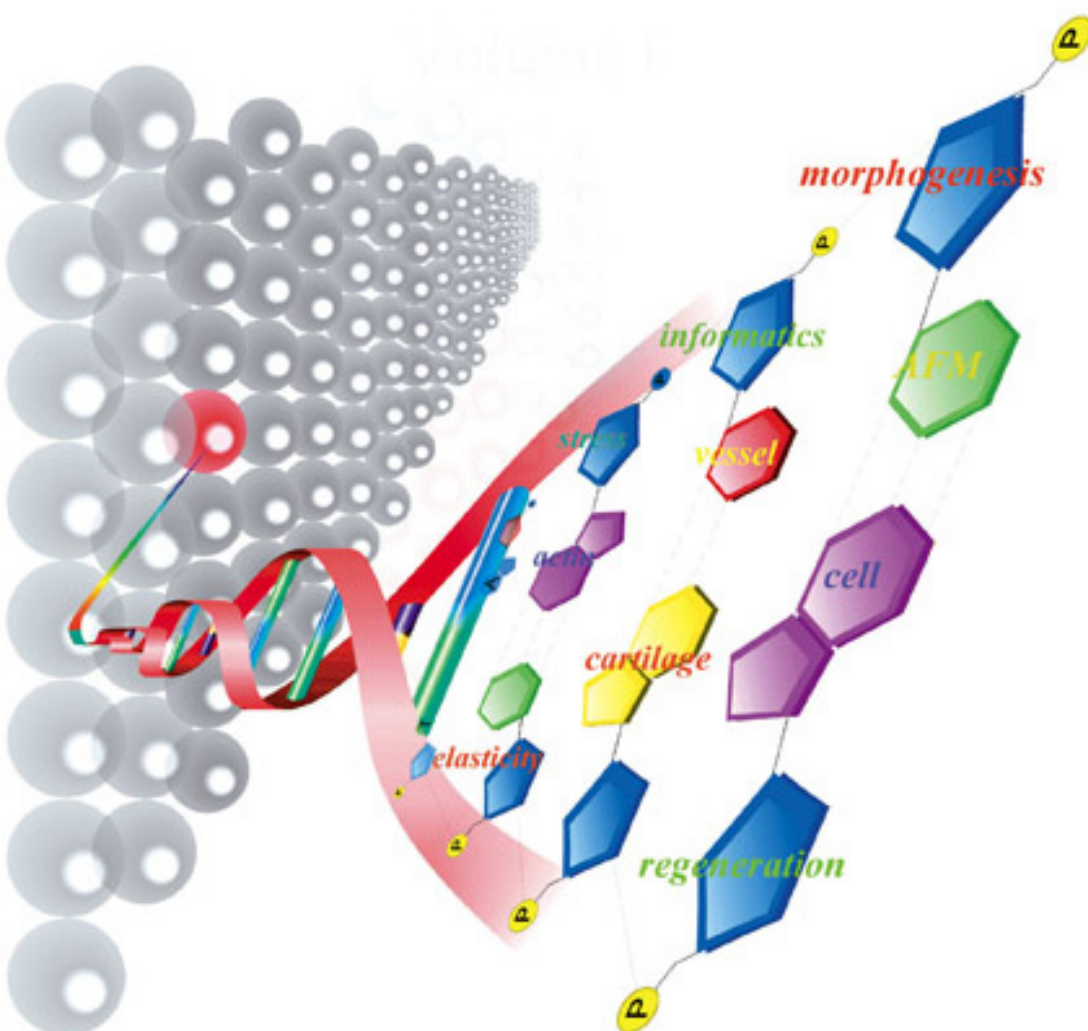


BIOMECHANICS AT MICRO- AND NANOSCALE LEVELS

Selected Papers from the Proceedings of the Fifth World Congress of
Biomechanics, Thread 3: Biomechanics at Micro- and Nanoscale Levels

VOL. III



Hiroshi Wada *editor*

BIOMECHANICS AT MICRO- AND NANOSCALE LEVELS

VOLUME III

Biomechanics at Micro- and Nanoscale Levels

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(Tohoku University, Sendai, Japan)

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Volume III

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PREFACE

A project on “Biomechanics at Micro- and Nanoscale Levels,” the title of this book, was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan in 2003, and this four-year-project is now being carried out by fourteen prominent Japanese researchers. The project consists of four fields of research, namely, Cell Mechanics, Cell Response to Mechanical Stimulation, Tissue Engineering, and Computational Biomechanics.

