Alterations of the apparent area expansivity modulus of red blood cell membrane by electric fields

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ABSTRACT Red blood cell membrane exhibits a large resistance to changes in surface area. This resistance is characterized by the area expansivity modulus, K, which relates the isotropic membrane force resultant, $\bar{T}$, to the fractional change in membrane surface area $\Delta A/A_o$. The experimental technique commonly used to determine K is micropipette aspiration. Using this method, E. A. Evans and R. Waugh (1977. *Biophys. J.* 20:307–313) obtained a value of 450 dyn/cm for the modulus. In the present report, it is shown that the value of K, as determined using this method, is affected by electric potential differences applied across the tip of the pipette. Using Ag-AgCl electrodes and current clamping electronics, we obtained values for K ranging from 150 dyn/cm with -1.0 V applied, to 1,500 dyn/cm with 1.0 V applied. At 0.0 V the modulus obtained was ~500 dyn/cm. A reversible, voltage- and pressure-dependent change in the cell volume probably accounts for the effect of the voltage on the calculated value of the modulus. The use of lanthanum chloride or increasing the extra- and intracellular solute concentrations reduced the voltage dependence of the measurements. It was also found that when dissimilar metals were used to "ground" the pipette to the chamber to prevent lysis of cells by static charge, values for K ranged from 121 to 608 dyn/cm. Based on measurements made at zero applied volts, in the presence of 0.4 mM lanthanum and at high solute concentration, we conclude that the true value of the modulus is ~500 dyn/cm.

INTRODUCTION

Because of its structure and physiological function, the red blood cell is an ideal system for studying the mechanical behavior of cell membranes. Biological membranes, in general, exhibit a large resistance to changes in surface area (1). The resistance of the membrane to area dilation is characterized by the area expansivity modulus, K. It relates the isotropic membrane force resultant, $\bar{T}$, to the corresponding fractional changes in surface area, α: $\bar{T} = K\alpha$.

Micropipette aspiration of single cells is a powerful and widely used technique for determining material coefficients of the membrane. Aspiration pressures applied to the surface of the cell produce force resultants (tensions) in the plane of the membrane which produce deformations of the membrane. Using this technique, Evans and co-workers (2, 3) determined a value for the expansivity modulus of 450 dyn/cm. Recently, we have observed that voltage differences between the shaft of the pipette and the suspending buffer can have a profound effect on the measured value of the modulus. In the present report we describe the effects of well-controlled electric fields on the measured value of the modulus. The likely mechanism responsible for the effect is a pressure- and voltage-dependent change in cell volume. We also describe conditions under which the "true" value of the modulus can be obtained.

MATERIALS AND METHODS

The methods for cell preparation and measurement were identical to those described in a companion paper (4), with the following additions:

Electrical system

To mimic typical conditions under which expansivity measurements have been performed in the past, current-clamping electronics were omitted in some cases during the present study. Pipette electrodes consisted of thin wires inserted into the back of the pipettes. Two types of chamber electrodes were used. One consisted of thin wires epoxied to the inside of the glass microscope chamber. The second consisted of "U" shaped metallic spacers which were sealed to microscope slides with vacuum grease to form the chamber. A variety of metals were used to make the electrodes: silver, platinum, steel, brass, copper, and Ag-AgCl. The chamber electrode was directly connected to the pipette electrode with an unshielded wire. For all measurements, the electrical continuity between the pipettes and the pressure transducer was disrupted by a section of nonconducting paraffin oil in the hydraulic line.

