Characterizing the Binding Mechanism of Fixed Sites on the Isolated Tectorial Membrane of the Mouse

by

Rosanne Rouf

Submitted to the Department of Electrical Engineering and Computer Science

in partial fulfillment of the requirements for the degree of

Master of Engineering in Electrical Engineering and Computer Science

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

July 1997

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ABSTRACT

Previous research has shown that the tectorial membrane (TM) swells in isosmotic bathing solutions when sodium is substituted for potassium. In an effort to determine whether sodium or potassium binds to the TM, solution exchange protocols were designed based on a polyelectrolyte gel model recently developed for the TM. Changes in the thickness and structure of the isolated tectorial membrane (TM) of the mouse were measured in response to isosmotic changes in the ionic composition of the bathing solution. Small changes in potassium concentration in the high potassium concentration range (174-134 mmol/L) resulted in larger changes in the thickness of the TM than those changes made in the low potassium concentration range (44-4 mmol/L). Changing the bathing solution from 174 mmol/L potassium to 89 mmol/L potassium and back results in a greater percent change in thickness of the TM than does changing the bathing solution from K89 mmol/L potassium to 4 mmol/L potassium. The results from these experiments suggest that the binding mechanism is a sodium binding mechanism, though analysis is confounded by slow responses, long experiments, and small excursions.

Thesis Supervisor: Dennis Freeman

Title: Assistant Professor of Electrical Engineering

Acknowledgments

First, I would like to thank Professor Denny Freeman for being patient and helpful this past year. Denny was always approachable and very willing to take the time to help me with my setup and explain things to me. I also appreciated his inspiring stories and sense of humor. He had a remarkable way of bringing our lab group together. It helped make my research experience a very enjoyable and memorable one.

Professor Tom Weiss's advice throughout my thesis has also been very helpful. His enthusiasm about my project was often times contagious. He was always interested in what I was doing with my research and eager to meet with me and Denny to discuss my progress. Our weekly meetings and brain-storming sessions with Denny were very productive, and I would like to thank him for convincing me they were a good idea.

I would also like to thank my labmates for their enormous amount of help in the lab. I thank Cameron for sharing all his surgery techniques with me, A.J. for teaching me how to get around the network, Zohair, for showing me how to install software, Quentin and Laura for helping me write gawk programs and shell scripts, Merm for waiting for me to finish up before moving me out of my lab, and Anthony for moral support. I also thank them for being as strange as me. It definitely made for interesting conversations, and I thank them all for sharing their cynicism, humor, and uniqueness with me.

I also thank my roommate, Kinu, for letting me keep our room a mess while I was working on my thesis and for putting up with my strange sleeping hours. Most of all, I would like to thank Sham for making me eat at least once every day, running errands for me, and in general, being supportive in my "crunch" period. The aesthetics of this thesis is largely credited to him, and without his help, I probably would not have survived these past couple of weeks.

Lastly, I thank my parents, brother, and sister, for being supportive throughout my M.I.T. career. Yes, Dad, I am finally finished.

Contents

Chapter 1

Introduction

The tectorial membrane (TM), found in the inner ear, is a connective tissue whose function is poorly understood. Recent research has revealed that the TM swells in isosmotic bathing solutions when sodium is substituted for potassium (Freeman et al., 1994; Shah et al., 1995; Weiss and Freeman, 1997). Two possible mechanisms have been proposed to explain this phenomenon. Potassium ions may bind to negative charges that are fixed on the macromolecules in the TM, or sodium ions may bind to neutral sites that are fixed on the TM (Weiss and Freeman, 1997). If ions from the bathing solution were to bind to the membrane, the fixed charge on the membrane would change. This in turn would cause a change in the concentration of mobile ions in the membrane in order to 0 bulk electroneutrality. As a consequence, the osmotic pressure in the TM would change, causing water to flow into or out of the TM to establish osmotic equilibrium— thus effecting the appearance of shrinking or swelling in isosmotic solutions. In an effort to determine which of the two cations, sodium or potassium, binds to the TM, I have based my experimentation on a proposed TM model.

1.1 The tectorial membrane—a physiological description

The mammalian tectorial membrane is a highly hydrated acellular matrix that lies in direct contact with the hair bundles of outer hair cells (Figure 1-1). The TM is immersed in endolymph, an ionic solution with a high potassium concentration, low sodium concentration, calcium, and uncharged solutes. Despite the TM's prominent position in the path of mechanical to electrical transduction in hearing, little experimental research has been done on the TM. The TM is microscopic, fragile, and nearly transparent— complicating its

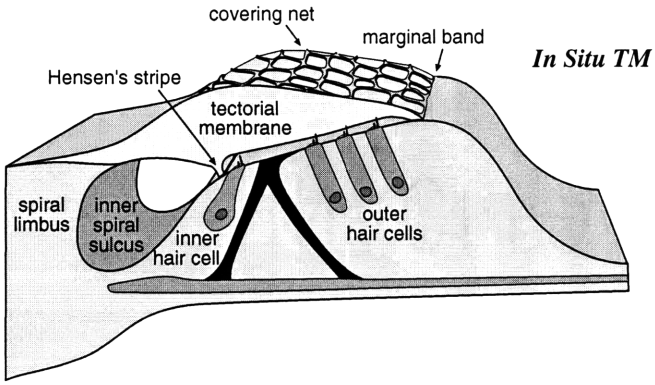

Figure 1-1: Schematic diagram of tectorial membrane in situ in inner ear. *In situ* the TM is attached to the spiral limbus and overlays the outer hair cells in the organ of Corti.

dissection and isolation. Thus, the TM's role in hearing has remained an enigma. Until recently, the most popular theory of the TM's function was that it played a purely mechanical role in sensory stimulation, acting as a load against which the hair bundles sheared. Such shearing was thought to open ion-gated channels in the hair bundles, thereby triggering an action potential. However, new evidence suggests that the TM might have a much more direct role in hearing. Because the TM is highly hydrated and has many charge groups, its material 0 are likely to depend on the composition of the bath which surrounds it— which may change with stimulus conditions or in pathologies.

1.2 Composition of the tectorial membrane

The TM contains about 97% water. The wet weight of the mouse TM is about 50μg, and its dry weight is about 1.5μg (Richardson et al., 1987). Fifty percent of the dry weight is protein, of which 40% is collagen type II (Thalmann et al., 1986). Fifteen to twenty percent of the TM dry weight is composed of glycosaminoglycans (GAGs), such as chondroitin sulfate and keratin sulfate (Thalmann et al., 1993). Structurally, the TM is made of radial fibers of two types: Type A protofibrils composed of collagen; and Type B protofibrils composed of proteoglycans (Kronester-Frei, 1978). Type A protofibrils form the radial structure of the TM that can be seen under light microscopy. They are straight, unbranched bundles of fibers which are 10 nm in diameter. Type B protofibrils form the matrix in which the Type A protofibrils are enmeshed. They are coiled, branched striated sheets of fibers that are 7 nm in diameter (Hasko and Richardson, 1988).

1.3 General connective tissues

The TM is a connective tissue. Connective tissues are composed of a network of insoluble proteins (such as collagen or elastin), soluble polysaccharides (such as GAGs), small solutes, ions and water (Grodzinsky, 1983). GAGs are polymers of disaccharides that combine to form larger molecules called proteoglycans, which in the TM, make up the underlying matrix. Each GAG contains 1-4 anionic charge groups which may be carboxyl groups, sulfate groups, or both. At physiological pH, these charge groups are ionized and impart fixed negative charges on the tissue. Fixed charges electrostatically attract mobile ions of opposite charge from the bath environment to achieve bulk electroneutrality, increasing the osmotic pressure inside the gel. This induces water to flow into the TM, and the tissue to swell.

As a result of such phenomena, connective tissues have been thought to resemble polyelectrolyte gels (Tanaka, 1981). Polyelectrolyte gel models have been very useful in interpreting physicochemical properties of a number of connective tissues, including cartilage, the cornea stroma, and the vitreous body (Weiss and Freeman, ress). Such gel models particularly emphasize the interactions between fixed charges and ions, and thereby provide links between molecular architecture, biochemical composition, and the material properties of gel-like tissues (Rička and Tanaka, 1984). Because the TM acts much like a polyelectrolyte gel (Weiss and Freeman, ress), a gel model has been developed for interpreting the osmotic and electrical responses of the TM.

Chapter 2

Binding Mechanism Theory

2.1 The gel model

A gel model of the tectorial membrane has recently been developed (Weiss and Freeman, ress). The model assumes that the the gel is immersed in an electrolytic bath of charged ions and uncharged solutes which resembles the composition of endolymph. The gel is also assumed to have macromolecules which have fixed ionizable groups. Based on the concepts of bulk electroneutrality, diffusive equilibrium, and osmotic equilibrium, the gel responds to the ionic environment in which it is immersed. To achieve bulk electroneutrality, fixed charges in the gel must be neutralized by soluble counterions in the bath. As soluble ions move into or out of the gel, the osmotic pressure changes. To achieve osmotic equilibrium, water flows between the gel and bath until the net flow is zero. Thus, changing the osmolarity, ionic strength, and composition of the surrounding bath, will cause the gel to adjust towards a new equilibrium in which all requirements are satisfied.