Our project can be summarized as follows. The essential diversity of phenomena in living organisms is controlled not by genes but rather by the interaction between the micro- or nanoscale structures in cells and the genetic code, the dynamic interaction between them being especially important. Therefore, if the relationship between the dynamic environment of cells and tissues and their function can be elucidated, it is highly possible to find a method by which the structure and function of such cells and tissues can be regulated. The first goal of this research is to understand dynamic phenomena at cellular and biopolymer-organelle levels on the basis of mechanics. An attempt will then be made to apply this understanding to the development of procedures for designing and producing artificial materials and technology for producing or regenerating the structure and function of living organisms.

At the 5th World Congress of Biomechanics held in Munich, Germany from 29th July to 4th August, 2006, we organized the following sessions:

Thread 3: Biomechanics at micro- and nanoscale levels

1. Cell mechanics
2. Molecular biomechanics
3. Mechanobiology at micro- and nanoscale levels
4. Computational biomechanics

We are planning to publish a series of books related to this project, the present volume being proceedings covering topics related to the sessions we organized. I would like to express my sincere gratitude to Professor Dieter Liepsch, the President of the 5th World Congress of Biomechanics, who granted us permission to publish these proceedings, as well as to the ten researchers who contributed to these proceedings.

Hiroshi Wada, PhD,
Project Leader,
Tohoku University,
Sendai,
December, 2006



5th World Congress of Biomechanics



FOREWORD

Munich, December, 2006

This volume represents the finest research of some of the best investigators in the world and I am very honored that these important and ground-breaking papers were presented at the 5th World Congress of Biomechanics in Munich, in 2006.

Between July 31st and August 4th, 2006, nearly 2600 scientists and 900 students from 64 countries, representing every continent, came to Munich, Germany to listen, learn and share their latest work in all fields of biomechanics. It quickly became obvious the newest trends lay in the fields of tissue and cellular biomechanics. It was also clear that the field was so rich and multi-faceted that it warranted an orthogonal ‘thread’ which would cut across all the various fields of research, showing the connections, interrelations and interdependencies. Under the guidance of Prof. Dr. Hiroshi Wada, Thread 3 “Biomechanics at micro- and nanoscale levels” incorporated cell mechanics, molecular biomechanics, mechanobiology at micro- and nano-scale levels and computational biomechanics.

When Prof. Hiroshi Wada first raised the possibility of incorporating micro- and nanoscale biomechanics into the 5th World Congress of Biomechanics, I knew that without this contribution, we could not hope to present to the international community the full scope and richness of this increasingly vital field of research. It was my pleasure and honor to be able to present to the international scientific world and to our students, the work of the scientists represented in this volume. This volume is the work of dedicated scientists who have earned the respect and esteem of their peers around the world and who have opened the door to new vistas of research for a new generation of students and young investigators. I am grateful to have played a small part in bringing this work to the international scientific community.

With best regards

Dieter Liepsch
Congress President



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I. CELL MECHANICS

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THE EFFECT OF STREPTOMYCIN AND GENTAMICIN ON OUTER HAIR CELL MOTILITY

B. CURRALL, X. WANG AND D. Z. Z. HE

*Department of Biomedical Sciences, Creighton University School of Medicine
Omaha, Nebraska, 68178, USA
E-mail: hed@creighton.edu*