Measurements and calculations

Osmotically swollen cells were aspirated into micropipettes (Fig. 1). This aspiration produced isotropic tensions in the plane of the membrane causing measurable surface area dilations. Two different pressure sequences were used depending on the behavior of the cells. With the first method, cells were initially aspirated at 1 cmHg and the pressure was increased in increments up to a maximum of 6 cmHg and then returned to its initial value of 1 cmHg. With the second method, cells were aspirated at low (~1.0 cmHg) and high (~6 cmHg) pressures in an

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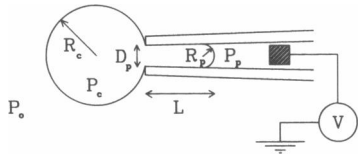

FIGURE 1 Schematic representation of a micropipette aspirated cell. The cell was osmotically swollen until nearly spherical. A negative pressure ($P_p - P_o$) applied to the back of the pipette draws the cell into the pipette a length L. Because the interior pressure of the cell P_c is greater than both the exterior pressure P_o and the pressure in the pipette P_p, the portion of the cell outside the pipette forms a sphere of radius R_c and edge of the cell projection inside the pipette forms a hemispherical cap of radius R_p (the inner diameter of the pipette, D_p, is optically measured at the tip and is constant along the length of the cell projection).

alternating sequence, with the final pressure being the same as the initial.

There is a linear relationship between the fractional surface area dilation of the membrane ($\alpha = \Delta A/A_o$) and the corresponding membrane isotropic tension ($\bar{T}$). The coefficient of proportionality that relates these two quantities is the area expansivity modulus K (2),

$$\bar{T} = K\alpha. \tag{1}$$

The relationship between the isotropic tension, $\bar{T}$, and the aspiration pressure, ΔP, is obtained by considering the balance of forces on the membrane (1):

$$\bar{T} = \frac{\Delta P\, R_o\, R_p}{2(R_o - R_p).} \tag{2}$$

The surface area was calculated from measurements of the initial outer cell radius (R_o), the length of the cell projection in the pipette (L), and the pipette inner radius (R_p) (Fig. 1) by the relationship

$$A = 2\pi R_p L + 4\pi R_o^2 - \pi R_p^2. \tag{3}$$

The change in surface area for a change in projection length is given by:

$$\Delta A \simeq 2\pi R_p \Delta L\,(1 - R_p/R_o) + 2\Delta V/R_o, \tag{4}$$

where ΔV is the calculated cellular volume change. The calculated change in volume is due to a reversible volume shift caused by the hydrostatic pressure imposed on the cell. In effect, the process of micropipette aspiration "squeezes" water from the cell. This effect has been described previously (2). The change in volume caused by the applied pressure is a function of the isotropic tension in the membrane ($\bar{T}$), the cellular surface area inside the pipette (A_i) and outside the pipette (A_o), the radius of the pipette (R_p), the radius of the cell outside the pipette (R_o), the intracellular ionic concentration (C_i), the fraction of osmotically active intracellular water (R_w), and the gas constant times the absolute temperature (β). The relationship takes the form (2):

$$\Delta V/V_o = -2R_w\,\bar{T}(A_i/R_p + A_o/R_o)/[\beta C_i(A_i + A_o)]. \tag{5}$$

An additional correction to the calculation of the area change is introduced because of a drift in the "unstressed" (reference) projection length, L_o. The origin of this drift is not known but it may be due to increased solute concentration in the suspending medium caused by evaporation, or to a gradual leak of cations from the cell. It is observed to be linear over the time of the expansivity measurements and only weakly dependent on the applied pressure (Fig. 2). The reference length at any time t during the measurement, $L_o(t)$, can be estimated by interpolation of initial (L_i) and final (L_f) reference lengths measured at times t_i and t_f, respectively:

$$L_o(t) = L_i + (L_f - L_i)\,(t - t_i)/(t_f - t_i). \tag{6}$$

For cells measured using an incremental pressure sequence, the modulus K was obtained by linear regression to the (α, $\bar{T}$) data pairs. These points were calculated from the measured values for pressure and the cell dimensions obtained from the video recordings of the experiments. Fig. 3 *a* shows a typical plot of $\bar{T}$ vs. α with (*solid circles*), and without (*open circles*) the correction for the time-dependent, irreversible projection length changes.

For cells measured using the alternating low-high pressure sequence the mean α at high $\bar{T}$, and the mean α at low $\bar{T}$, were calculated. The modulus K was calculated as the slope of the line connecting these mean values:

$$K = \frac{\langle \bar{T}_{high} \rangle - \langle \bar{T}_{low} \rangle}{\langle \alpha_{high} \rangle - \langle \alpha_{low} \rangle}. \tag{7}$$

The brackets $\langle\,\rangle$ indicate mean value. Fig. 3 *b* is a typical plot of $\bar{T}$ vs. α obtained by this method.

