Electrostatic properties of fiber cell membranes from the frog lens

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ABSTRACT The electrostatic properties of lens fiber cell membranes have been investigated by recording the electrophoretic mobility of membrane vesicles formed from isolated fiber cells. The vesicles appear to be sealed and have external surfaces that are representative of the extracellular surface of fiber cells. The average mobility of a vesicle in normal Ringer's solution was 0.9 μm/s per v/cm, which gives a zeta potential of -9 mV, a value similar to that reported for other cells (McLaughlin, S. 1989. *Annu. Rev. Biophys. Biophys. Chem.* 18:113–136.). There was no significant difference in the mobility of vesicles formed from peripheral, middle cortical, or nuclear fiber cells. Vesicle surface charges were titrated using Ca and Mg and each had a pK of ~2, which is similar to that for the most common phospholipids. We also titrated these charges with varying pH and found the most significant changes in mobility at pH values between 5 and 6. The majority of lipids found in biological membranes are not titratable in this pH range, so the pH effect is probably through a membrane protein charged group. These experimental data in conjunction with the previously measured extracellular voltage gradient (Mathias, R. T., and J. L. Rae. 1985. *Am. J. Physiol.* 249:C181–C190) imply that electroosmosis can generate a fluid velocity of ~0.6 mm/h, directed from the aqueous or vitreous toward the center of the lens, along intercellular clefts.

INTRODUCTION

The lens is an unusual organ in that it must be transparent to carry out its normal function. Light scattering elements, such as blood vessels, nuclei, mitochondria or other organelles, are therefore absent from the majority of the tissue. Moreover, the cells are tightly packed together with extracellular spaces that are smaller than the wavelength of visible light (Tardien and Delaye, 1988). Steady-state cell volume regulation is essential to maintain small extracellular spaces, but it is complicated owing to the lack of organelles and blood flow. Mathias (1985) proposed a model of steady-state volume and fluid flow in the lens (reviewed in Mathias and Rae, 1985). The model includes the possibility of electroosmotically driven fluid flow along the extracellular spaces between fiber cells. The fluid movement acts as an internal circulatory system, which convects glucose and other nutrients to the inner fiber cells.

Electroosmosis is the movement of fluid and ions due to a voltage gradient parallel to a charged surface (Bokris and Reddy, 1970). Most animal cell membranes have on their extracellular surface fixed negative charges, which concentrate mobile positive ions in the layer of solution adjacent to the membrane (McLaughlin, 1977). If two membranes are in close apposition, the narrow space between them will have a high electrical resistance, hence significant voltage gradients can exist in a direction parallel to the membrane surfaces. Voltage gradients will cause mobile positive and negative ions to migrate in opposite directions, consequently, in the layer of fluid where positive ions outnumber the negative, a net movement of solute occurs and drags fluid with it.

McLaughlin and Mathias (1985) showed that electroosmosis could move a significant amount of fluid along the lateral intercellular spaces of the renal proximal tubule. Mathias (1985) applied this analysis to the frog lens, where extracellular voltage gradients had been measured (Mathias and Rae, 1985) but the electrostatic properties of the fiber cell membranes were not known. The purpose of the work described here is to measure the electrostatic properties of the fiber cell membrane surface. As mentioned, these properties are important with regard to fluid movement. In addition, these measurements reflect the physical properties of lipids and protein of the fiber cell membrane.

METHODS AND MATERIALS

Solutions and materials

Bovine brain phosphatidylserine and egg phosphatidylcholine were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). We also used Tris (hydroxymethyl) aminomethane (Tris) and 2[*N*-morpholino] ethanesulfonic acid (MES) from Calbiochem-Behring Corp. (La Jolla, CA), 3-[*N*-morpholino]propanesulfonic acid (MOPS) from P-L Biochemicals Inc. (Milwaukee, WI), ethylenediaminetetraacetic acid (EDTA) from J. J. Baker Chemical Co. (Phillipsburg, NJ), wheat germ

agglutinin (WGA) and *N*-acetyl-D-glucosamine (NAG) from Sigma Chemical Co. (St. Louis, MO), 2',7'-bis-(carboxyethyl)-5-(and -6) carboxyfluorescein, acetoxymethy ester (BCECF-AM), and Texas Red wheat germ agglutinin (TR-WGA) from Molecular Probes Inc. (Eugene, OR), and bovine serum albumin (BSA) from Boehringer Mannheim Biochemicals (Indianapolis, IN).