The cochlear outer hair cell (OHC), which plays a crucial role in mammalian hearing through its unique voltage-dependent length change, has been established as a primary target of the ototoxic activity of aminoglycoside antibiotics. Although the ototoxicity eventually leads to hair cell loss, these polycationic drugs are also known to block a wide variety of ion channels such as mechanotransducer channels, purinergic ionotropic channels and nicotinic ACh receptors in acute preparations. The OHC motor protein prestin is a voltage-sensitive transmembrane protein which contains several negatively charged residues on both intra- and extracellular surface. The acidic sites suggest that they may be susceptible to polycationic-charged aminoglycoside binding, which could result in a disruption of somatic motility. We attempted to examine whether aminoglycosides such as streptomycin and gentamicin could affect the mechanical response of OHCs. Solitary OHCs isolated from adult gerbils were used for the experiments. Somatic motility and nonlinear capacitance were measured under the whole-cell voltage-clamp mode. Streptomycin and gentamicin were applied extracellularly or intracellularly. Results show that streptomycin and gentamicin, for the concentration range between 100 μ M and 1 mM, did not affect somatic motility or nonlinear capacitance. The result suggests that although streptomycin and gentamicin can block mechanotransduction channels as well as ACh receptors in hair cells, they have no immediate effect on OHC somatic motility.

1 Introduction

Aminoglycosides are low cost, high efficacy antibiotics, however, their use is limited by their nephrotoxic and ototoxic activity. Several toxic mechanisms have been associated with aminoglycosides. In genetically susceptible individuals, a mitochondrial mutation for an rRNA may be vulnerable to aminoglycoside interference [1]. It has also been shown that *N*-methyl-D-aspartate (NMDA) receptor, found in afferent neurons, may be affected by aminoglycosides, resulting in excitotoxicity followed by hair cell death [2]. Also, upon entry into the cell, whether via vesicle-mediated process [3] or the mechanoelectrical transduction channel [4], reactive oxygen species, free radicals and nitric oxide form, resulting in multiple signaling pathways that may lead to subsequent cell death [5-8]. While nephro- and ototoxicity seem to depend on intracellular accumulation of these antibiotics [9-10], numerous studies have demonstrated the ability of these polycationic drugs to acutely depress synaptic transmission at the neuromuscular junction, presumably by blocking presynaptic voltage-gated Ca^{2+} channels [11-12]. These polycationic drugs also block a wide variety of ion channels such as

mechanosensitive ion channels [13-14], purinergic ionotropic channels [15] and nicotinic ACh receptors [16-17].

In the cochlea, the ototoxic activity of aminoglycosides is characterized by a loss of outer hair cells (OHCs). The OHC is one of two receptor cells in the organ of Corti, and plays a critical role in mammalian hearing. OHCs are able to rapidly change their length [18-19] and stiffness [20] at acoustic frequencies when their transmembrane potential is altered. This fast somatic motility is believed to be the substrate of cochlear amplification [18, 21]. OHC electromotility is driven by voltage-sensitive molecules (or assemblies of molecules) able to change area when the membrane potential is altered. Both the motor and its sensor are located in the plasma membrane [22-24]. Recently, the gene *prestin* that codes the motor protein was identified [25]. The targeted deletion of prestin in mice results in the loss of electromotility *in vitro*, and a 40-60 dB loss in cochlear sensitivity *in vivo* [21]. The protein prestin is a voltage-sensitive transmembrane protein which contains several negatively charged residues on both intra- and extracellular surface [26]. The acidic sites suggest that they may be susceptible to aminoglycoside binding [27], which could result in a disruption of somatic motility. Therefore, the purpose of this study was to determine whether the aminoglycosides such as streptomycin and gentamicin could affect the mechanical response of OHCs.

