Role of the Membrane Cortex in Neutrophil Deformation in Small Pipets

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ABSTRACT The simplest model for a neutrophil in its "passive" state views the cell as consisting of a liquid-like cytoplasmic region surrounded by a membrane. The cell surface is in a state of isotropic contraction, which causes the cell to assume a spherical shape. This contraction is characterized by the cortical tension. The cortical tension shows a weak area dilation dependence, and it determines the elastic properties of the cell for small curvature deformations. At high curvature deformations in small pipets (with internal radii less than 1 μm), the measured critical suction pressure for cell flow into the pipet is larger than its estimate from the law of Laplace. A model is proposed where the region consisting of the cytoplasm membrane and the underlying cortex (having a finite thickness) is introduced at the cell surface. The mechanical properties of this region are characterized by the apparent cortical tension (defined as a free contraction energy per unit area) and the apparent bending modulus (introduced as a bending free energy per unit area) of its middle plane. The model predicts that for small curvature deformations (in pipets having radii larger than 1.2 μm) the role of the cortical thickness and the resistance for bending of the membrane-cortex complex is negligible. For high curvature deformations, they lead to elevated suction pressures above the values predicted from the law of Laplace. The existence of elevated suction pressures for pipets with radii from 1 μm down to 0.24 μm is found experimentally. The measured excess suction pressures cannot be explained only by the modified law of Laplace (for a cortex with finite thickness and negligible bending resistance), because it predicts unacceptable high cortical thicknesses (from 0.3 to 0.7 μm). It is concluded that the membrane-cortex complex has an apparent bending modulus from 1×10^{-18} to 2×10^{-18} J for a cortex with a thickness from 0.1 μm down to values much smaller than the radius of the smallest pipet (0.24 μm) used in this study.

In the resting or passive state, the human neutrophil is shaped like a sphere. However, the surface area of the sphere is not smooth, but consists of many small folds and projections that collectively form an "excess surface area" that is about twice as large as the projected surface area of the sphere (Evans and Yeung, 1989; Ting-Beall et al., 1993). This excess surface area allows the passive cell to deform into an elongated sausage-like shape as it flows into the small capillaries in vitro (Bagge et al., 1977; Evans and Yeung, 1989; Needham and Hochmuth, 1990) and in vivo (Warnke and Skalak, 1992). Because the cytoplasm of the cell behaves as a liquid rather than a solid, the spherical shape of the cell is caused by a persistent cortical tension (Evans and Yeung, 1989; Needham and Hochmuth, 1992) at a thin domain adjacent to the cell surface rather than some kind of internal elastic structure. The cortical tension pulls the deformed neutrophil, within about 15 s to 1 min (Sung et al., 1988; Tran-Son-Tay et al., 1991; Hochmuth et al., 1993), into a sphere just as the surface tension at an air-water interface causes small water drops (fog) to form spheres. The cortical tension for the neutrophil has been measured successfully in several experiments and found to be quite small — on the order of 10^{-2} mN/m (Evans and Yeung, 1989; Needham and Hochmuth, 1992). It has a very small area dilation dependence, with an apparent area expansion modulus on the order of 0.04 mN/m (Needham and Hochmuth, 1992). Because of the negligible apparent area expansion modulus, when a neutrophil is aspirated into a pipet with increasing suction pressures, it resists the deformation until it forms a hemispherical projection inside the pipet (the corresponding suction pressure is called "critical suction pressure"). Then the cell flows completely into the pipet at constant suction pressure.

It is not known what causes the cortical tension. The existing experimental results suggest that the region where the cortical tension is generated has a much smaller thickness than the cell radius. It is known that the neutrophil resistance for deformation in large pipets is described by the law of Laplace (Evans and Yeung, 1989). Evans and Kukan (1989) proposed the so called "contractile surface carpet" model, where the cortical tension is assumed to arise from contractile elements tangent to the membrane surface. They hypothesize that the cytoplasm membrane is associated with a relatively thick cortical shell (cortex), which is the source of the cortical tension.