All solutions were prepared with water purified in a Millipore/Continental Water Systems (Bedford, MA) Super A system. Solutions for the electrophoretic mobility studies were further distilled in a Heraeus-Amersil Inc. (Sayreville, NJ) quartz still. Our standard frog lens Ringer's solution contains, in millimolar: NaCl, 108.5; NaH_2PO_4, 0.85; Na_2HPO_4, 0.35; KCl, 2.5; $MgCl_2$, 1.2; $CaCl_2$, 2.0. This solution was adjusted to a pH of 7.4 with 2 M NaOH. Electrophoretic mobility did not vary when 1 mM MOPS was substituted for the phosphate buffer. In the "sidedness of vesicles" studies we used two modified Ringer's solutions: solution 1 was half concentrated lens Ringer's solution supplemented with 2 mg/ml BSA and 200 mM glucose, and solution 2 was half-concentrated lens Ringer's solution supplemented with 2 mg/ml BSA and 200 mM NAG.

In experiments where pH was varied, we titrated to more acid levels with concentrated HCl or bubbling with CO_2 and buffering with MES. We titrated to more basic levels with concentrated NaOH and buffering with MOPS.

In experiments where Ca^{++} or Mg^{++} were varied, we substituted either $MgCl_2$ or $CaCl_2$ for NaCl on a charge for charge basis, thereby maintaining a constant ionic strength but allowing some change in osmolarity. Measurements made on vesicles in zero Mg^{++} or Ca^{++} were not influenced by the presence or absence of EDTA. At divalent concentrations above 8 mM, MOPS buffer was used exclusively. At lower concentrations, similar results were obtained with either phosphate or MOPS buffer. In all instances the pH was 7.4.

Measurement of electrophoretic mobility

Adult grass frogs (*Rana pipiens*) were sacrificed in accordance with procedures approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and their lenses were extracted atraumatically. The capsule was removed and fibers at various depths were teased from anterior to posterior sutures using microsurgical techniques. Areas designated "outer" and "middle" cortex were spherical shells whose thickness averaged 0.25 mm. The "inner" cortex was an ~0.75-mm shell. It was impossible to remove fibers from the innermost, nuclear region of ~0.25-mm radius.

It is not feasible to measure the electrophoretic mobility of intact lens fiber cells, hence, we formed vesicles and measured their velocity in an electric field. Fibers were gently triturated in physiological Ringer's solution until a suspension of vesicles was formed (see Fig. 1). Electrophoretic mobilities were measured at 25°C with a Rank Brothers (Bottisham, Cambridge, UK) Mark II machine as described by Cafiso et al. (1989). We focused at the stationary layer (Henry, 1974), where convection does not contribute to the mobility.

Proper calibration of the technique was accomplished by first using multilamellar vesicles, prepared according to Bangham et al. (1974), of phosphatidylcholine and phosphatidylserine. These synthetic vesicles have well-known electrophoretic mobilities (McLaughlin et al., 1981). The electrophoretic mobilities were reported in terms of the zeta potential using the Helmholtz-Smoluchowski relation:

$$\zeta = \frac{\mu\eta}{\epsilon_r\epsilon_0}, \qquad (1)$$

where μ is electrophoretic mobility, ϵ_r is dielectric constant of the saline solution, ϵ_0 is permittivity of free space, η is viscosity of fluid, and ζ is zeta potential.