2 Materials and Methods

2.1 Preparation of isolated OHCs

Gerbils (*Meriones unguiculatus*) ranging in age between 4 and 8 weeks were anesthetized with an intraperitoneal injection of a lethal dose of sodium pentobarbital (150 mg/kg) and then decapitated. Cochleae were dissected out and kept in cold culture medium (Leibovitz's L-15). L-15 (Gibco) was supplemented with 10 mM HEPES (Sigma) and adjusted to 300 mOsm and pH 7.4. After the cochlear wall was removed, the BM-organ of Corti complex was unwrapped from the modiolus from the base to the apex. The organ of Corti was dissected out from the apical turn of the cochlea. The tissue was then transferred to the enzymatic digestion medium [L-15 supplemented with 1 mg/ml collagenase type IV (Sigma)]. After 10 minutes incubation at room temperature ($22\pm 2^\circ\text{C}$), the tissue was transferred to the experimental bath containing fresh L-15 medium. To obtain solitary OHCs, gentle trituration of the tissue with a small pipette was applied. A cell was selected for experimentation only if its diameter was approximately constant throughout its length and if it showed no signs of damage, such as swelling, blebbing, and dislocation of the nucleus. Cells were rejected if visible signs of damage and appearance changes occurred during the experiment.

2.2 Whole-cell voltage-clamp recording

Isolated OHCs were placed in the experimental chamber containing extracellular

fluid (pH 7.2, 320 mOsm, in mMol: NaCl₂ 120, TEA-Cl 20, CoCl₂ 2.0, MgCl₂ 2.0, CaCl₂ 1.5, HEPES₂ 10, Glucose 5.0) on the stage of an inverted microscope (Olympus IX-71). The patch electrodes were pulled from 1.5 mm glass capillaries (A-M System) using a Flaming/Brown Micropipette Puller (Sutter Instrument Company, Model P-97). The electrodes were back-filled with solution containing (in mM) CsCl 140; CaCl₂ 0.1; MgCl₂ 3.5; Na₂ATP; 2.5; EGTA-KOH 5; HEPES-KOH 10. The solution was adjusted to pH 7.4 with CsOH (Sigma) and osmolality adjusted to 300 mOsm with glucose. These solutions enabled to block K⁺ and Ca²⁺ conductances to isolate gating currents associated with somatic motility. The pipettes had initial bath resistances of 2-4 MΩ. The access resistance, that is, the actual electrode resistance obtained upon establishment of the whole-cell configuration, typically ranged from 6 to 12 MΩ. Series resistance was corrected off-line after data collection. Aminoglycosides were applied either extracellularly, with aminoglycosides placed in separate perfusion pipette, or intracellularly, with aminoglycosides placed in the patch pipette. Extracellular perfusion pipettes were placed within 60 μm of cell, after achieving whole-cell configuration, and perfusion started by opening gravity fed T-tubule switch. Streptomycin sulfate, and gentamicin sulfate were diluted to concentration specified in figures captions.

2.3 Somatic motility measurements

Somatic motility was measured and calibrated by photodiode-based measurement systems [28] mounted on the Olympus inverted microscope. The OHC was imaged using a 40x objective and magnified by an additional 20x relay lens. The magnified image of the edge of the cell was then split into two paths: one path projected onto the photodiode (Hamamatsu) through a slit and another projected onto a CCD camera so that the edge of the cell could be viewed at all times on a television monitor. During measurements, the magnified image of the edge of the cell was positioned near the edge of the slit. The slit was rotated, based on the orientation of the cell. The photodiode system had a cutoff (3-dB) frequency of 1,200 Hz. The signal was then amplified by a 60-dB fixed-gain dc-coupled amplifier. The amplified signal was then low-pass filtered (400 or 1,100 Hz) before being delivered to one of the A/D inputs of a Digidata (1322A, Axon Instruments) acquisition board in a Window-based PC. The measurement system was capable of measuring motions down to ~5 nm with 100 averages. Calibration was performed by moving the slit a known distance (1 μm).