Evidence for the existence of a thick cortex is mixed. Transmission electron micrographs have shown a relatively thick 0.05–0.1 μm cortex (Bray et al., 1986; Esaguy et al., 1989; Sheterline and Rickard, 1989). Other micrographs show no apparent cortex in that granules are observed to touch the surface of the membrane: they are not bounded away by a cortex. Fluorescent probes that are specific to F-actin sometime show significant fluorescence at the membrane surface (Sheterline et al., 1986) and other times show only a diffusive cytoplasmic fluorescence (Wallace et al., 1984).

If a relatively thick cortex exists, then the cortical tension will be an apparent characteristic of its middle surface (or "neutral surface"). When neutrophil is deformed into a pipet, the radii of curvatures of the middle surface of the cortex will

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be smaller than the pipet radius. Then the predicted critical suction pressures, when the pipet radius is used to estimate the curvature radii, will be smaller than the measured ones. The difference between the predicted and the measured critical suction pressures will be negligible for large pipets (because the pipet radius is much larger than the cortical thickness), but it can become significant for small pipets (if the pipet radius is on the same order of magnitude as the cortical thickness). It is possible, therefore, by measuring the critical suction pressure for different size pipets and comparing these values with the values predicted by the law of Laplace (where the radii of curvature are chosen to be equal to the pipet radius), to establish the existence of a cortex.

The purpose of this work is to determine, by mechanical means, whether there is a cortex. To do this, a portion of the neutrophil is aspirated into a large pipet (with radius above 1.2 μm) and the cortical tension is measured by the method of Evans and Yeung (1989). Then the critical suction pressure for the same cell is measured for pipets of increasingly smaller internal diameters (down to the limit of resolution of the light microscope, ~0.5 μm). If a relatively thick cortex exists, the surface where the apparent cortical tension acts will be shifted from the cell surface. The membrane-cortex complex can also have a resistance to bending. In the first case, when a cortex with a finite thickness exists, the suction pressure necessary to deform the neutrophil into the pipet will be larger than its estimate from the law of Laplace. In the second case, when the membrane-cortex complex also has a bending resistance, the estimates of the cortical tension for large and small pipets will be different (the estimates for large pipets will be smaller than that for small pipets).

It is found that the estimated suction pressures are smaller than the experimentally measured ones for pipets with radii less than 1 μm. These elevated suction pressures can be explained only if a 0.3–0.7 μm thick cortex exists. This is larger than the radius of the smallest pipet (0.24 μm) used in this study and the thicknesses of the cortex (from 0.05 to 0.1 μm), measured by the electron microscopy (Esaguy et al., 1989; Sheterline and Rickard, 1989). In addition, it is found that the estimate of the cortical tension for large pipets is from 0.024 mN/m to 0.035 μm, whereas for small pipets it is 0.051 mN/m. These results suggest that there is a cortex with a finite thickness and bending rigidity. The apparent bending modulus of the cortex is from 1×10^{-18} to 2×10^{-18} J for a cortical thicknesses from 0.1 μm down to the thickness of the plasma membrane. These values are an order of magnitude larger than the bending moduli for bilayer membranes and the red blood cell membrane (see Table 1). In a companion paper (Zhelev et al., 1994), it is shown that small diameter pipets can measure the known bending modulus of lipid bilayer, therefore providing an independent validation of the method used here. Thus, it is shown by mechanical means that a cortex exists on the inner surface of the neutrophil.

MATERIALS AND METHODS

Cell Preparation

The preparation of neutrophils has been described in detail elsewhere (Needham and Hochmuth, 1992). Briefly, venous blood was drawn from one of four healthy adult donors into vacutainers containing EDTA as an anticoagulant (in one of the experiments the anticoagulant was lithium heparin). The neutrophils were separated on a Ficoll-Hypaque gradient (Sigma Histopaque-1077 and -1119) at 800 × g for 20 min. The cells were collected at the 1077/1119 interface and washed once with Ca^{2+} and Mg^{2+} free modified Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO). Finally, the cells were resuspended in a 50% autologous plasma/HBSS solution. In the final cell suspension were some red blood cells that were used for an estimate of the excess suction pressure caused by shear elasticity. All of the procedures and the experiment were done at 23°C.