We realize there may be no simple relationship between the electrophoretic mobility and the electrostatic potential at the surface of a complicated biological membrane such as the lens fiber cell (see the review by McLaughlin, 1989). However, Eq. 1 allows us to report an "apparent" zeta potential.

Sidedness of vesicles

Sidedness was determined by observing fluorescence of vesicles labeled with TR-WGA and incubated in the membrane permeant dye BCECF-AM. WGA binds to extracellular oligosaccharides of membrane glycoproteins (Lennarz, 1983; Kornfeld and Kornfeld, 1985; Bloemendal, et al., 1972). BCECF-AM enters the vesicles, and if there are intracellular esterases present, it is cleaved to its fluorescent form, BCECF, which is membrane impermeant. TR and BCECF have different wavelengths for both excitation and emission so binding and/or loading could be observed in each vesicle. All steps were carried out at room temperature.

Isolated vesicles were incubated in 8 μM BCECF-AM for 30 min. The vesicles were pelleted at 2,000 *g* for 10 min. The pellet was resuspended in either solution 1 or 2 and layered onto a glass cover slip where the vesicles attached and could be superfused with the appropriate solution. The vesicles were then subjected to one of two protocols: (*a*) Sidedness. Vesicles suspended in solution 1 were incubated in 20 μg/ml TR-WGA for 30 min, superfused with solution 1 for 30 min, photographed and counted. (*b*) Control. Solution 2 was used to control for nonspecific binding. The lectin binding site on WGA binds NAG, thus preventing WGA from binding the extracellular oligosaccharides (Nagata and Burger, 1974). TR-WGA was preincubated in 200 mM NAG (solution 2) for 1 h. Vesicles were incubated in this solution for 30 min, superfused for 30 min, then photographed and counted.

Brightfield and fluorescent images were recorded using a Diaphot microscope (model 78919, Nikon Inc. Instruments Div., Garden City, NY). Fluorescent images were obtained by passing the halogen source through either a fluorescein (λ_{ex} 450–490 nm, λ_{em} 520 nm) or rhodamine (λ_{ex} 510–560 nm, λ_{em} 590 nm) filter cube (Nikon Inc. Instruments Div.) for BCECF or TR, respectively. Exposure times of 15 s for fluorescein and 45 s for rhodamine were used with Kodak P3200 film, ASA 1800, pull developed to 1600.

RESULTS

Fig. 1 shows a light micrograph of a lens fiber cell vesicle suspension. The largest of these vesicles is ~10 μm. We often see vesicles aggregated in pairs, which may represent two vesicles coupled by gap junctions. Fig. 1 *A* shows a vesicle suspension in a 220 mosM Ringer's solution. In Fig. 1 *B*, the same vesicles are seen in a 430 mosM Ringer's solution (2 × normal NaCl) and are obviously shrunken. When the solution is diluted back to 220 mosM the vesicles resume their original shape and are indistinguishable from those in Fig. 1 *A*. Hence, vesicles appear to be sealed, inasmuch as they demonstrate reversible osmotic volume changes. And, as described below, they retain BCECF dye for hours.

Our aim was to generate vesicles whose external surfaces were representative of the surface of the intercel-

