt-

Fig. 1. Identification of viable and damaged outer hair cells by simultaneous staining with fluorescein diacetate and propidium iodide. **a:** Viable outer hair cell exhibiting intracellular green fluorescence when excited by blue light. **b:** Outer hair cell with a bright red fluorescent complex in the nucleus indicating loss of membrane integrity.

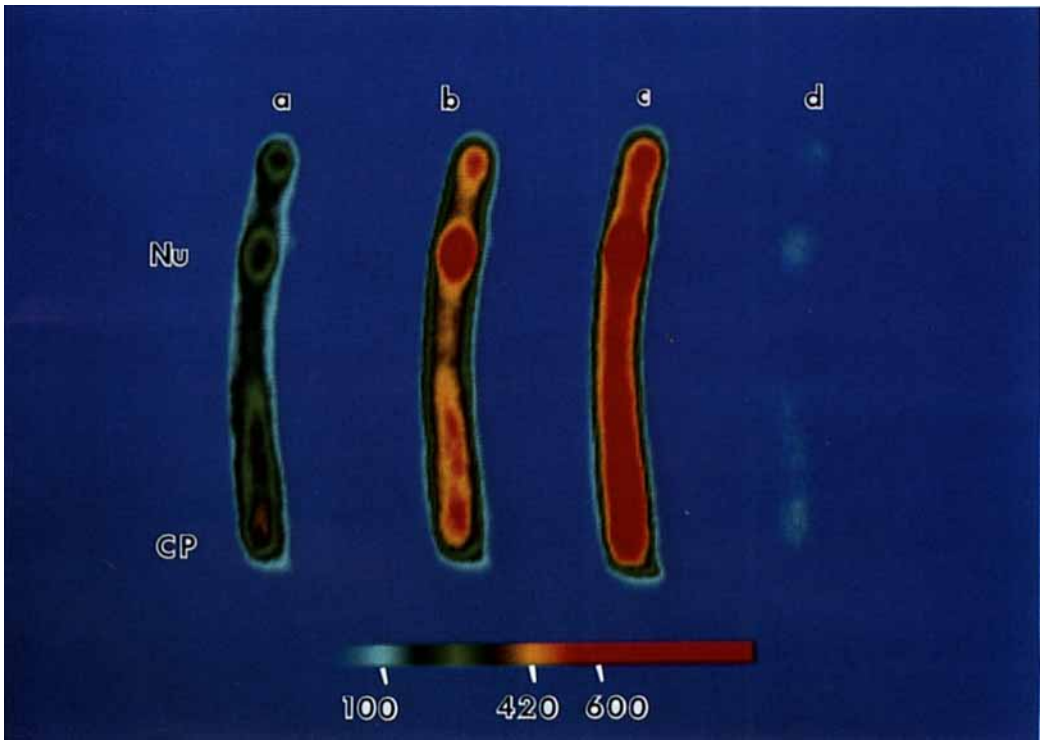

Fig. 4.

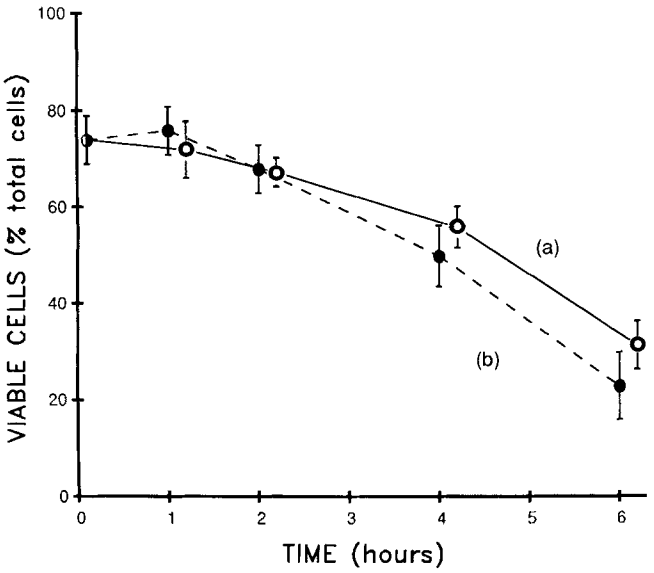

Fig. 2. Viability of isolated outer hair cells as a function of time: a) in normal HBSS (○) and b) in HBSS supplemented with 5 mM gentamicin (●). Isolated cells were incubated with fluorescein diacetate and propidium iodide as described in Materials and Methods. Cells were scored as viable (green fluorescein fluorescence) or not viable (red propidium iodide fluorescence), and the percentage of viable cells was calculated. Data are means (in % of viable cells) ± SD of 3 to 5 experiments in each of which approximately 100 cells were observed.

age-gated calcium channels in the plasma membrane of outer hair cells. The notion that the elevated intracellular calcium is due to calcium influx is supported by the fact that the increase was inhibited by the known calcium channel antagonist, methoxyverapamil, and that it was not significant in the absence of extracellular calcium. Although further electrophysiological and pharmacological investigations are needed in order to characterize these calcium channels, their demonstration complements the recent electrophysiological study of Santos-Sacchi and Dilger (1988) which proposed that outer hair cells possess a voltage-dependent Ca^{2+} current.