Micromanipulation

An inverted Leitz microscope with a 100× oil immersion objective was used in the experiments. The experimental chamber was 3 mm thick and open on both sides for micromanipulation. The chamber was trans-illuminated at 435 ± 4 nm, which reduced the diffraction patterns around the pipet. Micropipets were made from 0.75 mm capillary glass tubing pulled to a fine point with a vertical pipet puller. The tip of the pipet was immersed in a melted glass bead made of a glass with a lower melting temperature than the pipet. Then the bead was allowed to cool and the pipet was pulled until it broke randomly in some distance from the bead. The procedure was repeated for different pipets until a pipet with a flat end was found when observed with the light microscope. This gives pipets with a consistent ratio between the inside and outside radii. The pipets were filled with the same

TABLE 1 Bilayer membrane bending elastic moduli for model membrane and for red blood cell membrane, determined by different methods (the apparent bending modulus for neutrophils is added for comparison)

$k_c \times 10^{19}$ J	Membrane composition	Method	Reference
2.3	Egg lecithin	Fluctuation of lipid tubular structures	Servuss et al. (1976)
1.5	Egg lecithin	Vesicle thermal fluctuations	Schneider et al. (1984)
0.4–0.5	Egg phosphatidilcholine	Vesicle thermal fluctuations	Faucon et al. (1989)
1.15	DMPC and egg phosphatidilcholine	Vesicle thermal fluctuations	Duwe et al. (1990)
4.0	DMPC with 30% cholesterol	Vesicle thermal fluctuations	Duwe et al. (1990)
0.8	Egg lecithin	Thermal fluctuations of large sheets	Mutz and Helfrich (1990)
0.7	Dimyristoil-phosphatidylethanolamine	Thermal fluctuations of large sheets	Mutz and Helfrich (1990)
1.2	Phosphatidilserine	Tethers	Waugh et al. (1992)
0.44	DAPC or DGDG	Thermal tension	Evans and Rawicz (1990)
0.56	DMPC	Thermal tension	Evans and Rawicz (1990)
0.9	SOPC	Thermal tension	Evans and Rawicz (1990)
2.46	SOPC with 50% cholesterol	Thermal tension	Evans and Rawicz (1990)
0.3	Red blood cell	Flickering	Brochard and Lennon (1975)
1.8	Red blood cell	Buckling	Evans (1983)
0.3–0.7	Red blood cell	Thermal fluctuations	Duwe et al. (1990)
10–20	Neutrophil	Deformation in small pipets	This study

suspension media, in which the cells were resuspended and kept in the chamber for 15 min before starting the experiment. This prevents cell adhesion to the glass surface (Evans and Kukan, 1984). The pipets were connected to a manometer system, with which the pipet-chamber pressure difference could be controlled between 0.5 and 100 Pa using the micrometer-driven displacement of a water-filled reservoir, or up to 2000 Pa using a syringe. The pressures were measured with a differential pressure transducer (Validyne DP15–24). Experiments were recorded on video tape, digitized with an image grabber (Neotech Ltd., Hampshire, England) in a Macintosh II computer and analyzed with NIH Image 1.43. A scanning electron microscope (Philips 501) was used for measuring the inside and outside pipet radii.

In the experiments with neutrophils, a single "passive" cell was chosen, showing an optically smooth membrane surface and a visually uniform diffraction pattern around the cell. (The first sign of cell activation, when observed with the light microscope, was the change of the refractive index in a membrane domain where a pseudopod eventually forms.) The cell was gently aspirated into a large pipet (with radius $R_p \geq 1.2\ \mu m$), and the suction pressure was increased every 3 min in steps of 0.5 or 1 Pa until the cell began to flow into the pipet. The pressure at this point, which was the pressure required to form a hemispherical projection in a pipet and subsequent flow into it, is called the "critical pressure." The cell continued to flow into the pipet because of its liquid-like nature (Evans and Yeung, 1989). After determining the critical suction pressure for cell flow into a large pipet, the cell was allowed to recover to its initial spherical shape and was held in the large pipet with a suction pressure that was half of the critical value. Then a small pipet was pressed against the cell membrane (see Fig. 1), and its