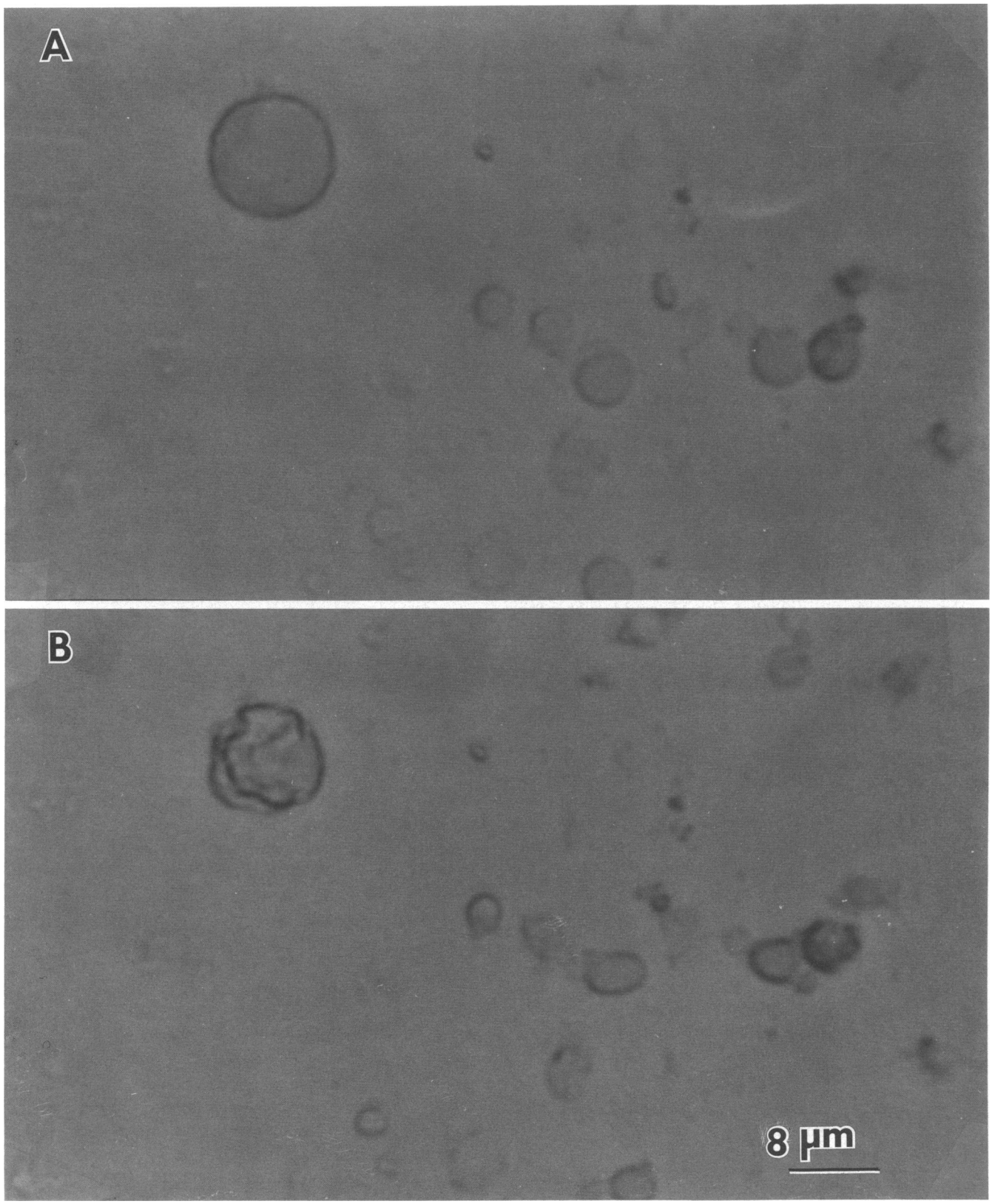

FIGURE 1 A light micrograph of vesicles formed by gentle trituration of isolated lens fiber cells. The 8-μm scale bar applies to both *A* and *B*. (*A*) Vesicles in normal Ringer solution, osmolarity 220 mosM. (*B*) Vesicles in twice normal NaCl Ringer solution, osmolarity 430 mosM. The vesicles have noticeably shrunk in the 430-mosM solution, suggesting that their membranes are intact. On return to normal Ringer solution, the vesicles resume the shapes seen in *A*.

lular clefts of the in vivo lens. One method of determining the orientation of a membrane vesicle is to look at the binding of fluorescently labeled wheat germ agglutinin (TR-WGA), which binds to the extracellular face of the cell membrane, where oligosaccharides of membrane glycoproteins are found. (WGA also binds to the membrane face lining the lumen of intracellular organelles, however, the lens fiber cells we used have no organelles.) Another method is to observe fluorescence of vesicles incubated in BCECF-AM. The BCECF-AM will enter all vesicles, but it is cleaved to its fluorescent form only if intracellular esterases are present in the vesicle. Vesicles that pinch off from the long, thin fibers may maintain varying degrees of esterase activity, depending on how long they are open, whereas broken pieces of membrane that form inside out vesicles would contain no esterase.