In some cases when the modulus was extremely large, the small change in projection length was near the limit of our ability to resolve it optically. Small errors in measurement could result in calculated values of K that were infinite, or even negative. To limit the inclusion of erroneous values into the reported data set we established criteria for "goodness of fit" for the data to be included in the reported sample means. For the data obtained using method one (successive increments in pressure), we required that the correlation coefficient for the linear regression be >0.6. For method two (repeated high and low pressures), the standard deviation of K was used to screen out irregular cells. The standard deviation for K was calculated from the standard deviations of

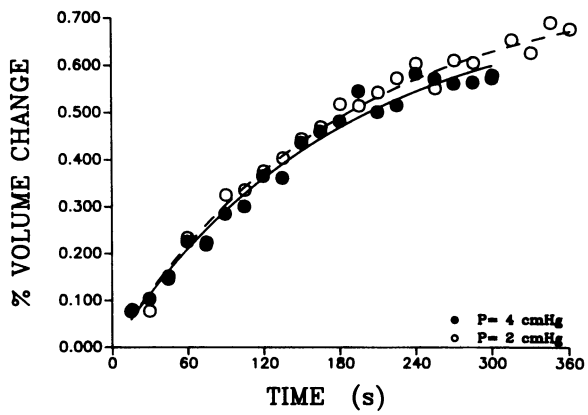

FIGURE 2 "Irreversible" changes in projection length with time at constant pressure and (zero) voltage. Open and solid circles represent measurements made on cells aspirated at 2.0 and 4.0 cmHg, respectively. Changes in projection length are normalized with respect to cell volume. Curves are first-order exponentials fit to the data: 2.0 cmHg, dashed; 4.0 cmHg, solid. The time constant for the response was ~3.0 min. Over the short duration of the area expansivity measurements (~2.0 min) the change in length with time is approximately linear.

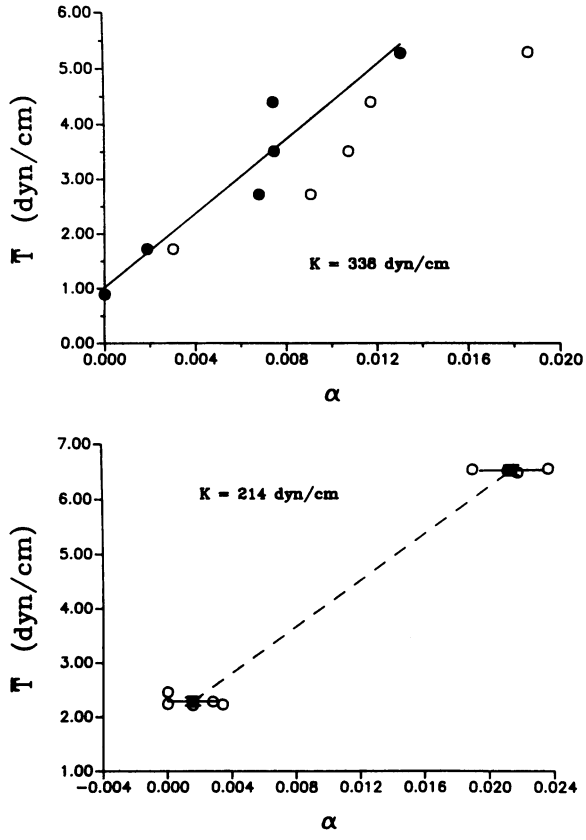

FIGURE 3 (*a*) Typical response of micropipette aspirated cell to incremental changes in aspiration pressure. The initial pressure was 1 cmHg. The pressure was increased in 1.0 cmHg increments to a maximum of 5 cmHg. The isotropic membrane tension was calculated via Eq. 2. The corresponding fractional area change was calculated according to Eqs. 3–6 (*solid circles*). The importance of the correction for unrecovered length change (Eq. 6) is illustrated by the open circles, which do not reflect this correction. The expansivity modulus, K, is determined by linear regression to the data (*solid curve*). (ϕ = 0 V) (*b*) Response of the cell to a repeated, alternating sequence of high and low pressures. The solid squares represent the means of the individual measurements (*open circles*) at each pressure. The slope of the dashed line connecting the means is the area expansivity modulus, K. (ϕ = 0 V)

the average $\bar{T}$'s and α's using the method of differentials.