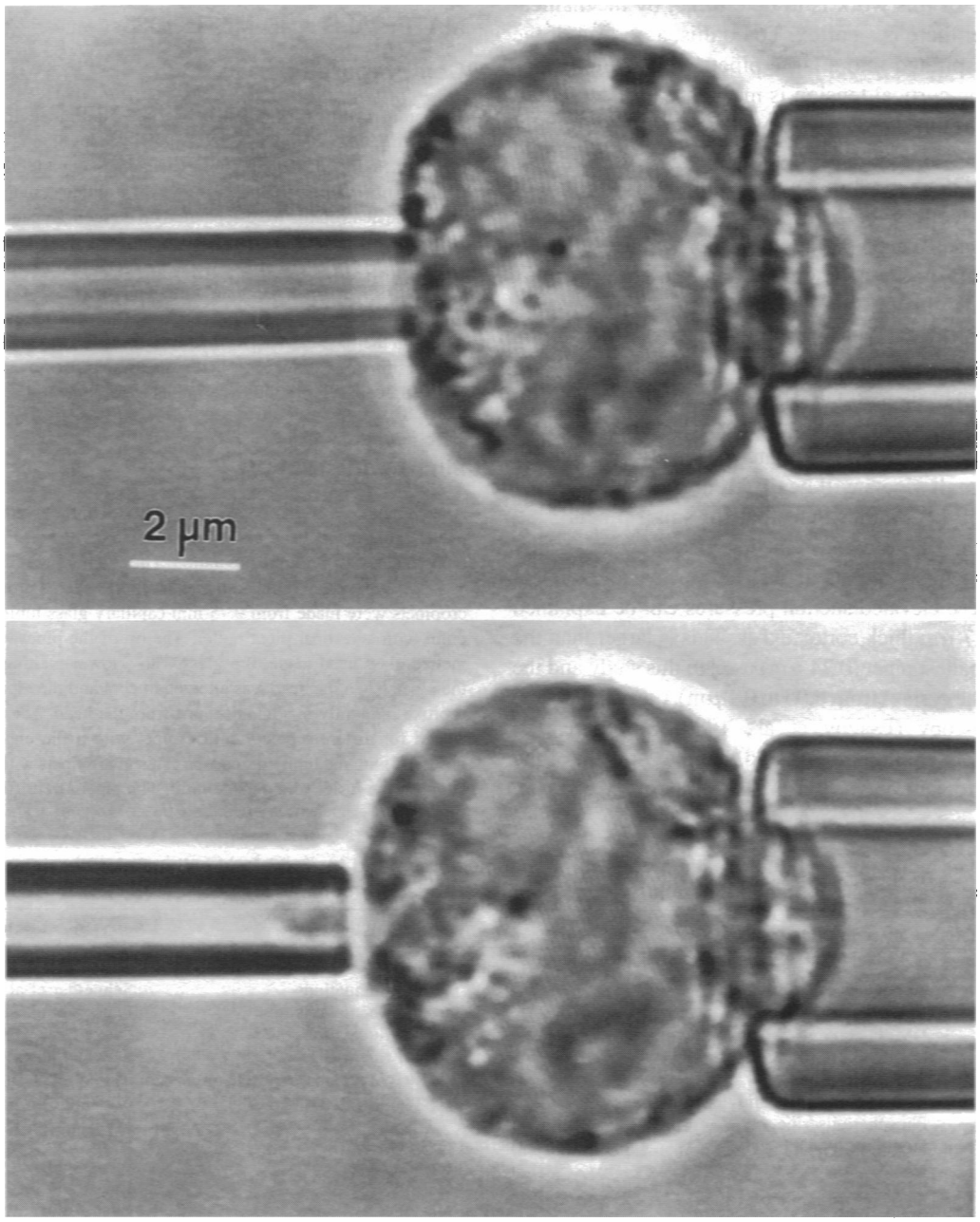

FIGURE 1 (*top*) Neutrophil held with a large pipet when the critical suction pressure for a small pipet is determined; (*bottom*) 3 min later, the small pipet is pulled out to show the neutrophil flowing into the pipet.

suction pressure was increased in steps of 50 Pa in a manner already described for large pipets until the cell moved into the small pipet. This measured the critical pressure in a small diameter pipet.