2.4 Nonlinear capacitance measurements

The AC technique was used to obtain motility-related gating charge movement and the corresponding NLC. This technique has been described in details elsewhere [29]. In brief, it utilized a continuous high-resolution (2.56 ms sampling) two-sine voltage stimulus protocol (10 mV peak at both 390.6 and 781.2 Hz), with subsequent fast Fourier transform-based admittance analysis. These high frequency sinusoids were superimposed on voltage ramp stimuli. The NLC can be described

as the first derivative of a two-state Boltzmann function relating nonlinear charge movement to voltage [30-31]. The capacitance function is described as:

$$C_m = \frac{Q_{\max} \alpha}{\exp[\alpha(V_m - V_{1/2})](1 + \exp[-\alpha(V_m - V_{1/2})])^2} + C_{lin}$$

where, Q_{\max} is maximum charge transfer, $V_{1/2}$ is the voltage at which the maximum charge is equally distributed across the membrane, C_{lin} is linear capacitance, and $\alpha = ze/kT$ is the slope factor of the voltage dependence of charge transfer where k is Boltzmann's constant, T is absolute temperature, z is valence, and e is electron charge. Capacitive currents were filtered at 2 kHz and digitized at 10 kHz using jClamp software (SciSoft Company), running on an IBM-compatible computer and a 16-bit A/D converter (Digidata 1322A, Axon Instruments).

3 Results

3.1 Extracellular application of streptomycin and gentamicin

OHC motility was measured from isolated cells before and after streptomycin and gentamicin were applied to the extracellular solution through a puffer pipette positioned 60 μm away from the cells. The cells were held at -70 mV and voltage steps varying from -120 mV to 60 mV were used to evoke motility. Fig. 1 shows examples of two OHCs before and 2-minutes after 100 μM streptomycin and gentamicin were applied. The motile response was asymmetric, with contraction being larger than the elongation. The response was also nonlinear, with saturation at both directions. We measured a total of 10 cells (5 cells each) for streptomycin and gentamicin at the concentration of 100 μM . Streptomycin and gentamicin did not change the magnitude nor the asymmetry of the response as shown in Fig. 1.

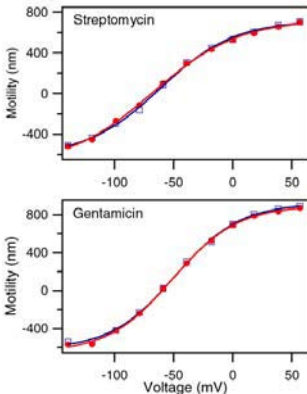


Figure 1. Voltage to length change function measured before (in black) and 2-minutes after 100 μM streptomycin and gentamicin were applied (in red). The cells were held at -70 mV under whole-cell voltage-clamp mode. Voltage steps varying from -120 mV to 60 mV were applied to evoke motility. Motility magnitude was measured from the steady-state responses. Voltage error due to series resistance was compensated. Note that neither the magnitude nor the response characteristics were changed after the treatment.

Associated with the OHC electromotility is an electrical signature, a voltage-dependent capacitance or, correspondingly, a gating charge movement [30-31], similar to the gating currents of voltage-gated ion channels [32]. The gating currents are thought to arise from a redistribution of charged voltage sensors across

the membrane. This charge movement imparts a bell-shaped voltage dependence to the membrane capacitance [30-31]. Measures of nonlinear capacitance (NLC) have been used to assay OHC's motor function [25, 31]. We measured NLC before and after streptomycin and gentamicin were applied to the cells. Fig. 2 shows some representative responses from two OHCs. NLCs were measured before and 2-minutes after 100 μ M streptomycin and gentamicin were applied through a perfusion pipette positioned 60 μ m away from the cells. As shown, the capacitance

function was bell-shaped with respect to stimulating voltage. The NLC exhibited a peak around -50 mV for both cells showed in the example. As shown, the magnitude of the peak capacitance did not change significantly after the treatment. We compared the maximum charge transfer (Q_{max}), $C_{non-lin}$, and slope factor (α). None of them changed significantly after the treatment.