Given the above model, it stands to reason that changing the concentration of the fixed charge on the gel can also cause the gel to adjust to a new equilibrium. A change in fixed charge concentration on the gel can result from the binding of ions in the bath to fixed sites. With past observations in shrinking and swelling experiments, we have noticed two phenomena when isosmotic solutions perfuse the TM: the addition of sodium (with accompanying subtraction of potassium) causes the TM to swell; and the addition of potassium (with accompanying subtraction of sodium) causes the TM to shrink (Freeman et al., 1994; Shah et al., 1995). Assuming that the TM may act like the polyelectrolyte gel model described, the most likely hypotheses to explain the observed behavior are as follows:

1) the sodium-binding hypothesis: The TM is neutrally charged in physiological pH, but becomes more positively charged with the binding of sodium to neutral sites on the TM. With this increased positive fixed charge, the TM attracts mobile ions into the TM to counter the increased fixed charge. This thereby increases the osmotic pressure in the TM causing water to flow in. Thus, when we increase the concentration of sodium in the bathing solution, more sodium binds to the TM, increasing fixed charge, and thus causing it to swell.

2) the potassium binding hypothesis: The TM is negatively charged in physiological pH, but becomes less negatively charged with the binding of potassium to negative sites on the TM. With this decrease in negative fixed charge, the TM needs fewer mobile ions to counter the charge, and these ions leave the TM. This decreases the osmotic pressure in the TM, causing water to flow out. Thus, when we increase the concentration of potassium in the bathing solution, more potassium binds to the TM, decreasing the fixed charge, and causing it to shrink.

The difficulty in determining which binding mechanism actually occurs lies in the fact that in past protocols, changes in potassium concentrations accompanied equal but opposite changes in sodium concentrations, in an effort to make the solutions isosmotic and equal in ionic strength. These protocols involved making a transition from a highly concentrated potassium solution to a new solution which had the same concentration of sodium. Thus previous research did not indicate clearly if the TM responds to solutions as a result of a sodium binding mechanism, a potassium binding mechanism, or perhaps a dual binding mechanism since both explanations 1) and 2) could fit the observations.

2.2 Quantitative analysis of binding model

The polyelectrolyte gel model proposed by Weiss and Freeman offer two quantitative ways to examine the potassium and sodium binding mechanisms. If potassium were to bind to negatively charged sites, the following chemical equilibrium would hold:

$$\mathrm{FK} \longleftrightarrow \mathrm{F}^- + \mathrm{K}^+ \tag{2.1}$$

where K^+ is the mobile potassium cation, and F^- is the fixed charge on the TM. FK is the bound complex which is neutral in charge. The dissociation constant K_K is

$$K_K = \frac{C_{F^-}C_{K^+}}{C_{FK}}. \tag{2.2}$$

During equilibrium, the volume of the gel is constant so the total concentration of ionizable fixed sites, C_T, is also constant, where

$$C_T = C_{F^-} + C_{FK}. \tag{2.3}$$

Combining Eq. 2.2 with Eq. 2.3 results in

$$C_{F^-} = C_T \frac{K_K}{K_K + C_{K^+}} \tag{2.4}$$

where C_{K^+} is the concentration of the potassium cation in the bathing solution.

If sodium ions were to bind to neutral sites, similar relationships would hold:

$$FNa^+ \longleftrightarrow F + Na^+ \tag{2.5}$$

where Na^+ is the mobile sodium cation, and F is the fixed neutral charge on the TM. FNa^+ is the bound complex which is positive in charge. The dissociation constant K_{Na} at equilibrium is

$$K_{Na} = \frac{C_F C_{Na^+}}{C_{FNa^+}}. \tag{2.6}$$

During equilibrium, the total concentration of ionizable fixed sites is C_T, so

$$C_T = C_F + C_{FNa^+}. \tag{2.7}$$

Combining Eq. 2.6 with Eq. 2.7 results in

$$C_{FNa^+} = C_T \frac{C_{Na^+}}{K_{Na} + C_{Na^+}} \tag{2.8}$$

where C_{Na^+} is the concentration of free sodium cation in the bathing solution.

Because the total concentration of ionizable fixed sites C_T is not known for the TM, C_{F-} and C_{FNa^+} are normalized with respect to C_T. If we assume to a first approximation

that the volume, V, of the TM is proportional to fixed charge density then,

$$V \propto \frac{K_K}{K_K + C_{K+}} \tag{2.9}$$

if potassium binding is important or

$$V \propto \frac{C_{Na+}}{K_{Na} + C_{Na+}} \tag{2.10}$$

if sodium binding is important. Let V_o represent the volume of the TM in an endolymph-like solution with C_{K+}=174 mmol/L and C_{Na+}=2 mmol/L Na^+. If a solution with different C_{K+} and C_{Na+} is introduced, the fractional change in volume predicted by Eq. 2.9 is

$$\frac{V - V_o}{V_o} = \frac{174 - C_{K+}}{K_K + C_{K+}} \tag{2.11}$$

and the fractional change in volume predicted by Eq. 2.10 is

$$\frac{V - V_o}{V_o} = \frac{K_{Na}}{2} \frac{C_{Na+} - 2}{K_{Na} + C_{Na+}}. \tag{2.12}$$

These relations are plotted for $K_K = K_{Na} = 20$ mmol/L in Figure 2-1 with the assumption that $C_{K+} + C_{Na+} = 176$ to maintain isosmotic changes in solution.

Both of these relations predict swelling at low potassium concentration. However the potassium binding relation predicts little swelling for change in C_{K+} from 174 to 89 mmol/L (half normal concentration) while the sodium binding relation predicts large swelling for the same change. Furthermore, the slopes of the swelling relations are quite different in the high and low potassium ranges. The potassium binding relation predicts big changes in volume as potassium concentration changes from 174 mmol/L to 0-20 mmol/L, while the sodium binding relation predicts small changes in volume over this range. As potassium concentration changes from 174 to 174-154 mmol/L, the opposite predictions are seen. Over this range, the sodium binding relation predicts large changes in volume, while the potassium binding relation predicts small changes. Thus, making small changes in the low and high potassium ranges may differentiate between sodium and potassium binding.

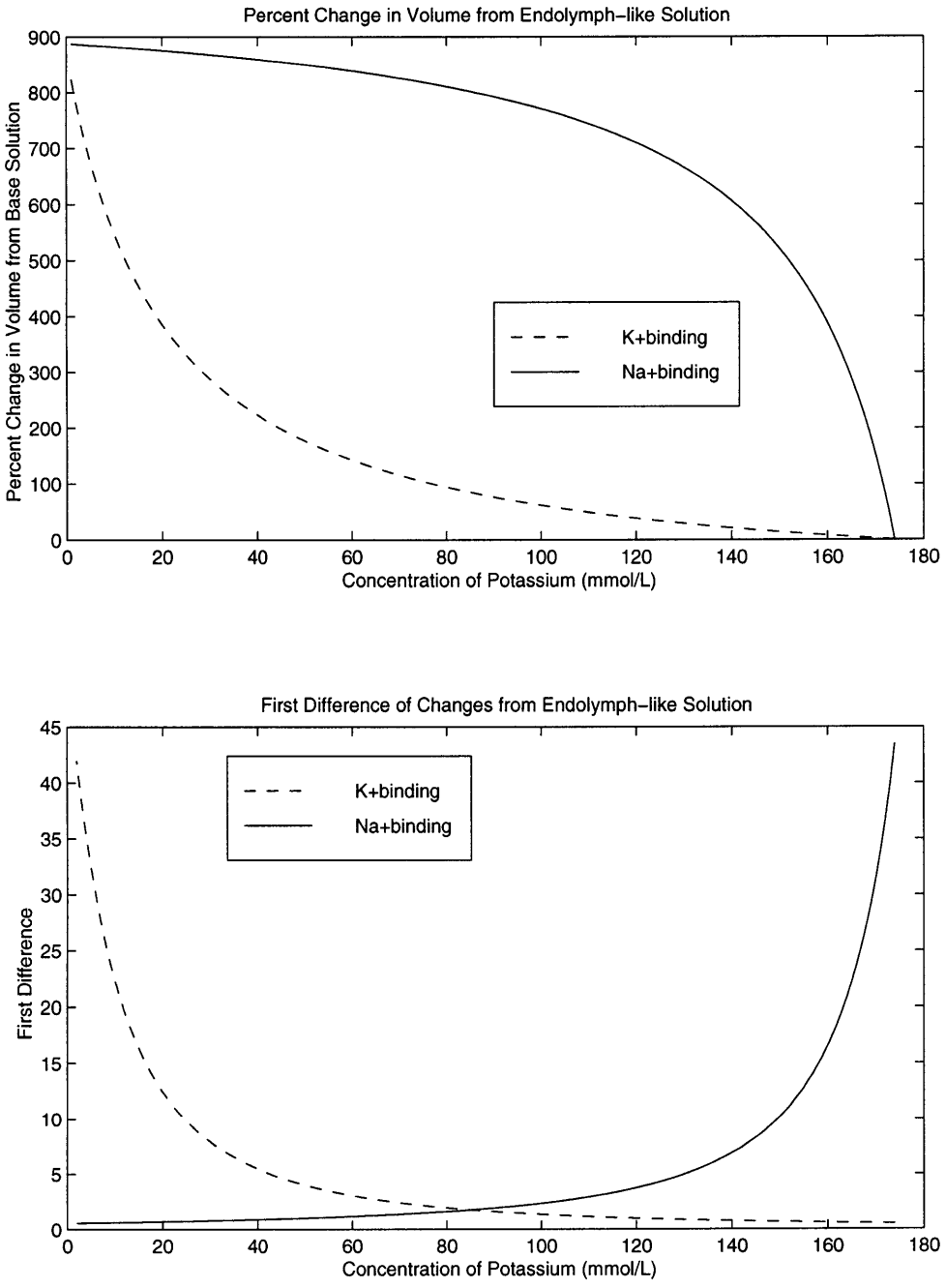

Figure 2-1: Volume relations for the potassium and sodium binding mechanisms. $K_K = K_{Na}$=20 mmol/L, and $C_{K^+}+C_{Na^+} = 176$. The top panel shows percent change in volume as the solution is changed from $[K^+] = 174$ and $[Na^+] = 2$ (base line) to smaller concentrations in potassium (abscissa). The dotted line assumes the potassium binding model with Eq. 2.11 and the solid line assumes the sodium binding model with Eq. 2.12. $K_K = K_{Na}$=20 mmol/L, and $C_{K^+} + C_{Na^+} = 176$. The bottom panel shows the slopes of these curves which indicate steep slopes in the low and high potassium ranges.