In experiments with red cells, a nonadherent cell was chosen. To do this, a large pipet was positioned a few microns from the cell without touching it and a suction pressure was applied. A cell was considered nonadherent if there was no observable shape change during movement over a distance that was greater than its diameter. Then a small pipet was pressed against the surface of the cell, and the suction pressure was increased to 50 Pa in steps of 2.5 Pa and the projection length versus suction pressure was measured.

DETERMINATION OF THE PIPET RADIUS

The inside pipet diameter (ID) was measured with a scanning electron microscope (SEM). For this, the pipets used in the experiments were saved, washed in a surfactant (sodium dodecylsulphate, BDH Chemicals, England), and observed by the microscope. In some cases, the plasma proteins plugged the tip of the pipet. In this case, the ID could not be measured with the SEM, so we developed a technique for determining the ID using only the light microscope. For this technique, the light intensity profile for the outside pipet diameter (OD) was determined first. Then the distance between the middle of the diffraction patterns at both sides of the pipet was related to the actual OD measured with the SEM (Fig. 2). At the same time, the SEM was used to measure the ID, and the relation between ID and OD was found (Fig. 3). Making the pipets in a consistent way gives a very consistent relation between ID and OD as shown in Fig. 3. Thus, in a solution, the pipet ID is determined with Figs. 2 and 3 and a light intensity profile of the OD.

RESULTS AND ANALYSIS

The experiment illustrated in Fig. 1 is to determine the necessary suction pressure to form a hemispherical projection in pipets with different internal radii. This pressure is called the critical suction pressure. Neutrophils in a resting state flow into the pipet when the suction pressure exceeds the critical one (Evans and Kukan, 1984), similarly to lipid vesicle membranes under constant tension (Zhelev et al., 1994). Deformation of neutrophils in pipets with large radii (1 μm or more) shows that the cell surface is under an isotropic contraction characterized by a cortical tension, with a value ranging from 0.035 mN/m (Evans and Yeung, 1989) to 0.024 mN/m (Needham and Hochmuth, 1992). The relationship between the critical suction pressure and the pipet radius for large pipets is (Evans and Yeung, 1989)

$$\Delta P = 2T\left(\frac{1}{R_p} - \frac{1}{R_{out}}\right), \tag{1}$$

where ΔP is the pressure difference between the chamber and the pipet, T is the isotropic cortical tension, R_p is the pipet radius, and R_{out} is the radius of the outside portion of the cell.

From the experiments for measuring the cortical tension in large pipets, it is known that the cortical tension is a property of a domain close to the cell surface rather than a property of the bulk part of the cytoplasm. If a thick cortex exists, the cortical tension will be a characteristic of the neutral ("middle") surface of the membrane-cortex complex. Then the law of Laplace, when a cortex with a finite thickness exists, is

$$R_p = T\left(\frac{(1 - R_p/R_{out})}{\Delta P/2}\right) + d, \tag{2}$$

where d, ($d \ll R_{out}$) is the distance of the neutral surface from the pipet wall. When the pipet radius is much larger than the thickness of the membrane-cortex complex, Eq. 2 gives Eq. 1.

The cortical tension T and the distance to the neutral surface d can be determined from the slope and the intercept of the regression line, when the experimentally measured parameters are presented in accordance with Eq. 2. Fig. 4 shows the experimental data in these coordinates. The slope and the