Table 1 lists the four possible outcomes of our double label experiment and the results we observed. >95% of the vesicles show varying degrees of fluorescence of BCECF; these vesicles have membrane integrity and esterase activity. Of those vesicles that labeled with BCECF, >60% also show some degree of fluorescence with TR, usually as a faint annular ring. If the TR-WGA molecules are first bound to NAG, none of the vesicles fluoresce with the rhodamine excitation filter. The 37.3% of the vesicles that do not label with TR could still be right side out, as discussed later. A few percent of the vesicle population label only with TR indicating either the lack of esterase activity or the disruption of the membrane and consequential loss of BCECF. <2% of the vesicles did not label with either fluorochrome.

Fig. 2 illustrates the electrophoretic mobility in standard lens Ringer's solution of vesicles formed from fiber cells at various radial depths of the frog lens. The electrophoretic mobility is reported in terms of the zeta potential using Eq. 1. Approximately 50 measurements were made at each of three radial depths. The data suggest no significant difference in electrostatic properties of fibers taken from any of these three radial locations. In normal lens Ringer, $\zeta = -9$ mV, a value similar to that obtained for other biological membranes studied (McLaughlin and Mathias, 1985; Pasquale et al., 1986). This value established that the intercellular clefts between lens fiber cells are lined with fixed negative charges.

TABLE 1 Summary of the sidedness experiments

	No fluorescence	BCECF only	BCECF and TR-WGA	Only TR-WGA
Counted (249)	3	93	147	6
Percentage (%)	1.2	37.3	59.1	2.4

The 249 vesicles observed were grouped according to their fluorescence.

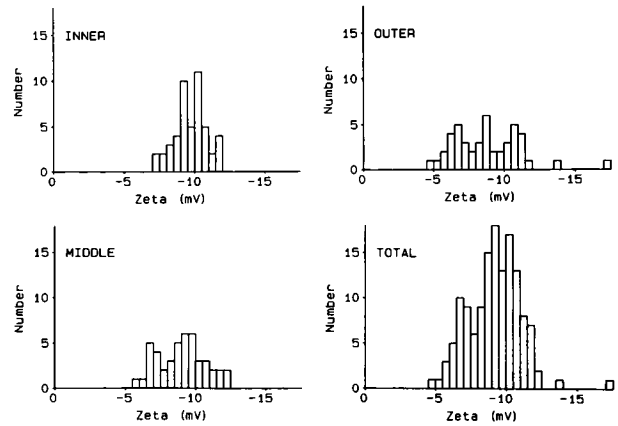

FIGURE 2 Histograms of the zeta potential (membrane surface potential) of vesicles made from fiber cell membrane. The zeta potential is calculated from the measured electrophoretic mobility using Eq. 1. The lenses were ~3 mm in diameter; the "other cortex" included fiber cells from the surface to ~0.25 mm into the lens; the "middle cortex" results are from fiber cells located between 0.25 and 0.5 mm from the surface; the "inner cortex" results are from cells located 0.5 to 1.25 mm from the surface. There is no significant difference in average the zeta potential measured in these three locations so we lumped all results together in the "total" histogram and found an average zeta potential of -9 ± 2 mV.

There did not appear to be a bimodal distribution of electrophoretic mobilities suggesting that all vesicles have the same orientation.