The aminoglycoside antibiotics, represented here by gentamicin and neomycin, block these voltage-gated calcium channels. Such a block may have several acute consequences on the physiology of the cell. The resting potential of outer hair cells is determined by calcium-activated K^+-channels (Ashmore and Meech, 1986). Thus, the lack of increased intracellular calcium during cell depolarization may reduce this $[K^+]$-outward current and prevent cell hyperpolarization. Secondly, calcium-dependent neurotransmitter release may be impaired. The acute and reversible effects on the cochlear microphonic potential (Takada and Schacht, 1982) may therefore reflect the calcium channel-blocking property of these ototoxic drugs. On the other hand, it should be noted that transduction channels in hair cells are also potential targets for aminoglycoside antibiotics (Hudspeth and Kroese, 1983).

The finding that aminoglycoside antibiotics can behave as Ca^{2+}-channel antagonists is consistent with the blockage of calcium influx through N-channels by these drugs in other excitable cells (Suarez-Kurtz and Reuben, 1987; Knaus et al., 1987). The concentrations of neomycin leading to impairment of calcium influx in hair cells (IC_{50}, approx 50 μM) are similar to those effective in isolated nerve terminals (IC_{50} 30 μM; Atchison et al., 1988).

While gentamicin clearly blocks calcium channels, the distribution of FTC-gentamicin should not be taken to imply the location of the channels. The pattern of calcium influx does not indicate that these channels should be preferentially localized to the base of the cell as is the FTC-gentamicin. Rather, the lack of visualization of bound FTC-gentamicin along the cell body may reflect the sensitivity of the fluorescent drug and suggests that the strong basal fluorescence is contributed by intracellular sites of residual nerve endings. It is, however, intriguing to consider in this context that uptake mechanisms have been suggested to be present at the base of outer hair cells (Lim, 1986).

Importantly, however, it is apparent that aminoglycosides do not readily penetrate cell membranes. The absence of fluorescently labeled gentamicin in intact cells and the lack of apparent changes in the morphology and viability of the outer hair cells in the presence of gentamicin or neomycin fit well with the pattern of aminoglycoside ototoxicity. Clinically, aminoglycoside-induced hearing loss is manifest only after chronic treatment, and in experimental animals the destruction of the sensory cells is delayed although the drugs rapidly enter the fluid and tissue spaces of the inner ear (Tran Ba Huy et al., 1986; Dulon et al., 1986). Futhermore, a slow toxic action of the aminoglycosides has also been observed in cultured fibroblasts and pig kidney epithelial cells in vitro: only after 2 to 3 days were changes in cellular metabolism apparent, involving lysosomal enzymes (Oshima et al., 1986), protein synthesis (Buchanan et al., 1987), and phosphoinositide metabolism (Schwertz et al., 1986). The lack of a direct effect on cell viability suggests that the chronic irreversible ototoxicity requires an additional mechanism, e.g., internalization, whereupon intracellular metabolism becomes the target of these drugs (Schacht, 1986; Williams et al., 1987).

The exclusion of gentamicin from viable cells ap-

Fig. 4. [Out of sequence due to color] Imaging of intracellular calcium with the fluorescent calcium indicator fluo-3. Isolated outer hair cell: **a)** before, **b)** after 30 sec of depolarization (70 mM $[K^+]$), **c)** 30 sec after the application of 10 μM ionomycin, and **d)** 5 min after the addition of 2 mM $MnCl_2$ and lysis of the cell. The intensity of fluorescence of fluo-3 is imaged in pseudocolor. The color scale indicates the corresponding values of calcium concentrations (nM) determined as described in Materials and Methods. Nu and CP indicate the position of the nucleus and the cuticular plate, respectively.