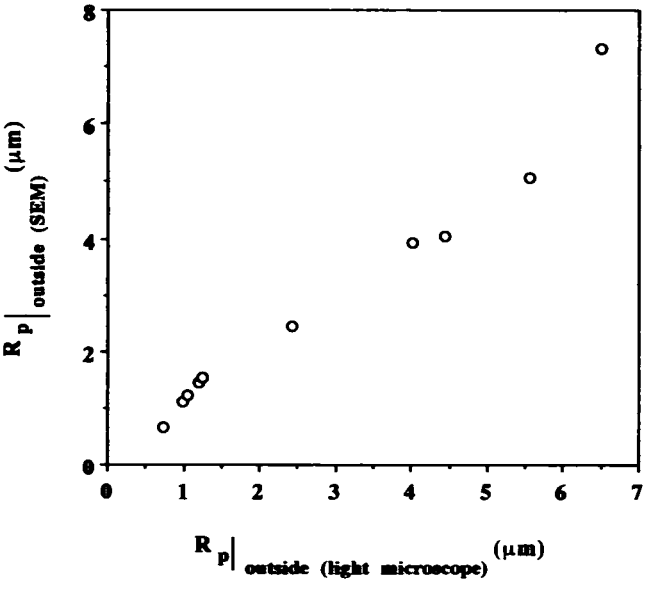

FIGURE 2 Outside pipet radii for the same pipets measured by the scanning electron microscope, $R_{p\,|\,outside(SEM)}$, and found from intensity profiles, $R_{p\,|\,outside(light\ microscope)}$.

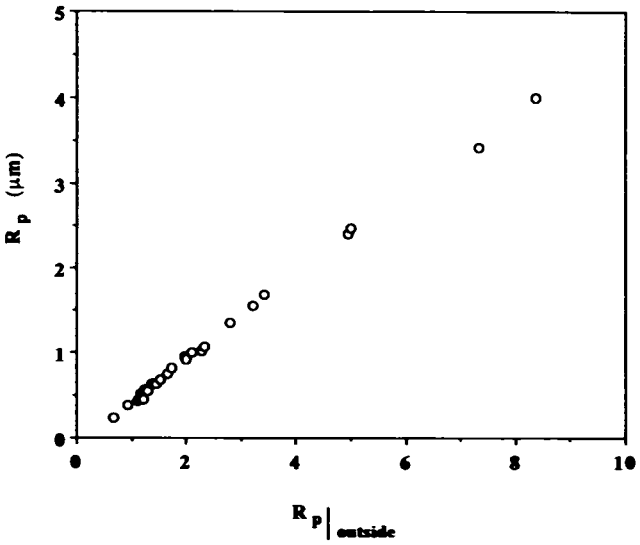

FIGURE 3 Inside pipet radii R_p versus outside pipet radii $R_{p\,|\,outside}$ for the same pipets, as determined by the scanning electron microscope.

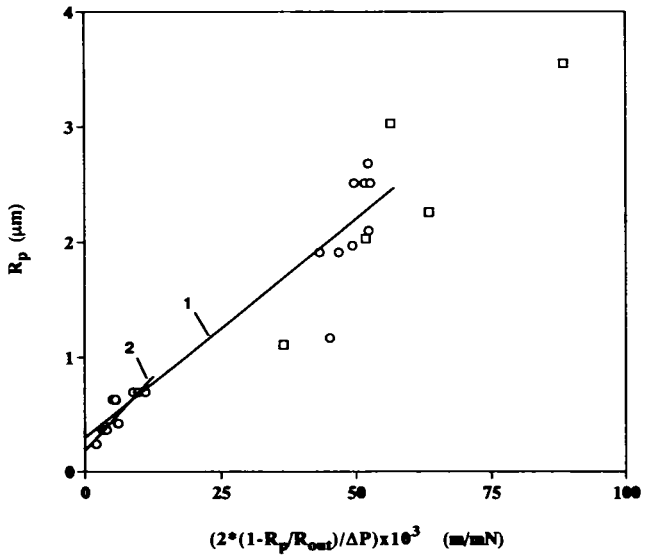

FIGURE 4 Experimental data of pipet radii and corresponding critical suction pressures presented in accordance with Eq. 2. Open circles are the individual measurements from this study; the squares are the data reported in Evans and Yeung (1989). The slope of the line approximating the experimental data gives the cortical tension and the intercept gives the distance of the neutral surface from pipet wall (which is approximately half of the cortical thickness). The slope found from all data is 0.038 mN/m, and the intercept is 0.3 μm (only data from this study were used). The slope and the intercept for small pipets (with radii less than 0.9 μm) are 0.051 mN/m and 0.17 μm, respectively.