The fixed negative charges on the lens fiber cell vesicles were titrated with varying concentrations of Ca^{++}, Mg^{++}, and H^+. Fig. 3, *A* and *B*, shows ζ as a function of bulk Ca^{++} and Mg^{++} concentrations ranging from 1 to 32 mM. As described in the legend, the surface concentrations differ from bulk, but vary over the same order of magnitude. Each point represents an average of at least 80 electrophoretic mobility determinations ± SD. The effective dissociation constants of Ca^{++} and Mg^{++} with lens fiber cell vesicles are similar to those measured for vesicles made of mixtures of phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (McLaughlin et al., 1981). Broekhuyse and Veerkamp (1968) reported the phospholipid composition of fiber cell membranes from several animals. A typical fiber cell had 30% PC, 13% PS, 30% PE, 20% sphingomyelin (S), and the remainder was several minor components. The head groups of PC and S are the same, so our data on Ca^{++} and Mg^{++} can be most simply explained as a titration of lipid head groups. Binding did not vary as a function of radial depth, so the data shown represent averages from all of the measured mobilities.

Fig. 3 *C* shows the effect of pH on ζ. There is a dramatic reduction of surface negative charge when bulk pH is

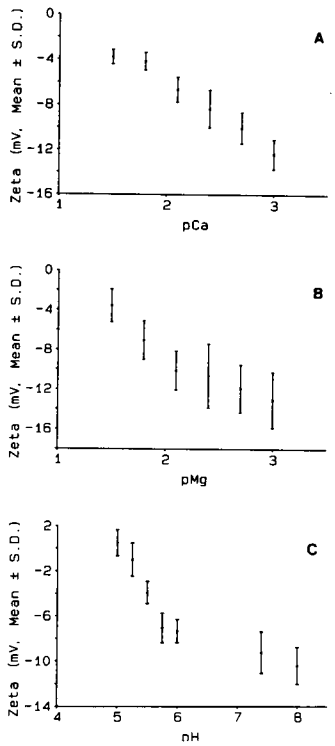

FIGURE 3 Titration of fixed negative charges on the outer membrane surface of lens fiber cells. Each titration curve was run using outer, middle, and inner cortical fiber cell membrane vesicles. However, no significant differences were observed, so we have presented averaged data from all three locations. For the curves in *A* and *B*, we replaced NaCl with either $CaCl_2$ or $MgCl_2$ on a charge for charge basis, thus allowing small changes in osmolarity. The screening of surface charges with Ca^{++} or Mg^{++} produces binding curves that are characteristic of the common lipid head groups. Whereas the binding of H^+ requires a more complex explanation and probably represents binding to charged groups on some membrane protein, and perhaps a conformational change in the protein.

The data are reported as a function of bulk concentration, whereas the concentration at the membrane surface will differ from bulk whenever $\zeta \neq 0$. If we assume the charges are uniformly smeared at the plane of the membrane surface, then the Boltzman relationship predicts the surface concentration to equal bulk concentration times exp $(-\zeta FZ/RT)$. Thus, we estimate that as the bulk Ca^{++} or Mg^{++} concentrations vary between 1 and 32 mM, the surface concentrations vary between 2.6 and 44 mM. For H^+, the bulk concentration varies between 10 nM and 10 μM and we estimate that the surface concentration varies between 15 nM and 10 μM.

changed between 5 and 6. Although the pH at the vesicle surface differs from bulk pH, as described in the legend, the vesicles experience essentially the full range of bulk pH change. Titration of common lipid head groups (PC, PE, or PS) does not occur over the pH range of 5 to 7 (Tsui et al., 1986), so the effect of H^+ on lens vesicles is evidently through some membrane protein.

DISCUSSION

We have shown that lens fiber cell membranes have fixed negative charges on their outer surface. Mathias and Rae (1985) reported an electric field of ~0.2 V/cm along the intercellular clefts of the small grass frog lens. Our experimental data demonstrate a zeta potential in normal Ringer's solution of −9 mV (electrophoretic mobility = 0.9 μm/s per V/cm). The product of the electric field and electrophoretic mobility gives the electroosmotic fluid velocity at distances from the cleft surface greater than a few Debye lengths. Thus, electroosmosis can generate a fluid velocity of ~0.6 mm/h, directed from the aqueous or vitreous toward the center of the lens, along the extracellular clefts. This flow is in the same direction as that hypothesized by Mathias (1985) based on transmembrane sodium fluxes. As described below, these data support the model that the lens generates an internal circulation of fluid, which convects metabolites to and from fiber cells, thus acting as a miniature circulatory system for this avascular organ.