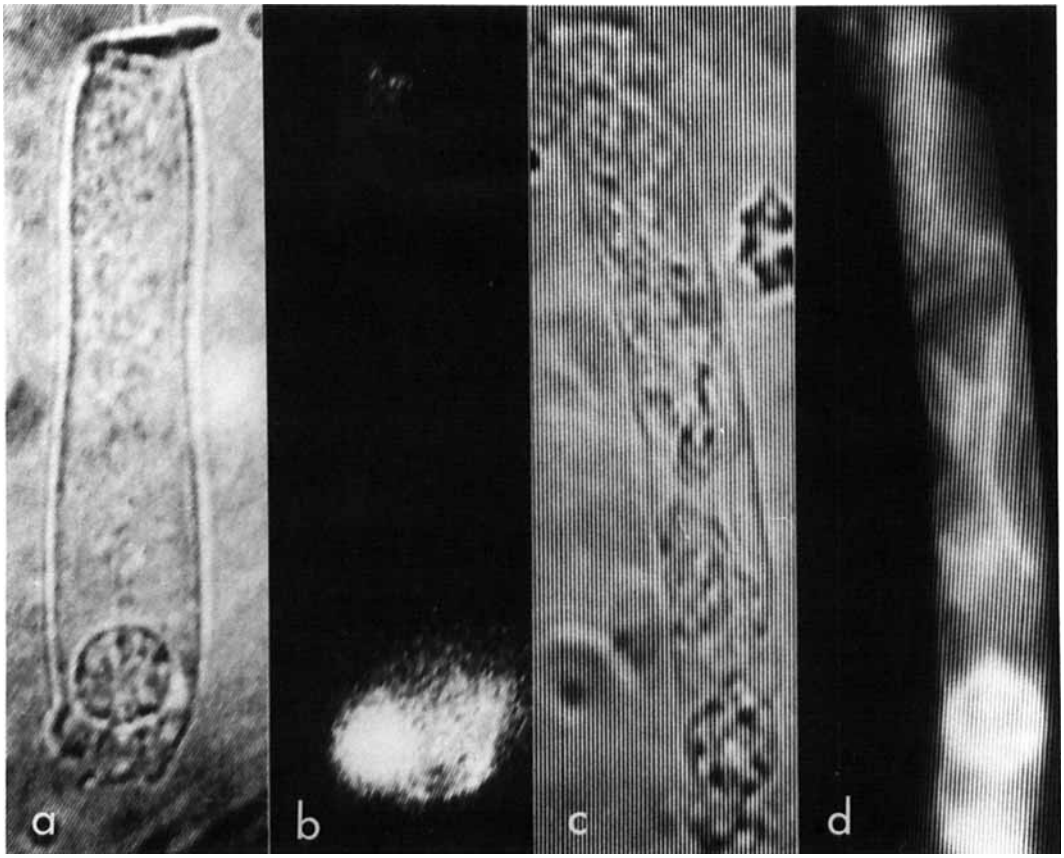

Fig. 3. Uptake of FTC-gentamicin. a,b: Live cell after 3 hr of incubation with 0.2 mM FTC-gentamicin at room temperature, in transmitted light **(a)** and in fluorescence **(b)**. Note that the fluorescence is associated with the synaptic region and the cuticular plate. c,d: Non-viable outer hair cell under similar conditions of incubation with FTC-gentamicin. **c:** Light micrograph shows severe alterations in morphology such as cytoplasmic granulations and loss of birefringence. **d:** Intracellular fluorescence is present.

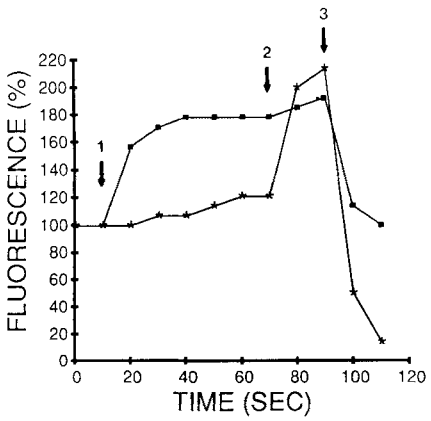

Fig. 5. Kinetics of calcium entry in isolated hair cells. Intracellular free calcium was imaged as described in Materials and Methods in response to $[K^+]$-depolarization in the absence (■) or presence (*) of 1 mM neomycin: 1) addition of HBSS with 70 mM KCl; 2) addition of the calcium ionophore ionomycin (10 μM); 3) addition of the calcium chelator (10 mM EGTA).

pears, however, in contrast to the reported rapid in vitro uptake of radiolabeled gentamicin in isolated outer hair cells (Williams et al., 1987). It is difficult to reconcile this difference, but two possibilities exist. The first is a higher sensitivity of the radioactive method. The other is suggested by the marked presence of FTC-gentamicin in non-viable cells: a confounding contribution by non-viable cells may have existed in the study by Williams et al., (1987).