$$dK = K\left[\frac{d\bar{T}_h - d\bar{T}_l}{\bar{T}_h - \bar{T}_l} - \frac{d\alpha_h - d\alpha_l}{\alpha_h - \alpha_l}\right]. \quad (8)$$

A standard deviation of >60% of the value of K was criteria for rejection of the cell from the sample.

Generally, less than one cell in 20 was excluded by these criteria, except at positive voltages of 0.5 V and higher. For large positive voltages the sample size reported is a subset of ~20 cells measured.

RESULTS

Positive voltages (chamber electrode is ground) applied across aspirated red cells increased the apparent resistance of the cell membrane to surface area dilation. With 1.0 V applied between the pipette and chamber electrodes there appeared to be an increase in the expansivity modulus of the aspirated cell membrane to ~1,500 dyn/cm. Applying negative potentials decreased the apparent resistance of the membrane to surface area dilations. The calculated value for K with −1.0 V applied across the two electrodes was ~150 dyn/cm. At zero applied volts, a value of 500 dyn/cm was obtained for the modulus (Fig. 4). Data points represent sample means from 20 measurements except for the 0.5 V and 1.0 V points, where n = 14 and n = 5, respectively. It must be emphasized that the voltage reported here is the total potential difference between the electrodes. Because the resistance of the "seal" between the cell and the pipette is low, the potential drop across the cell itself is likely to be considerably smaller. The exact potential across the cell cannot be calculated because of anomalous changes in resistance when the cell is aspirated in the pipette ([4], Appendix A).

To test the dependence of the measurement on solute concentration, cells were loaded with cations using the drug nystatin so that swollen cells could be obtained with high intracellular solute concentrations. The modulus was measured as a function of concentration at zero volts. There appeared to be a decrease in the calculated value for K as the intracellular solute concentration was increased (Fig. 5). The extrapolated value of K at $1/Ci$ = 0, was 425 dyn/cm, in good agreement with the results of Evans and Waugh (2).

The presence of low concentrations of multivalent

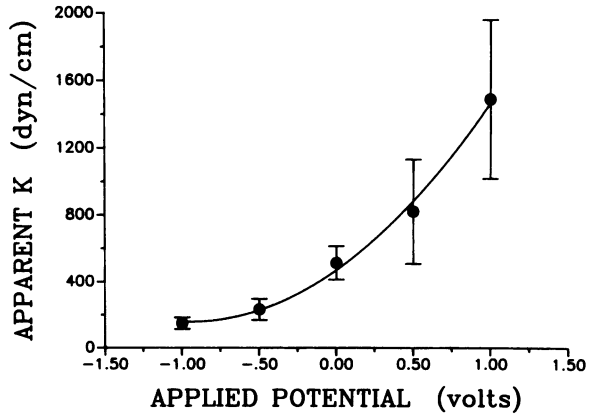

FIGURE 4 Calculated area expansivities as a function of electric potentials applied across the pipette and chamber electrodes. Closed circles represent sample means of twenty measured cells, except for the +0.5 V and +1.0 V points where n = 14 and n = 5, respectively. The error bars represent plus or minus one standard deviation of the mean. The solid line is an arbitrary curve drawn through the data. The empty pipette resistance (resistance between the electrodes without a cell aspirated into the pipette tip) ranged from 170–340 MΩ for these measurements.