Chapter 3

Methods

Methods used in this study were similar to those used in previous studies (Shah et al., 1995; Freeman et al., ress). In brief, a piece of the tectorial membrane was dissected from a mouse ear and allowed to adhere to the floor of an experimental chamber which contained artificial endolymph. Carboxylated latex beads were allowed to settle onto the tectorial membrane and were tracked with an automated system as different test solutions were perfused through the experimental chamber. A total of 30 tectorial membrane preparations were studied, of which 10 were selected for further analysis.

3.1 Solutions

The compositions of the test solutions used are described in Table 1. The test solutions were mixed from two stock solutions which we call K174 and K4. The K174 solution closely resemble mammalian endolymph. The K4 solution was composed similarly except that the KCl concentration was 4 mmol/L and the NaCl concentration was 172 mmol/L. The pH of the stock solutions was adjusted to 7.3 +/- .05 by adding approximately 2 mmol/L of KOH (for K174) or NaOH (for K4). Calcium contamination was minimized by using reagent grade salts (Sigma ACS or Mallinckrodt AR) and ultrapure water (prepared using a Millipore MilliQ UV Plus). All test solutions are nominally isosmotic and of the same ionic strength.

Table 3.1: Compositions of stock solutions

	K174	K4	K164	K154	K144	K134	K44	K34	K24	K14
Conc. (mmol/L)										
KCl	174	4	164	154	144	134	44	34	24	14
NaCl	2	172	12	22	32	42	132	142	152	162
$CaCl_2$	.02	.02	.02	.02	.02	.02	.02	.02	.02	.02
D-glucose	3	3	3	3	3	3	3	3	3	3
HEPES	5	5	5	5	5	5	5	5	5	5
NaOH	-	2	-	-	-	-	2	2	2	2
KOH	2	-	2	2	2	2	-	-	-	-
pH (±0.05)	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3

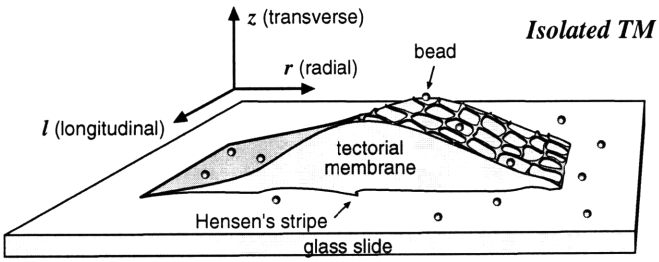

Figure 3-1: The isolated TM fixed to the glass surface of a chamber and covered with beads. The axes labeled in the drawing indicate the coordinates used to describe changes in bead position.

3.2 Preparation

The cochlea was dissected in the manner described elsewhere (Shah et al., 1995). In short, adult male mice (38-50g body weight) were asphyxiated with CO_2 and decapitated. The cochlea was dissected and transfered to a 5 ml K174 (AE) bath, in which the bone overlying the apex of the cochlea was chipped away. Each of the tectorial membranes used in these experiments was obtained from the apical turn. A piece of the tectorial membrane was gently removed from the second turn of the cochlea and attached to the glass floor of a K174-filled experimental chamber using tissue adhesive (Cell Tak, Collaborative Research, Bedford, MA) (Figure 3-1). Carboxylated latex beads in K174 solution were perfused through the experimental chamber and allowed to stick to the surface of the tectorial membrane. Ten were chosen for tracking, 8 of which were attached to the surface of the TM to measure osmotic responses and 2 of which were attached to the floor of the experimental chamber as reference beads. Measurements of bead position were taken every minute while pictures of the TM and beads were taken every 5 minutes.

3.3 Solution exchange protocols

Two different approaches were used in 10 experiments. One approach examined the response of the TM to small changes in potassium concentration in both the high potassium (low sodium) and low potassium (high sodium) concentration ranges. The other approach examined responses of the TM to larger changes in potassium concentration where the concentration was changed from one extreme (K174 or K4) to the midpoint concentration (K89) and then changed to the opposite extreme. Four experiments were done with the first approach, six with the latter. Time intervals of test solution perfusions varied slightly from experiment to experiment.

3.4 Perfusion system

The experimental chamber held approximately 0.2 ml of solution and was perfused with a system consisting of teflon tubing, 2 syringe pumps and a peristaltic pump. Test solutions were placed in the syringe pumps, one of which perfused the chamber with fresh solution at a rate of 10.1 ml/hr while the peristaltic pump removed effluent solution. Solution changes were made via a 4-way valve system which switched the source of perfusion from one syringe pump to the other. Transit time from the valve to the chamber was less than 1 minute (Shah et al., 1995).

3.5 Measurement system

Pictures and measurements of the tectorial membrane and beads were taken with a video microscopy system which consisted of a compound microscope (Zeiss WL), Newvicon camera (Hamamatsu C2400), video digitizer (Data Translation DT2862) and personal computer (IBM PC/AT compatible) (Figure 3-2). A 40X water immersion objective with numerical aperture of .75 was used to magnify the image of the tectorial membrane. X and y coordinates were calibrated with a stage micrometer. The z coordinate was determined from the output of an encoder attached to the fine-focus of the microscope. An automated procedure described elsewhere (Shah et al., 1995) allowed positions of beads to be tracked every minute. The repeatability of measured positions was determined by tracking the positions of beads glued to a glass slide. The standard deviation of these measurements were .082

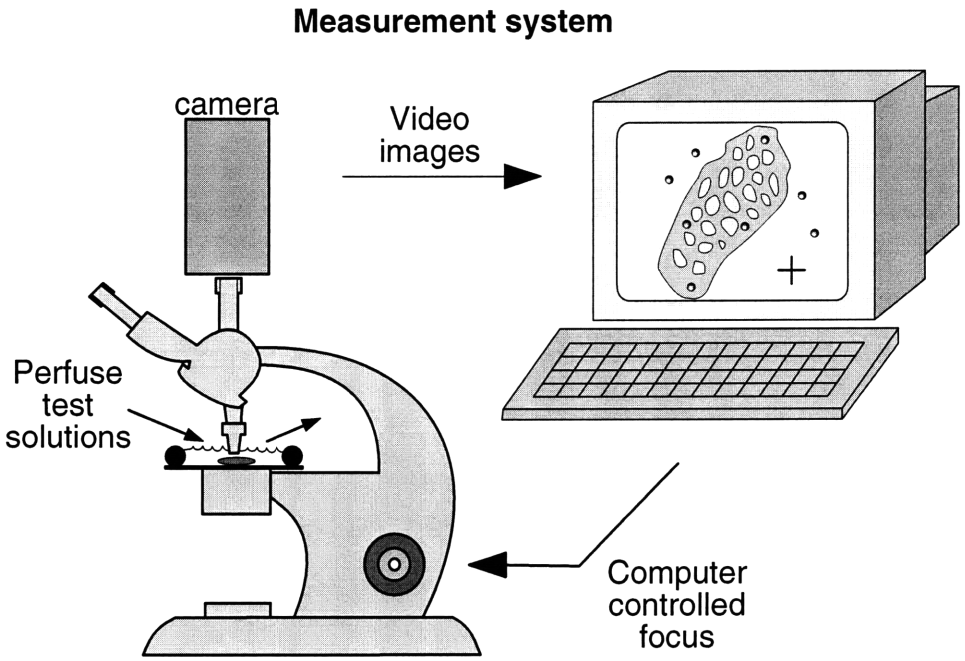

Figure 3-2: Measurement system consisting of compound microscope, Newvicon camera, video digitizer, and personal computer.

microns for the x position, .098 for the y position, and .20 for the z position and are shown graphically in Figure 3-3.