Another interesting issue of our study concerns the motility of the outer hair cells. Outer hair cells present several types of motile responses to depolarization, one which is triggered by electrical stimulation (Brownell et al., 1985; Kachar et al., 1986; Ashmore, 1987), another by potassium (Zenner et al., 1985; Dulon et al., 1988). Our results clearly show that the presence of the calcium channel blockers, gentamicin and methoxyverapamil, or the absence of extracellular calcium did not inhibit the shortening of the cells under $[K^+]$-depolarization while preventing the rise in intracellular calcium. This is in

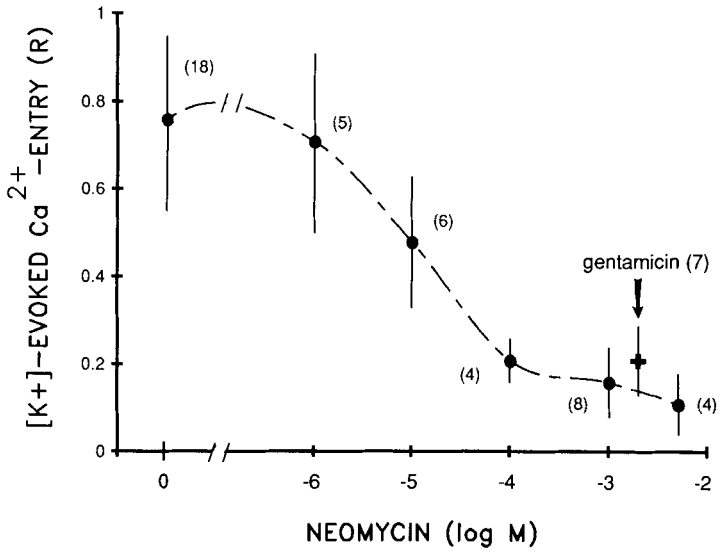

Fig. 6. Effect of the aminoglycoside antibiotics neomycin (●) and 1 mM gentamicin (+) on depolarization-induced calcium uptake in isolated outer hair cells. The intracellular calcium was monitored before and during 60 sec of $[K^+]$-depolarization (70 mM KCl) as described in Materials and Methods with the fluorescent indicator fluo-3. The value of R indicated in the graph represents the relative increase in fluorescence upon depolarization compared to the fluorescence maximum of the intracellular dye: $R = [F_k - F_0] / [F_{max} - F_0]$. F_0 is the radiance value of the cell before stimulation; F_k is the cell radiance measured after 60 sec depolarization; F_{max} is the radiance maximum obtained for each cell by the addition of the calcium ionophore ionomycin (10 μM) added just after the 60 sec depolarizing stimulation. The vertical bars indicate the standard deviation, and the numbers in parentheses are the number of samples studied for each concentration of aminoglycoside.

agreement with Zenner's argument (Zenner et al., 1985) against a muscle-like mechanism for $[K^+]$-induced shape changes and confirms our previous observations that this motile response is calcium-independent and based on an osmotic mechanism (Dulon et al., 1988).

On the other hand, an actomyosin-coupled irreversible contractile process has been postulated in response to the application of calcium and ATP to detergent-permeabilized cells (Zenner, 1986; Flock et al., 1986) or via passive intracellular microinjections (Dulon et al., 1988). In contrast to the arguments by Zenner et al., (1985) and Dulon et al., (1988), such a muscle-like contraction has also been proposed to be responsible for (Slepecky et al., 1988) or at least underlie in part (Ulfendahl, 1988) the $[K^+]$-induced shape changes. If this were the case, calcium influx should enhance the $[K^+]$-induced axial shortening by activating a calcium-dependent contraction. Calcium influx, however, exerted the opposite effect: it counteracted the shortening of the cells since a larger amplitude of shortening was observed when the intracellular rise of calcium was prevented by channel blockers or the absence of extracellular calcium. Consistent with this observation, the entry of calcium through the ionophore ionomycin elongated rather than shortened the cells. This novel effect of calcium, implying its involvement in elongation (i.e., cortical contraction) rather than axial contraction of outer hair cells, will require further detailed investigations.

In summary, our observations describe the presence of voltage-activated calcium channels in outer hair cells and suggest that their block by aminoglycoside antibiotics may be part of the acute ototoxic mechanisms of these drugs. They also suggest that drug entry into hair cells may be a rate-limiting step in chronic aminoglycoside toxicity. Furthermore, our results not only confirm the calcium independence of $[K^+]$-induced hair cell motility but imply a new role for calcium in inducing a cortical contraction in outer hair cells.

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