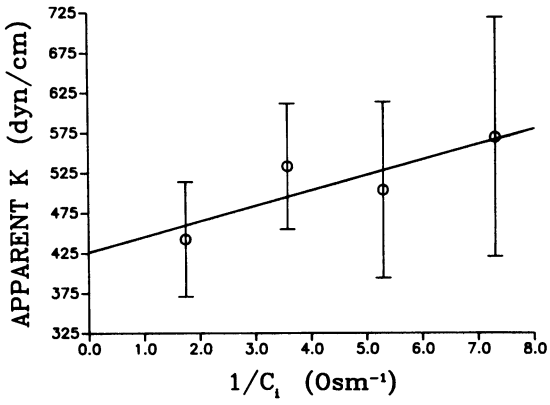

FIGURE 5 Area expansivities of nystatin-treated red cells as a function of the inverse of solute concentration. The applied potential was held at zero volts by the WPI system. Open circles represent sample means for twenty cells. The solid line is a linear regression to these means. Error bars represent plus or minus one standard deviation. The extrapolated intercept at infinite solute concentration is 425 dyn/cm. The pipette resistance was 23 MΩ.

cations reduced the effects of the applied potentials on the measurements (Fig. 6). The concentrations of the different ions in the suspending solutions were those determined by Seaman and Pethica (5) to be the charge reversal concentration in electrophoretic mobility measurements. Lanthanum (0.4 mM) was more effective than cobalt (9 mM) or manganese (13.7 mM, not shown) at limiting the effect of applied potentials on the calculated expansivity modulus. At these concentrations, with zero applied volts, the value of K in the presence of $LaCl_3$ (550 dyn/cm) was closer to the literature value (450 dyn/cm) than the value obtained in the presence of cobalt (760 dyn/cm) or manganese (860 dyn/cm).

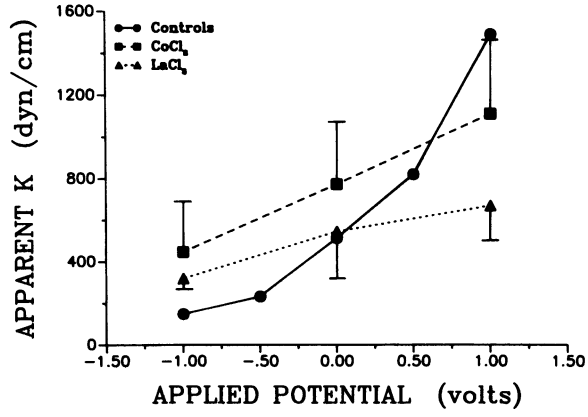

FIGURE 6 Apparent area expansivity as a function of potential for cells in the presence of charge reversal concentrations of lanthanum (0.4 mM, *triangles*) and cobalt (9.0 mM, *squares*). Pipette impedance was 77–83 MΩ. Controls are indicated by solid circles (pipette impedance was 150 MΩ). Each point represents the mean of 20 measurements and error bars represent one standard deviation.

Measurements were performed that illustrate the magnitude of the errors that can occur under conditions previously employed in our laboratory, and typically employed in other laboratories when measuring the membrane expansivity modulus. The influence of the type of metal and glass used to make the chamber, the type of metal used to make electrical contact in the pipette, and the type of glass used to make the micropipette were investigated. Under these "typical" conditions, current clamping electronics are not used, and a simple unshielded wire was used to make an electrical connection between the shaft of the pipette and the chamber. The results of these measurements are summarized in Tables 1 and 2.

The measured value of the modulus was strongly influenced by the materials used to make the chamber and pipette electrodes (Table 1). The calculated modulus varied from 120 dyn/cm to 608 dyn/cm depending on the combination of metals used to make the electrodes. The glass used to make the chamber can also influence the value of K (Table 2). The value depended not only on the type of glass in the chamber where the measurements were made, but also on the composition of the chamber in which the cells were stored before measurement. In a set of experiments, cells were suspended in one chamber, then a single cell was transferred to a second chamber to perform the measurement. Silver wire was used for both the pipette and chamber electrodes. Cells suspended in a chamber made of siliconized glass and transferred to a chamber also made of siliconized glass had a mean modulus of 249 dyn/cm. Cells suspended in a chamber made of "nesa" glass (tin oxide coated) and transferred to a chamber made of siliconized glass for the measurement exhibited similar behavior with a mean modulus of 245 dyn/cm. Cells suspended in a "nesa" glass chamber and transferred to a "nesa" glass chamber appeared to have much smaller expansivity moduli, K = 180 dyn/cm, and cells stored in a siliconized glass chamber and transferred into a "nesa" glass chamber exhibited the smallest expansivity moduli, 81.6 dyn/cm.