3.6 Quantification of bead motion

At the onset of a solution change, bead positions were observed to change quickly at first and approach a steady state value as time progressed. These motions were fit to exponential functions of time. To simplify the interpretation of such motions, x and y coordinates were translated to r and l coordinates via a transformation based on the position of Hensen's stripe, as shown in Figure 3-1 and Figure 3-4, panel 2. R and l are physiologically significant as radial and longitudinal directions on the TM. Fits made with the r, l, and z coordinates

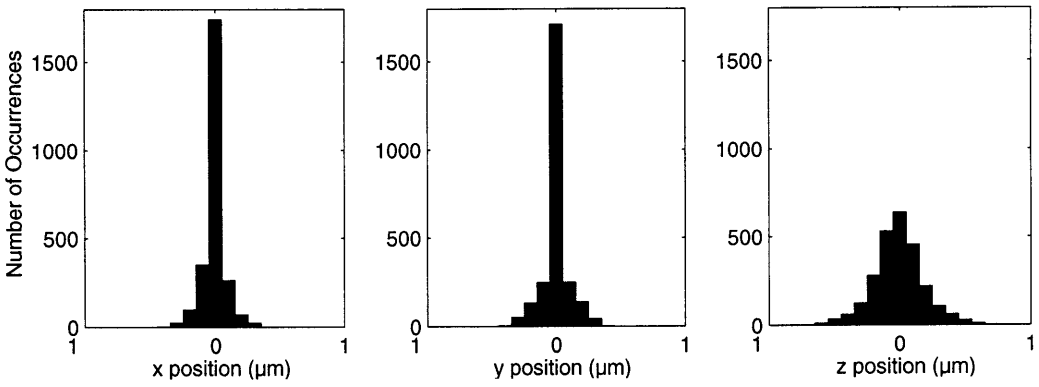

Figure 3-3: Repeatability of measurements of bead position. The position of beads attached to the floor of a glass slide were measured every minute. The histograms depict changes between successive measurements from 8 beads. The standard deviations of these measurements are .082, .098, and .20 μm for x, y, and z, respectively.

were of the form

$$r(t) = \begin{cases} r_d & ; \ t < t_d \\ r_d + r_e(1 - e^{-\alpha(t-t_d)}) & ; \ t \geq t_d \end{cases}$$

$$l(t) = \begin{cases} l_d & ; \ t < t_d \\ l_d + l_e(1 - e^{-\alpha(t-t_d)}) & ; \ t \geq t_d \end{cases}$$

$$z(t) = \begin{cases} z_d & ; \ t < t_d \\ z_d + z_e(1 - e^{-\alpha(t-t_d)}) & ; \ t \geq t_d \end{cases}$$

and were determined for each test interval in which a different solution was being perfused (Freeman et al., ress). Excursions were determined by the total difference in thickness from beginning to end of an interval, as determined by the exponential fit.

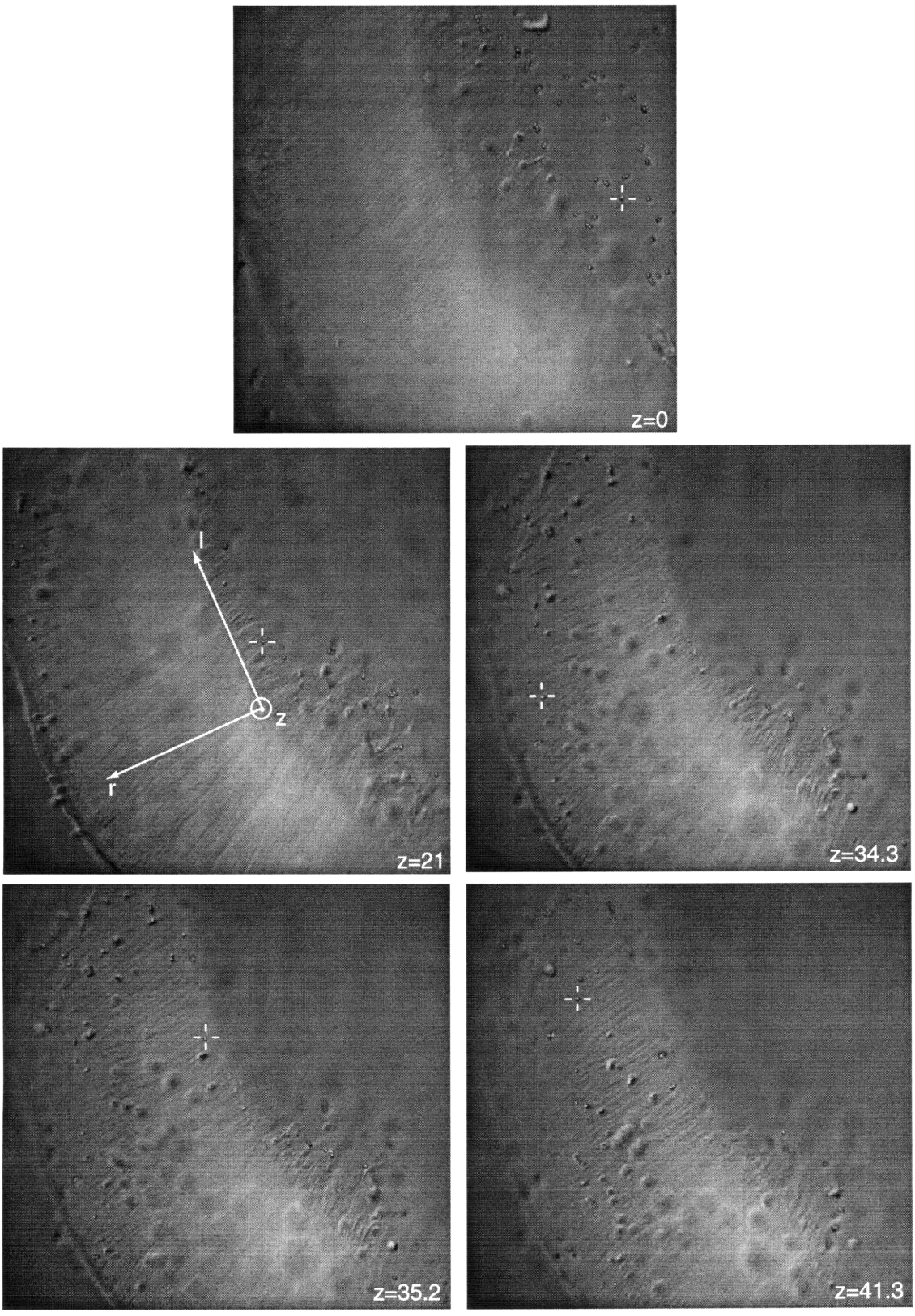

Figure 3-4: Each panel is a picture of the TM taken at different z planes. The beads tracked in each plane are marked. The r, l, and z coordinates based on Hensen's stripe are shown in panel 2.

Chapter 4

Results

While images of the TM were obtained, ionic solutions varying in potassium and sodium concentration were perfused for pre-determined intervals of time. Osmotic responses to these solution changes were measured by tracking the positions of 8 beads attached to the surface of a TM preparation every minute. The results described in this chapter are from 10 TMs, each isolated from a different mouse.

4.1 Effect of small changes in potassium and sodium concentration at high potassium concentrations

Previous experiments have described the effects of substituting potassium with sodium and vice versa (Shah et al., 1995). However, the results of such experiments have not indicated which change, potassium or sodium, is the predominant cause of the observed response. The experiments described in this section were devised to take a closer look at the responses to small changes in potassium at high potassium concentrations. Results shown are summarized from 3 experiments.

Figure 4-1 shows the results for one bead from such an experiment. The z position represents the position of the bead relative to the glass floor of the slide, and therefore approximates the thickness of the TM. The TM was dissected in and initially perfused with K174 for 112 minutes. Interval 1 shows the stability of the TM's thickness in this solution during the latter half of this time period. In the second interval, K134 was perfused and the TM swelled. In order to measure the magnitude of swelling, the data points in each interval

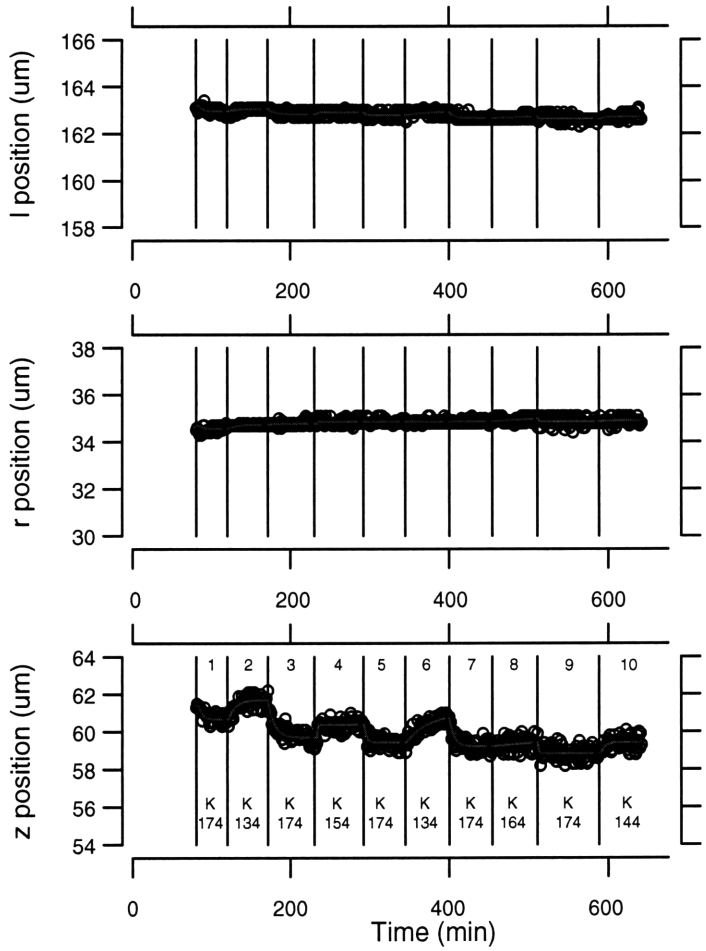

Figure 4-1: Tracking of a bead on the TM as solutions with varying sodium and potassium concentrations were perfused. Vertical lines represent the times at which the perfusing solution was changed. Circles represent measurements of position of the bead as a function of time. Measurements were taken every minute, and each interval (between vertical lines) was fit to an exponential. All perfusates were isosmotic (Table 1). The lines which overlay the plotted data between intervals, indicates the fitted exponential. The solution perfused during the interval is indicated below the plot.