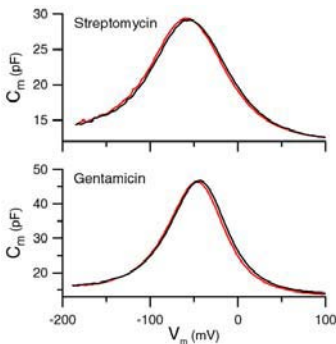
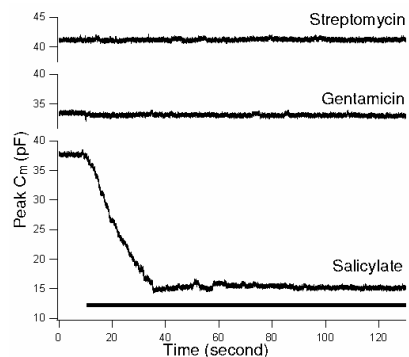


Figure 2. Capacitance measured from two OHCs before (in black) and 2-minutes (in red) after 100 μ M streptomycin and gentamicin were applied to the extracellular solution. Note that neither the magnitude of the peak capacitance nor the $V_{1/2}$ changed after perfusion.

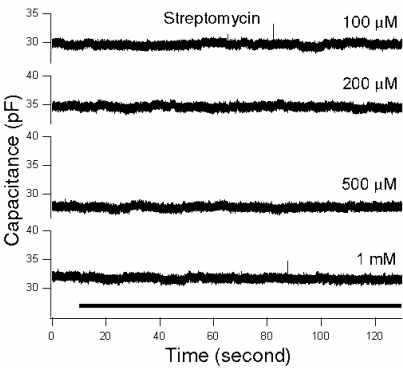
The changes in peak capacitance ($C_{m_{pk}}$) have been used to examine the effects of certain agents on OHC motility [33]. We also monitored $C_{m_{pk}}$ during application of streptomycin and gentamicin to further determine their influence on motility. For positive control, we monitored the change in $C_{m_{pk}}$ after salicylate was applied extracellularly. Salicylate is known to significantly reduce OHC somatic motility and NLC [26, 33]. $C_{m_{pk}}$ was monitored using the software in the jClamp (version 12.1) package over the course of 2 to 4 minutes after aminoglycosides or salicylate was applied. Fig. 3 shows an example of such recordings. As shown, 5 mM salicylate caused significantly reduction in $C_{m_{pk}}$. However, neither streptomycin nor gentamicin had any affect on $C_{m_{pk}}$.

Figure 3. Peak capacitance ($C_{m_{pk}}$) monitored after streptomycin and gentamicin was applied to the extracellular solution. For positive control, 5 mM salicylate was applied. The cells were held at -40 mV under whole-cell voltage-clamp condition. $C_{m_{pk}}$ was measured using the software in the jClamp package. Bar indicates the duration that streptomycin was perfused. Note that $C_{m_{pk}}$ was significantly reduced after 5 mM salicylate was applied. However, neither streptomycin nor gentamicin had any affect on $C_{m_{pk}}$.



While it has been demonstrated that 100 μ M streptomycin or gentamicin is enough to block mechanoelectrical transducer current as well as ACh receptor in

hair cells, we inquired whether it requires even higher concentration to be effective to affect OHC somatic motility. Peak capacitance was monitored using the software



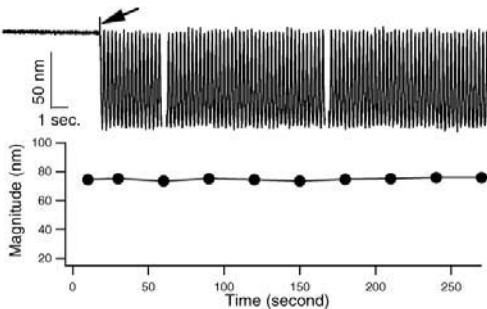
in the jClamp package over the course of experiments when streptomycin with concentration of 0.1, 0.2, 0.5 and 1 mM was applied. Fig. 4 illustrates the peak capacitance measured from OHCs in response to different concentrations of streptomycin applied to the extracellular solution. As shown, the peak capacitance did not change for all the concentrations applied.

Figure 4. Peak capacitance monitored with different concentration of streptomycin applied to the extracellular solution. Peak capacitance was measured using the software in the jClamp package. Bar indicates the duration when streptomycin was perfused.