Pipette electrode	Chamber electrode	n	Apparent K (dyn/cm) mean	(SD)
Silver	Steel	20	413.0	(184.0)
Silver	Glass-silver	20	370.0	(93.8)
Silver	Glass-steel	20	332.2	(67.2)
Silver	Brass	20	273.3	(88.1)
Silver	Copper	22	120.5	(39.4)
Platinum	Steel	20	608.0	(184.0)
Platinum	Glass-steel	20	487.0	(108.0)
Platinum	Glass-platinum	20	271.0	(176.0)
Steel	Steel	60	502.0	(110.0)

TABLE 2

Suspension chamber	Measurement chamber	n	Apparent K (dyn/cm) mean	(SD)
Siliconized glass	Siliconized glass	24	249.0	(16.5)
Nesa glass	Siliconized glass	23	245.0	(107.0)
Nesa glass	Nesa glass	20	180.0	(129.0)
Siliconized glass	Nesa glass	20	81.6	(10.1)

In contrast to the strong dependence of the apparent modulus on the chamber composition, the modulus was relatively insensitive to modifications of the pipette used to aspirate the cell. Neither substitution of Boralex glass for Kimax, nor treatment of the Kimax glass with a siliconizing agent (Surfasil; Pierce Chemical Co, Rockford, IL) had a significant effect on the measurement.

DISCUSSION

In assessing the mechanism for the voltage dependence of the area expansivity measurement, we are hampered by an inherent limitation of the micropipette technique. Measurements of aspirated cell deformations cannot distinguish directly between changes in surface area and changes in cell volume. This limitation arises because changes in the outer radius of the cell during the measurement are too small to be resolved optically. Only changes in the projection length (L) can be detected accurately. Consequently, changes in surface area cannot be calculated without making assumptions about what is happening to the cell volume. It is essential to know the contribution of volume changes to the net change in projection length to calculate the modulus accurately.

In a separate report, we describe a reversible, voltage-induced change in the volume of micropipette-aspirated red cells (4). The volume of the cell decreases (projection length increases) when negative voltages are applied, and the volume increases (projection length decreases) when positive voltages are applied. The magnitudes of these changes depend on the aspiration pressure used to hold the cell in the pipette. The larger the aspiration pressure, the larger the change. Additionally, the magnitude of the change is inversely proportional to the solute concentration, and is reduced by the presence of multivalent cations in the suspending medium.

Because the change in volume with voltage is pressure dependent, it is likely that this is the mechanism that accounts for the apparent change in K with voltage. If the change in cell volume with voltage were not pressure dependent, only the initial length of the projection during constant voltage measurements would be affected by the applied field. Changes in the projection length in response to changes in pressure would not be affected by the voltage. However, because the field-induced change in the cell volume is pressure dependent, a change in volume is expected to occur at fixed voltage and changing pressure. Thus, the change in projection length with pressure will reflect a contribution from the change in cell volume, and the volume contribution will depend on the voltage applied. For example, if a negative voltage is applied, the volume of the cell will decrease as the pressure is increased, causing a larger increase in the projection length than would occur without the applied voltage. This results in an overestimate of the change in surface area (thus leading to a calculated modulus that is too small). On the other hand, if a positive voltage is applied, the cell volume will increase as the pressure increases, causing a smaller change in the projection length and an underestimate of the change in surface area (thus leading to a calculated modulus that is too large).