are fit to an exponential function of time (as described in Methods). The difference of TM thickness from the beginning to the end of the interval as determined by this exponential fit is called the excursion. Thus, in interval 2, the z excursion is 1.05 μm. Since the position of the bead in z was 60.66 μm from the glass slide, this excursion corresponds to a Δ excursion of 1.72%. As the perfusate was changed back to K174 in interval 3, the z position decreased with a Δ excursion of -3.27%, implying that the TM shrunk during this interval. The second exposure to K134 which occurred in interval 6 resulted in a 2.21% increase in the z excursion with a subsequent Δ excursion of -2.53% as the solution was changed back to K174. This pattern of swelling then shrinking was noted to repeat, as perfusions of less concentrated solutions of potassium were preceded then followed by K174. Intervals 4, 8, and 10 show positive Δ excursions of 1.17%, .33%, and .97% respectively; whereas intervals 5 and 9 showed negative Δ excursions of -1.57% and -.94%. For further reference, the transition from K174 to a new solution is called the forward transition. The return from the new solution back to K174 is called the reverse transition. Notably, the smallest change in potassium concentration, K174-K164, resulted in the smallest percent excursions in both the forward and reverse solution transitions. Likewise, the largest change in potassium concentration, K174-K134, resulted in the largest percent excursions in both directions. It is also interesting to note that transitions from a less concentrated solution of potassium to K174 (intervals 3, 5, 7, 9) appeared to shrink the TM to a z position lower than the starting z position of the previous interval (intervals 2, 4, 6, 8).

Similar changes were found to occur in the other two experiments. Pooled results from all three experiments are shown in Figure 4-2. Shown are the percent thickness changes for transitions between K174 and a solution with less potassium concentration. In Figure 4-3, both the forward and reverse transitions are plotted in the same column for each set of solution changes. Note that for these sequences, the interquartile ranges of the responses for the forward and reverse transitions do not overlap for the three largest changes in potassium concentration (columns 2, 3, 4), indicating that substituting a less-potassium solution in place of a high-potassium solution results in swelling, and the reverse transition results in shrinking. Only the interquartile ranges of the smallest change in potassium concentration overlap (column 1). Furthermore, in Figure 4-2 the interquartile ranges of all swelling transitions (columns 1, 3, 5, 7) overlap, suggesting that a transition from K174 to a solution with less concentration of potassium results in similar magnitudes of swelling.

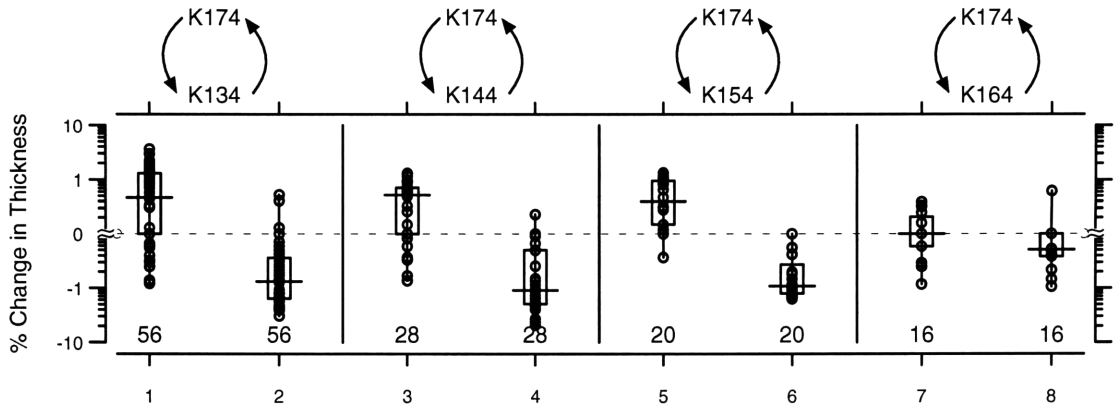

Figure 4-2: Percent change in TM thickness during transitions of the high potassium solutions. Each column represents the transition between two solutions as labeled above the column. Percent changes in thickness were obtained from the z excursions of exponential functions that were fit to the data. The box plots represent the interquartile ranges. The number below each column indicates the number of data points in that column.

Only the interquartile range of the K174-K164 reverse transition in column 8 is disjoint from those of the other shrinking transitions of columns 2, 4, and 6, perhaps suggesting that there is a minimum change in potassium concentration needed to elicit a measurable shrinking response.

4.2 Effect of small changes in potassium and sodium concentration at low potassium concentrations

At low potassium concentrations, solution transitions parallel to those described in the above section were made in order to compare the results in the high potassium concentration range with those in the low potassium concentration range. Again, the dissection and initial perfusion were done in K174 to assure stability of TM thickness. Figure 4-4 shows the tracking of one bead. In interval 2, K4 was perfused for a period of 60 minutes, at the end of which the Δ z excursion was found to be 2.72%. In interval 3, K44 was perfused, and the TM continued to swell. Subsequent intervals involved transitions from this new base solution K44 to less concentrated solutions of potassium, followed by a transition back to K44. For instance, in the fifth interval, K44 was perfused. This interval was then followed

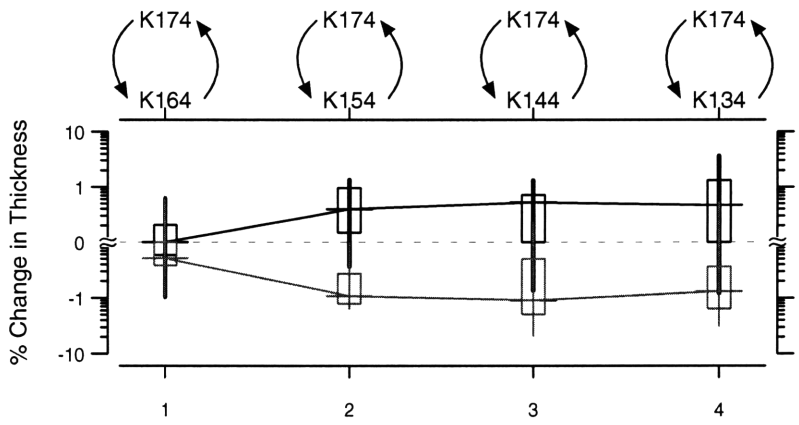

Figure 4-3: Percent changes in thickness for both the forward and reverse transitions in high potassium solutions. Each column describes the solutions that were used. In black, is the pooled forward responses. In gray, is the pooled reverse responses.

by a perfusion of K24 which resulted in an Δ excursion of .22%. Transition back to K44 resulted in a -.16% Δ excursion, indicating shrinking of the TM. In general, excursions in this experiment were extremely small (less than 1 micron) and not as large as those in the high potassium experiments described earlier.

The results for this experiment were pooled over all eight beads and all transitions and are shown in Figure 4-5. Figure 4-6 plots the forward and reverse transitions in the same column for each set of solutions. For the highest and lowest changes in potassium concentration (columns 1 and 4), the interquartile ranges of the forward and reverse responses overlap, while the less extreme changes in potassium (columns 2 and 3) show completely disjoint distributions. The greatest transition in potassium concentration results in small percent changes in thickness in the TM, as does the smallest transition in potassium concentration; moderate changes in potassium concentration result in larger swelling and shrinking.

A comparison of the swelling responses between those of the high potassium and low potassium experiments (Figure 4-7) reveals that the median Δ excursion of the responses at high potassium concentration (columns 1, 3, 5) were higher than those of their parallel transitions at low potassium concentration (columns 2, 4, 6). Only the smallest transition (columns 7 and 8) does not hold this trend.