3.2 Intracellular application of streptomycin

Aminoglycosides can enter hair cells through the open mechanotransducer channels [4]. So we inquired whether aminoglycosides disturbed somatic motility when they were applied intracellularly. We examined such possibility by monitoring somatic motility immediately after rupturing the cells and throughout the entire course when the streptomycin (together with normal intracellular solution) in the patch electrode diffused to the cytosol of the cells. Fig. 5 illustrates an example of such recordings from a gerbil apical turn OHC. The cell was held at -40 mV and a 5 Hz sinusoidal voltage command with peak-to-peak amplitude of 30 mV was continuously applied to the cell to evoke motility. Motility was measured using a photodiode-based displacement system. Since the streptomycin in the patch electrode diffused to the cytosol took time (normally it would take 20 to 30 second to equilibrate), the motility measured immediately after rupturing was used as control [34]. Motility

Figure 5. Motility measured after streptomycin was applied intracellularly through the patch electrode. Arrow indicates the moment when the cell's membrane was ruptured and streptomycin started to diffuse to the cytosol of the cell. The cell was held at -40 mV and 5 Hz sinusoidal voltage stimulus with peak-to-peak magnitude of 30 mV was continuously delivered to the cell to evoke motility. Three representative responses in the top panels were acquired at 10 seconds before and after the cell was ruptured, and at 30 and 200 seconds after the cell was ruptured. Steady-state responses (peak-to-peak) at different moments during perfusion were measured and plotted in the bottom panel.



was observable immediately after the cell was ruptured. We measured the magnitude of motility at different times during perfusion and the magnitude of motility is plotted in the bottom panel of Fig. 5. As shown, the magnitude of motility remained basically the same throughout the course of equilibrium. This suggests that motility is not affected by intracellular application of streptomycin.

4 Discussion

Aminoglycosides are large, lipid insoluble, polycationic molecules that are known to block a variety of ion channels including large-conductance Ca^{2+} -activated K^+ channels [35], Ca^{2+} channels [36] and ryanodine receptors [37]. In hair cells, aminoglycosides have been reported to block transducer channels [13-14], ATP receptors [15], nicotinic acetylcholine receptors [16] and large-conductance Ca^{2+} -activated K^+ channels [16]. Aminoglycosides have a strong propensity to associate with negatively charged lipid bilayers [38] and to compete at Ca^{2+} binding sites on the plasma membrane of OHCs [39].

Contrary to our expectations that streptomycin or gentamicin would be able to screen a significant proportion of fixed negative charges in prestin, we saw no reduction in either NLC or somatic motility by gentamicin or streptomycin. Though 100 μM of either streptomycin or gentamicin has been found potent enough to block mechanotransducer channels and ACh receptors (cite), we found no influence on somatic motility despite concentrations as high as 1 mM. We monitored the change in peak capacitance for over 4 minutes, long enough to see the effect if any. It is possible that such screening effect on negative charges do not affect the function of prestin.

Aminoglycosides enter hair cells via vesicle-mediated process [3] or the mechano-electrical transduction channel [4]. Since the hair bundle is usually damaged in isolated OHCs, it is possible that the concentration of streptomycin or gentamicin inside the cell was too low to produce any effect. However, since we also did not see any effect when streptomycin was applied intracellularly, such possibility could be ruled out.

Despite the high concentrations used, this study does not eliminate the possibility that aminoglycosides may have some effect on somatic motility in the long term. Hearing loss as a result of aminoglycoside dosage is delayed and may require an accumulation of aminoglycosides over a period of time [3]. A recent paper also suggests that the MET may act as a one-way valve for aminoglycosides, resulting in high concentration of cytosolic aminoglycosides [4]. Although it is possible that higher concentrations of aminoglycosides may accumulate inside the cells which may disturb motility through secondary processes, therapeutic levels of aminoglycosides are expected to be well below the concentrations used in this study. This study suggests that aminoglycosides do not have any immediate or direct effect on OHC somatic motility.