To test this hypothesis, we used the measurements of the change in cell volume with voltage at different (constant) aspiration pressures (4) to estimate the change in cell volume that would be expected for a given pressure change at a given voltage. We then used this change in volume in Eq. 4 to calculate the "real" change in area. As is illustrated in Fig. 7, this correction eliminated the apparent potential dependence of the modulus. It is important to recognize that this result does not prove the hypothesis that it is a volume change that mediates the effect. (One could just as easily argue that it was the voltage dependence of the modulus that accounted for the pressure dependence of the change in projection length with voltage.) However, it does demonstrate that these two phenomena are different manifestations of the same

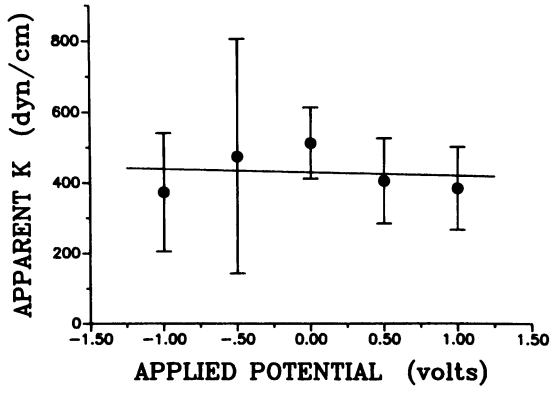

FIGURE 7 Dependence of the area modulus on potential. The data presented in Fig. 4 were "corrected" for changes in cell volume with applied potential. The corrections were obtained from measurements of the change in projection length with voltage at different pressures (4). Filled circles represent the means of 20 calculations; error bars represent plus or minus one standard deviation. The solid line is a linear regression to the means.

effect, and that they probably arise from the same underlying mechanism. The dependence of the effect on the inverse of the solute concentration leads to the conclusion that it is a change in cell volume that accounts for these phenomena. The influence of lanthanum and cobalt on the voltage dependence of the modulus measurement is consistent with this reasoning. That is, under conditions in which the voltage-dependent change in volume is reduced, the voltage dependence of the modulus measurement is also reduced.

The present results indicate a slight dependence of the expansivity modulus on salt concentration (Fig. 5). This is in contrast to the results previously reported by Evans and Waugh (2). In that report it was found that inclusion of the volume correction (Eq. 5) in the calculation of the expansivity modulus produced values for K that were independent of solute concentration. The present results show a decrease in the modulus from ~550 dyn/cm at 140 mOsmol to ~430 dyn/cm at 575 mOsmol. Evans and Waugh (2) did not control for possible electric fields at the pipette tip, and it is likely that the difference between those early observations and the present results is due to small fields present in the early studies. The present results are consistent with observations that the relationship between surface pressure and area per molecule in lipid monolayers depends on electrolyte concentration (6).

Despite the lack of a concrete mechanism to explain the cellular behavior observed in this study, conclusions can be made about the "real" value for the expansivity modulus of the red blood cell membrane. A value of ~500 dyn/cm was obtained under all conditions in which the voltage-dependent change in cell volume was minimized (4): when the aspirated cell was exposed to zero applied volts, in the presence of 0.4 mM La, and as the solute concentration became very high.

The variability in the values obtained for K under "typical" conditions (Tables 1 and 2) indicates that the values obtained by other investigators under conditions in which electric fields are not well controlled might be in serious error. This variability is almost certainly the result of contact potentials between the different metals and the suspending buffer, additional potentials from environmental sources (60 cycle noise), and, possibly, effects of dissolved metal ions in the suspending phase. Our intention is not to perform a detailed study of these artifacts, but simply to demonstrate the magnitude of the errors that are possible. Clearly, caution must be exercised when evaluating results of micropipette aspiration experiments used to determine the area expansivity modulus for red blood cell membrane in which electric fields at the pipette tip have not been carefully controlled. To avoid cell lysis by static charge, it is necessary that pipettes be electrically grounded to the chambers during such measurements. Because such grounding completes an electrical circuit containing the high impedance pipette tip, proper shielding from extraneous electric fields and careful monitoring of the transcellular potential are essential. Conclusions drawn from results obtained without taking these precautions may be erroneous because of the influence of electric fields on the measurements.

These results demonstrate that the combined application of electric fields and mechanical forces can have significant and unexpected effects in studies using micropipettes or microelectrodes. Identification and characterization of the mechanism underlying these effects should be of interest to all investigators engaged in such studies.

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