The shrinking responses of the high and low potassium concentrations also show similar trends. For each of the transitions, the one that occurred at the high potassium concentra-

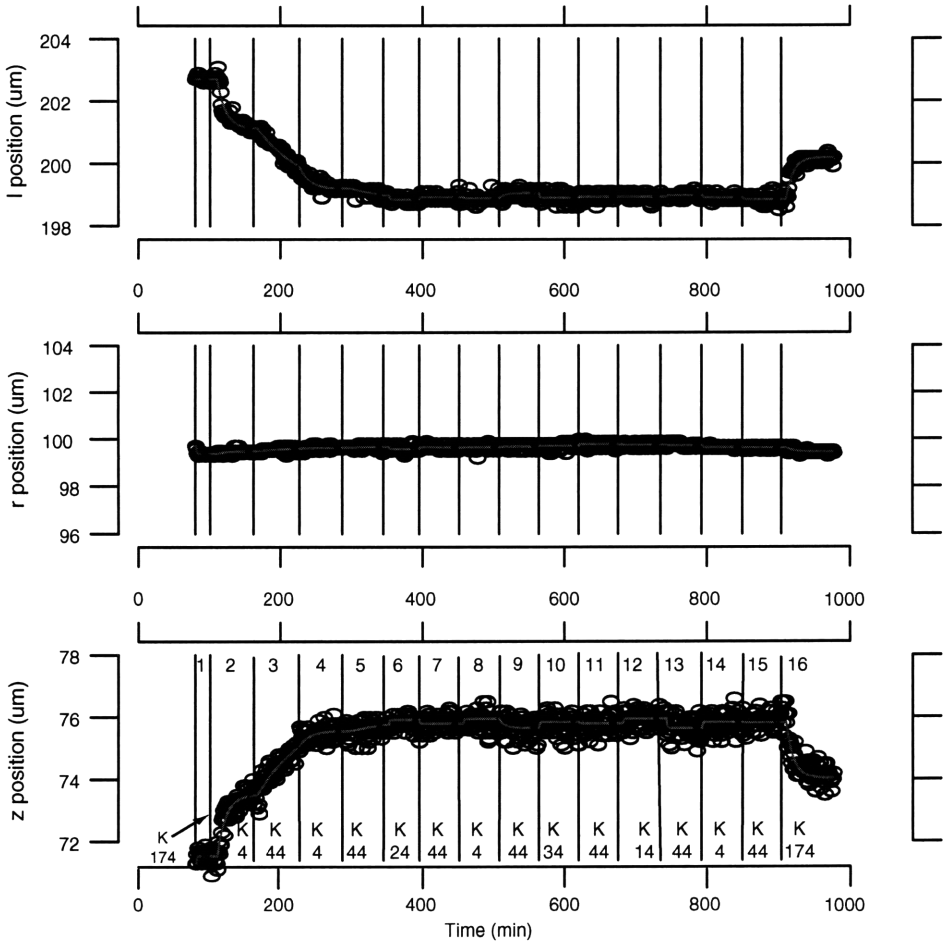

Figure 4-4: Effects of changes in the low potassium concentration region. Aspects of this figure are described in Figure 4-1.

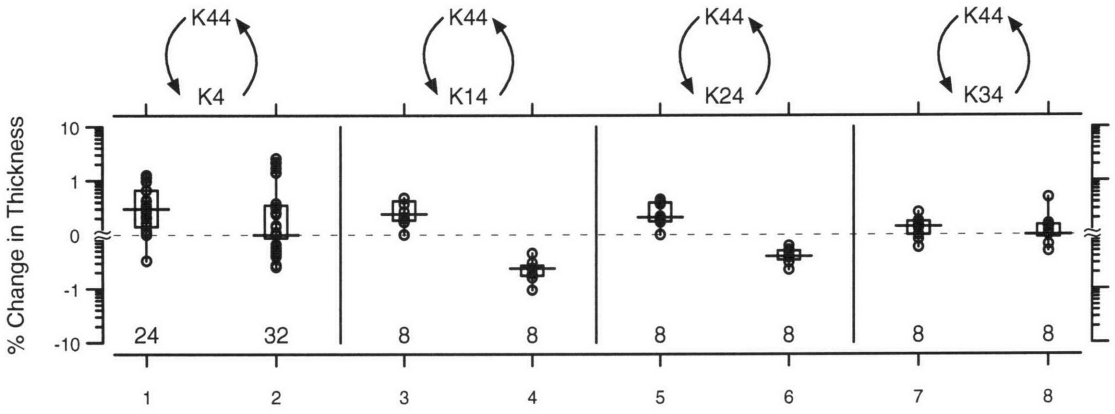

Figure 4-5: Percent changes in thickness during transitions in low potassium solutions. Aspects of this figure are described in Figure 4-2.

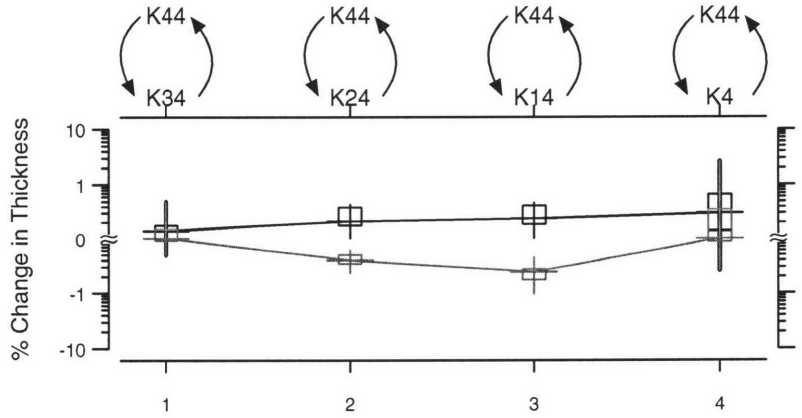

Figure 4-6: Percent changes in thickness for both the forward and reverse transitions in low potassium solutions. Each column describes the solutions that were used. In black, is the pooled forward response. In gray, is the pooled reverse response.

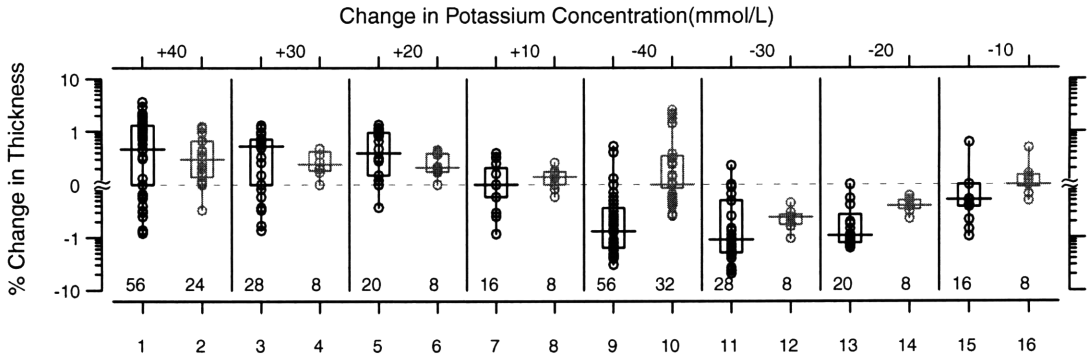

Figure 4-7: Comparison of swellings and shrinkings in the high potassium range vs. low potassium range. In the left half of the figure are the swellings, with Δ in potassium concentration (mmol/L) labeled above the columns. The right half are the shrinkings. Plotted in black are the pooled results from the high potassium range experiments. Plotted in gray, are the the pooled results from the low potassium range experiment. Aspects of this figure are described in Figure 4-2.

tion always had the greater magnitude response (columns 9, 11, 13, 15). Thus for the most part, these results suggest that changes in concentration which occur in the high potassium concentration range have a greater effect on the TM than the same changes in potassium concentration in the low potassium concentration range.

4.3 Effect of changing potassium concentration in two equal transitions from K174 to K4

In order to determine whether most of the change from K174 to K4 occurred in the upper half or lower half of the potassium concentration range (described in Chapter 2), six experiments were performed to determine the extent of swelling and shrinking that occurred from one extreme (K4 or K174) to the midpoint (K89). Four experiment protocols followed a "triangle" format, in which solution changes were made from one extreme (K4) to the midpoint (K89) to the other extreme (K174) and back through the midpoint. The following results were obtained from all six experiments.

Figure 4-8 depicts the results of one bead from a "triangle" experiment. As in the other experiments, the TM was dissected in and allowed to stabilize in K174. The second

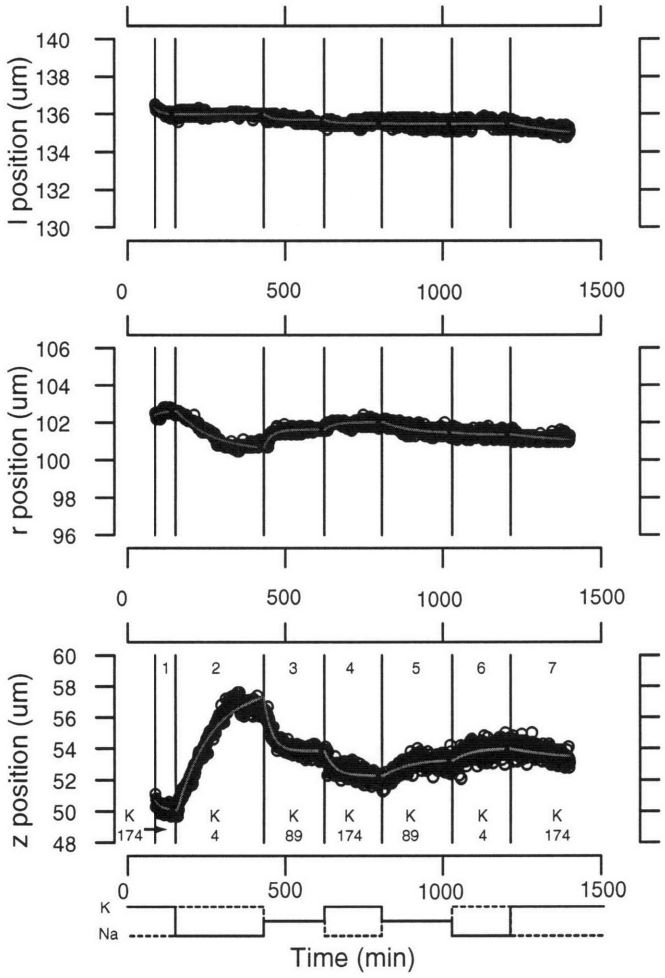

Figure 4-8: Effects of changes in solutions going from K174 or K4 to the midpoint solution, K89. The ionic composition of the perfusates are indicated by line plots in the lower panels. Aspects of this figure are described in Figure 4-1.