The evidence for fluid circulation in the lens is indirect (Mathias et al., 1985; Mathias, 1985; Mathias and Rae, 1985), but the data presented here have some direct consequences. The measured electric field and zeta potential act as an electrophoretic force to move fluid along the extracellular clefts. If fluid flow does not exist, then a hydrostatic pressure must oppose the electrophoretic force. This would require a positive pressure between the innermost fiber cells. Structural data, however, suggest that the innermost cells are most tightly packed together, hence the existence of a positive hydrostatic pressure seems unlikely. We feel that the data presented in this paper are most consistent with the model proposed by Mathias (1985) where fluid movement is osmotically generated by a circulating sodium flux. Electroosmosis contributes to the extracellular flow but is not sufficient to drive an extracellular flux equal to the transmembrane fluid flux. Thus, there is a negative hydrostatic pressure in the clefts between fiber cells, and this pressure, together with the electrophoretic force, drives the movement of fluid along the extracellular spaces towards the lens center.

Titration studies

We have studied the binding of Ca^{++} and Mg^{++} and conclude that there are no high-affinity binding sites on lens fiber cell membranes for these cations, whereas the binding of hydrogen appears to be to some membrane protein. The large change in vesicles surface charge observed in the range of pH 5–6 is probably not a simple

titration. It is more likely that the binding of H^+ to some protein induces a conformational change that amplifies the effect on surface charge. Red blood cell membranes are not affected by H^+ in the range of pH 5–7 (Pasquale et al., 1986). One difference between lens vesicles and red blood cells is the gap junctional component of membrane in the fiber cell vesicles. But some fraction of the vesicles should have no junctional membrane, whereas the electrophoretic mobility of every vesicle we examined was strongly affected by pH. Another possibility is that the pH effect is mediated by the main intrinsic polypeptide (MIP or MP 26) of fiber cell membranes. MIP is found in both junctional and nonjunctional membranes (Zampighi et al., 1989). There are likely to be other possibilities that we have not considered. Further work is needed to identify the mechanism of the pH effect.

Sidedness studies

There is no unequivocal experiment to determine sidedness, but all of our data suggest that the vesicles were predominantly right side out. The procedure of gently triturating the long thin fiber cells was designed to produce vesicles whose orientation was right side out. The cleavage of BCECF-AM to BCECF by intracellular esterases is consistent with vesicles that pinched off from fibers and sealed within 1 s, thus trapping the esterases. The few vesicles that did not show fluorescence from BCECF could be (*a*) inside out, (*b*) leaky, or (*c*) right-side out and sealed, but have lost their esterase activity during formation. The binding of WGA to a large percent of the vesicles also suggests they are right side out, particularly when the same vesicle contained BCECF. There are several possible explanations for those vesicles that did not show TR fluorescence. We overlaid photographs of the vesicles seen in bright field with fluorescent images from BCECF and TR. WGA-TR binding appears as a faint annular ring of fluorescence around the periphery of the vesicles, so we could easily have missed a TR labeled vesicle that was not in focus when photographed. Moreover, many vesicles should have been formed from membrane domains that were predominantly gap junctional and these domains may not bind WGA. The histograms in Fig. 2 show no obvious bimodal distribution of surface charge and the mean value of $\zeta = -9$ mV is similar to that found for the extracellular surface of other cells. Moreover, the titration studies in Fig. 3 did not reveal two populations of vesicles. Because biomembranes are generally not symmetric, our data suggest that essentially all of the vesicles were right-side out.

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