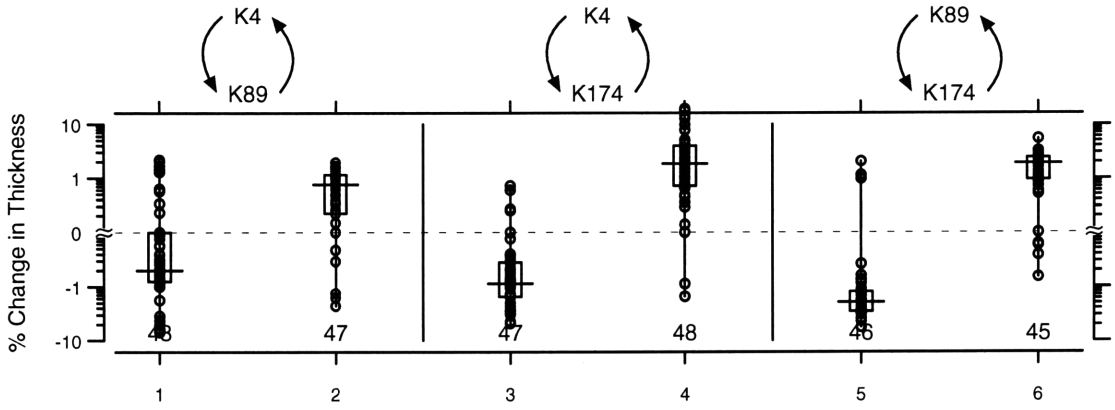

Figure 4-9: Percent changes in thickness during the interval described above the column. Aspects of this figure are described in Figure 4-2

interval in which K4 was perfused for four hours, shows that the z position for this bead increased by 14.40%. During the third interval, K89 was perfused and the z position was shown to decrease by -6.02%. During the 4th interval, K174 was perfused. This resulted in a shrinking of the TM by -2.93%. The following intervals resulted in swellings of 1.86% and 1.37% when K89 then K4 were again perfused, with the last interval of K174 perfusion ending in a -.82% Δ excursion.

Results for six experiments are pooled in Figure 4-9. Note that for the lower half of the potassium titration range (K4-K89), the median of the shrinking and swelling columns (1 and 2), respectively were -.5% and .76%. For the upper half of the potassium titration range (K89-K174), the medians for shrinking and swelling were -1.955% and 1.88%, respectively. For the full range of potassium (K4-K174), the medians were -.88% for shrinking and 1.84% for swelling.

These results are better summarized in Figure 4-10 and Figure 4-11. Figure 4-10 shows all the shrinking responses side by side. Note that the interquartile ranges of the responses to the K4-K89 and K4-K174 transitions overlap to a greater extent than the K89-K174 and K4-K174 transition. It appears that these responses are very similar, and statistically not different from each other. However the K89-K174 transition also overlaps with the K4-K174 transition, perhaps suggesting that this transition, too, is similar to the K4-K174 transition.

Figure 4-11 shows that the interquartile ranges of the responses to transitions K174-

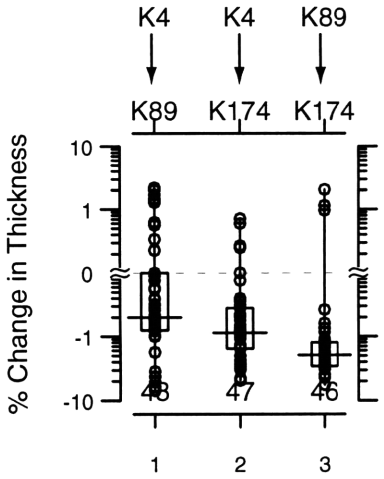

Figure 4-10: Percent changes in thickness in the intervals during which the solution was changed from a low potassium solution to a high potassium solution.

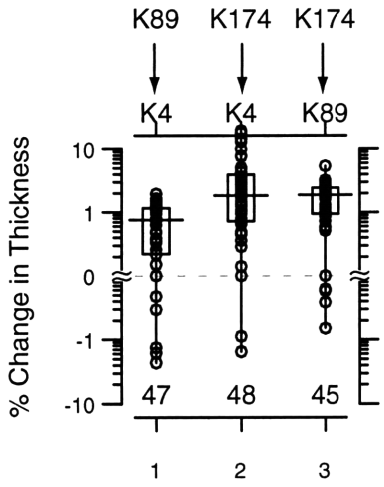

Figure 4-11: Percent changes in thickness in the intervals during which the solution was changed from a high potassium solution to a low potassium solution.

K4 and K174-89 overlap to a greater extent than the K89-K4 and K174-K4 responses do. However, all interquartile ranges do overlap.

Furthermore across both shrinking and swelling responses the K4-K89 transition had the least median magnitude response and the K89-K174 transition had the greatest median magnitude response. This would suggest that the greater response occurs in the upper half of the potassium concentration range.

These experiments indicate that the greatest magnitude of response occurred in the 89 to 174 mmol/L concentration of potassium, i.e.. the upper half of potassium concentrations. Figure 4-10 and Figure 4-11 both demonstrate this with their median values for shrinking and swelling.

4.4 Analysis of reversibility

In past experiments (Freeman et al., ress; Shah et al., 1995), it was noted that the response to solution changes were not always reversible. This phenomenon was attributed to the possibility that mechanical swelling might result in the loss of GAG's or stretching of collagen fibers, that would not allow the TM to return to its original thickness.

It appears from these experiments, however, that responses to small changes in potassium concentration are reversible to a first order approximation. Figure 4-12 shows the pooled results of each solution change. Each column shows the data from both the forward and reverse transition. Ideally, if responses were perfectly reversible, these pooled results should be centered around zero. However, column 1, the K44-K4 transition, is obviously skewed positively. The reason for this can be seen in Figure 4-4. Theoretically, in intervals 3 and 5, the transition from K4 to K44 should have resulted in shrinking, as it did in intervals 9 and 15. Instead, in intervals 3 and 5, the TM swelled, possibly because the K4 interval in both cases (interval 2 and 4) had not reached steady state, as it had in intervals 8 and 14. All other solution changes at the low potassium concentrations, however, are very much centered around zero.

An interesting observation was also made in the experiments with small changes in potassium concentration in the high potassium concentration range. Though all the responses to the solution changes in columns 5, 6, 7, and 8, are centered close to zero, solution changes, K174-K134 and K174-K144, show a slight skewing in the negative direction. Further examination of Figure 4-1 reveals why. Intervals 3 and 7, where K134 was replaced by K174, show that the TM shrunk more in these intervals than it had swelled in the previous interval where K174 had been replaced by K134 (intervals 2 and 6). This type of observation was also made in another experiment, where it seemed, that the shrinking intervals had a greater magnitude response than the swelling intervals.

The issues of reversibility were more complicated to analyze in the experiments where mid-point changes in potassium concentration were made in the bathing solution because these experiments followed the triangle format where the sequence of perfusions was:

$$K174 - K4 - K89 - K174 - K89 - K4 - K174$$

Forward and reverse transitions were not immediately repeated after each other, in the

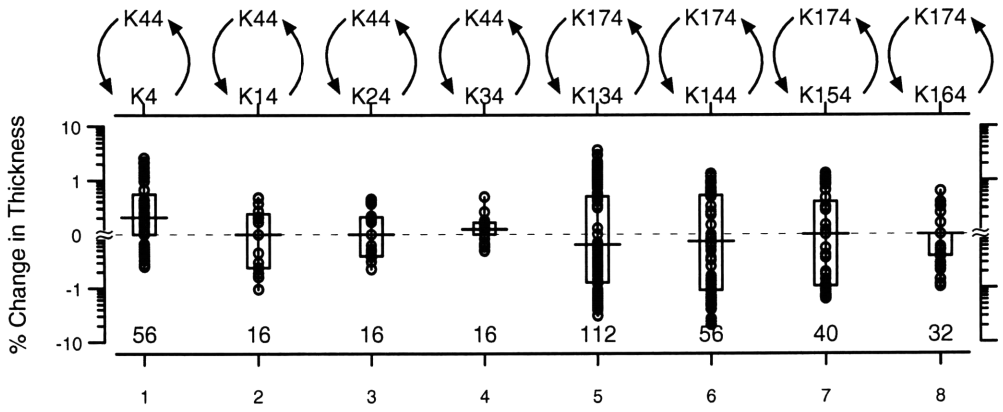

Figure 4-12: Pooled results of the small changes in potassium, in both the forward and reverse directions.

K174-K4 and K4-K89 transitions, and therefore, reversibility could not be determined from these experiments.

4.5 Structural changes in TM over solution changes and time

The fibrillar microstructure of the TM seemed to become much less prominent as time progressed during the experiment. Figure 4-13 shows a part of the TM at the beginning of each solution change, and then finally, its appearance at the end of the experiment. Note in panel 3, how much of the fibrillar structure disappears after K4 is perfused, perhaps because of swelling. The following K89 and K174 perfusions fail to bring the TM back to its original appearance (panels 4 and 5). By the end of the experiment, much of the structure seems faded and barely visible. This indicates that the TM changes structurally as the perfusate changes and may also change over time.

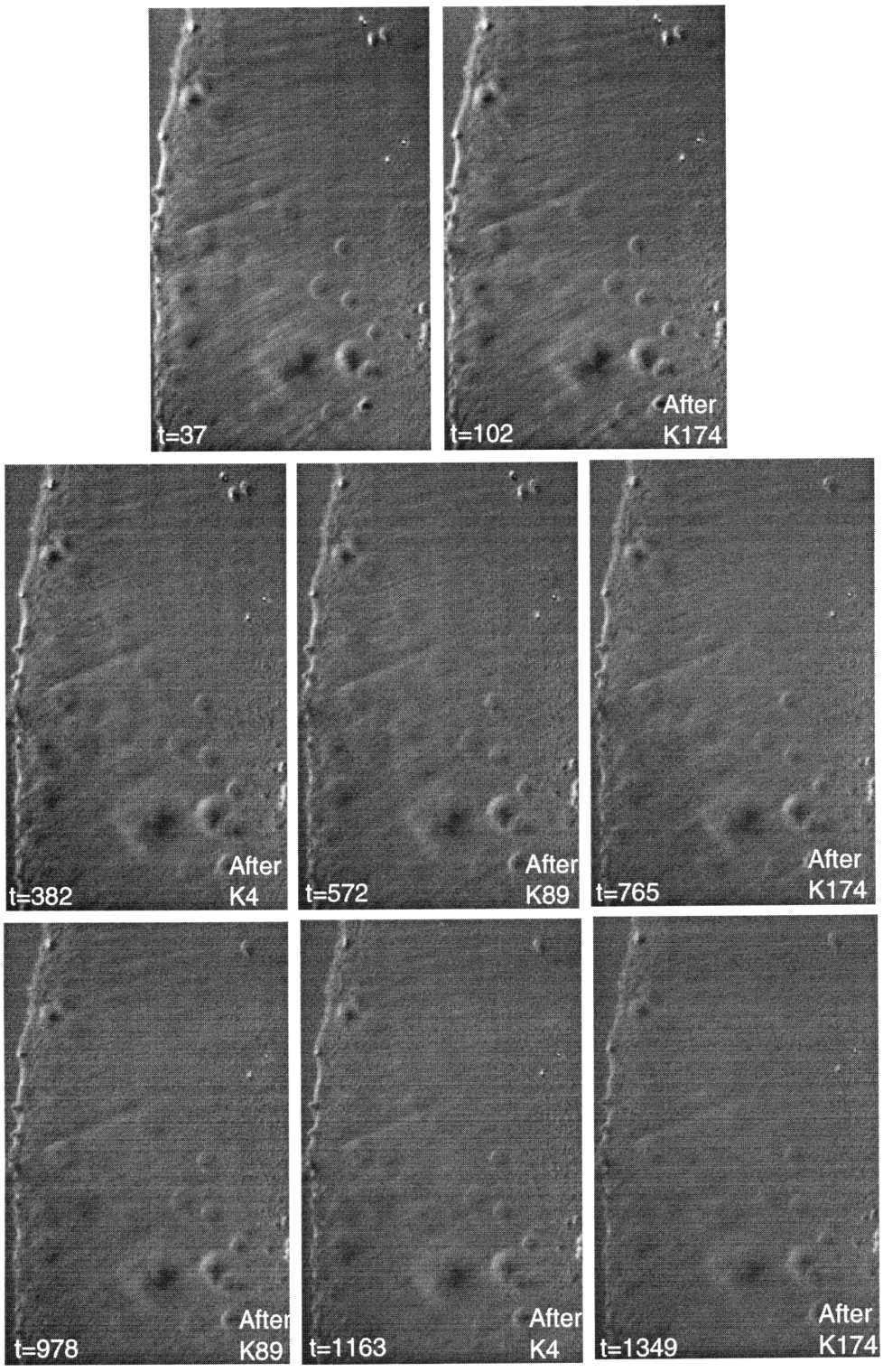

Figure 4-13: Each panel is a picture of the TM. Panel 1 is a picture taken at the beginning of the first interval. The time the picture was taken is labeled in each panel, as is the bathing solution.

Chapter 5

Discussion

5.1 Interpretation difficulties

There were a number of issues in these experiments that complicate interpretation of the results. This section describes these issues briefly.

5.1.1 Responses are slow

Swelling of the TM in high sodium solutions is slow. Consider the second interval in Figure 4-4. The time constant for this interval appears be greater than 100 minutes. Interval 2 was one of the longer intervals in any experiment. With smaller intervals, the observation that the swelling had not reached steady state was even more apparent. This phenomenon occurred most often in the lower half of the potassium concentration range when the change in potassium was + or - 85 mmol/L between two intervals. In the first of these intervals, swelling usually occurred without reaching a steady state value. If swelling occurred in the second interval as well, the exponential rise seemed to follow the same time constant as that of the previous interval, indicating perhaps, that the TM had continued to be in some sort of quasi-state of change. This offers a possible explanation for why the direction of the response was not always clear for the +85 mmol/L transitions where K4 was replaced with K89. One experiment in which the K4 interval had lasted only 2 hours, resulted in continued swelling in the following K89 interval which was also 2 hours long. In the other three experiments, the K4 solution had been perfused for four hours before K89 was perfused for another 3 hours, and the magnitudes of shrinking in these intervals seemed

directly related to how close to steady state the previous interval had ended.

Regardless of whether the TM had reached a steady state or not, transition to the K174 solution from either K89 or K4 consistently resulted in a very noticeable shrinking response of the TM. Even if the previous interval did not reach steady state, the TM shrank every time K174 was introduced. In light of the ambiguous results which occur when the same magnitude of change occurs in the low potassium region, an interpretation of this observation is that the magnitude of this response is so large, that transient effects have little impact on the observed response. This would be consistent with the observation that the both the K89-K174 and K4-K174 transitions result in similar responses. However, the result may be less significant if the swelling of the TM depends monotonically on the concentration of potassium, as we have assumed.

5.1.2 Experiments are long

Figure 4-10 and Figure 4-11 suggest that the K4-K89 swelling more closely resembles the K4-K174 swelling. However, this interpretation is confounded by the fact that the K4-K174 transition occurred late in the experiment, nearly 21 hours after experimentation had begun. The TM may have deteriorated somewhat during this time which may have reduced the responsiveness of the TM. If structural appearance of the TM is any indication of the state of the TM, Figure 4-13 seems to support that fact that the TM is different at the end of the experiment. Furthermore, in all cases, the K174-K4 transition was always the first change and by far the largest in magnitude. Previous studies have shown that the first interval is always the largest response and that subsequent intervals with the same transition going either forwards or backwards result in responses smaller than that seen in the first interval. Thus complete reversibility was not expected in the last K4-K174 interval, and it is not surprising that the TM did not completely return to its original thickness at the beginning of the experiment. It is likely that if the transition had been reversible, it would look more like the K89-K174 response. However, there is no direct evidence on how rapidly the TM deteriorates with time and to what extent responses at the end of 24 hours can be compared reasonably to the responses in the first six hours of the experiment.

5.1.3 Excursions are small

Not only are responses slow, but they are also small relative to changes that occur in the absence of solution changes. It is not unusual for the TM to shrink 1 or 2 microns over long periods of time. Many of the excursions were on the order of 1-2 microns in magnitude. Also, the irreversibilities were relatively large compared to the magnitude of the excursions. Thus, analyzing the results of such small excursions is a difficult task.

5.2 Comparison with the binding model

Results from these studies suggest that most of the swelling and shrinking response occurs in the upper half of the potassium concentration range which is more consistent with a sodium binding hypothesis than with a potassium binding hypothesis (Chapter 2). As observed in Figure 2-1, the response from K174-K89 is not very different from the response of K174-K4. If the binding mechanism had in fact been with potassium, the two responses would have to be statistically different from each other. This is not shown by the given data, as in both Figure 4-10 and Figure 4-11 the interquartile ranges overlap for the K174-K4 and K174-K89 sequences. Furthermore, the consistency with which the K4-K174, and K89-K174 transitions shrink the TM, may also support the sodium binding hypothesis. The ambiguity of the K4-K89 response can be interpreted as being so weak that the TM could appear to shrink or swell. However, the results do not rule out the fact that it may be a potassium binding mechanism, since there are many ambiguities due to the slow responses, long experiments, and small excursions.

5.3 Implications for sodium binding

Sodium binding suggests that the fixed charge of the TM in normal endolymph is neutral or positive to begin with. However, the biochemical nature of the TM suggests that it be negatively charged in endolymph, since at physiological pH, GAGs impart negative charges. Measurements of potential across the membrane (Steel, 1983) have also suggested that the TM has a negative fixed charge at physiological pH. These observations suggest that the binding mechanism is through potassium. Because these measurements may not be conclusive, it will be necessary to measure the TM potential especially for different sodium

concentrations in the bathing solution.

5.4 Future experiments to resolve issues

A number of changes could be made to the protocols, to better ensure more indisputable results. First of all, longer intervals are needed to achieve a steady state response especially for the swelling responses. However, this would lengthen the experiment and without knowing the effects of time on the TM, may still lead to ambiguous results. Thus, repetition of transition sequences and differing the order of transition sequences from experiment to experiment would also be necessary. Also, repetition of the last K4-K174 transition right after the first K4-K174 transition would establish early on, the reversibility of the TM thickness. A greater number of experiments (and thus data points) might also decrease the size of the interquartile ranges in the data and provide more statistically relevant results.

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