intercept for the whole range of pipet sizes used in the experiment are 0.038 mN/m and 0.3 μm, respectively. For small pipets (with radii less than 0.9 μm), they are 0.051 mN/m and 0.17 μm, respectively. The cortical tension found from all measurements is in the order of magnitude of the cortical tension measured with large pipets whereas its value for small pipets increases. The estimate for the thickness of the cortex determined from the intercepts is from 0.3 to 0.7 μm. The smallest pipet used in the experiments has a radius of 0.24 μm. (The diameter of this pipet was measured with the SEM.) Then the thickness of the cortex should not exceed the radius of this pipet. Thus, the thickness of the cortex is on the order of 0.24 μm or less. Fig. 5 shows the experimentally measured critical suction pressures as a function of the pipet radii. Curve 1 is for a cortical tension 0.038 mN/m and negligible thickness of the cortex (Eq. 1). Curve 2 is found from the law of Laplace for a finite cortex (Eq. 2) with a thickness of 0.24 μm and apparent cortical tension T = 0.038 mN/m. It is seen that for large pipet radii the predicted and the measured critical suction pressures are close for both curves. For small pipets, however, the measured values are larger than the predicted ones, even for a thickness of the cortex as large as 0.24 μm, which is larger than the values of 0.05 to 0.1 μm determined by electron microscopy methods (Essaguy et al., 1989; Sheterline and Rickard, 1989). Thus, the measured excess suction pressures cannot be explained by the existence of a cortex with a thickness of 0.24 μm or less. Two sources of additional resistance for deformation are considered: 1) the existence of a bending resistance, or 2) the existence of a small shear elasticity of the neutrophil surface. A model is developed in the Appendix to account for the bending resistance. Critical suction pressures for neutrophils, red blood cells, and SOPC vesicles in pipets with similar diameters are compared in order to estimate the role of shear elasticity.

The critical suction pressure for neutrophils in a 0.6 μm radius pipet is on the order of 230 Pa as determined from the line fit in Fig. 4. The maximum predicted value from Eq. 2 is 140 Pa (*curve* 2 in Fig. 5, for d = 0.12 μm). There is still an excess suction pressure of 90 Pa. The "critical" suction pressure for red cells in a 0.62 μm pipet is 19.6 ± 4.7 Pa (found from nine cells) and for free SOPC vesicles in a 0.58 pipet is on the order of 10 Pa (Zhelev et al., 1994). The lipid vesicles have zero shear elasticity above the phase transition temperature. Then the critical suction pressure for free SOPC vesicles depends only on the bilayer bending modulus and the membrane tension induced by thermal fluctuations. The "critical" suction pressure for red blood cells has two components: one related to the thermal excitations and another related to the shear elasticity. Because the bending modulus of the red blood cell membrane is on the same order as the bending modulus of the SOPC bilayer (see Table 1), the first component of the "critical" suction pressure will be on the same order as the critical suction pressure for free SOPC membrane (about 10 Pa). Then the component of the "critical" suction pressure related to the shear elasticity is on the order of 10 Pa. At the same time, the neutrophil has a much smaller shear elasticity than the red blood cell. (As a result of the shear elasticity, red cells do not flow into the pipet after the projection length exceeds the pipet radius (see Fig. 6), in opposition to what is observed with neutrophils in large pipets (Evans and Kukan, 1984; Evans and Yeung, 1989), and lipid vesicles (Zhelev et al., 1993)). Thus, the measured excess suction pressures for neutrophils are not a result of shear elasticity.

Thus, the additional resistance for deformation in small pipets is a result of bending rigidity. In the model used to study the role of the bending resistance, it is assumed that the membrane-cortex complex is a thin layer having a smaller thickness than the pipet radius. The apparent cortical tension and the apparent bending modulus are introduced as free energies per unit area of the neutral surface of the membrane-cortex. It is assumed, also, that for the small deformations in these experiments the membrane surface experiences a constant cortical tension. With this characteristic, the cortical tension is similar to an interfacial tension (Adamson, 1990). Then the cortical tension T is defined as an energy per unit area g_c of the neutral surface

$$g_c = T. \tag{3}$$

Equations 1 and 2 can be derived from Eq. 3 when the change of the free energy for a small displacement from the final position (when the inside portion of the cell forms a hemisphere) is assumed equal to the work that produces this displacement.

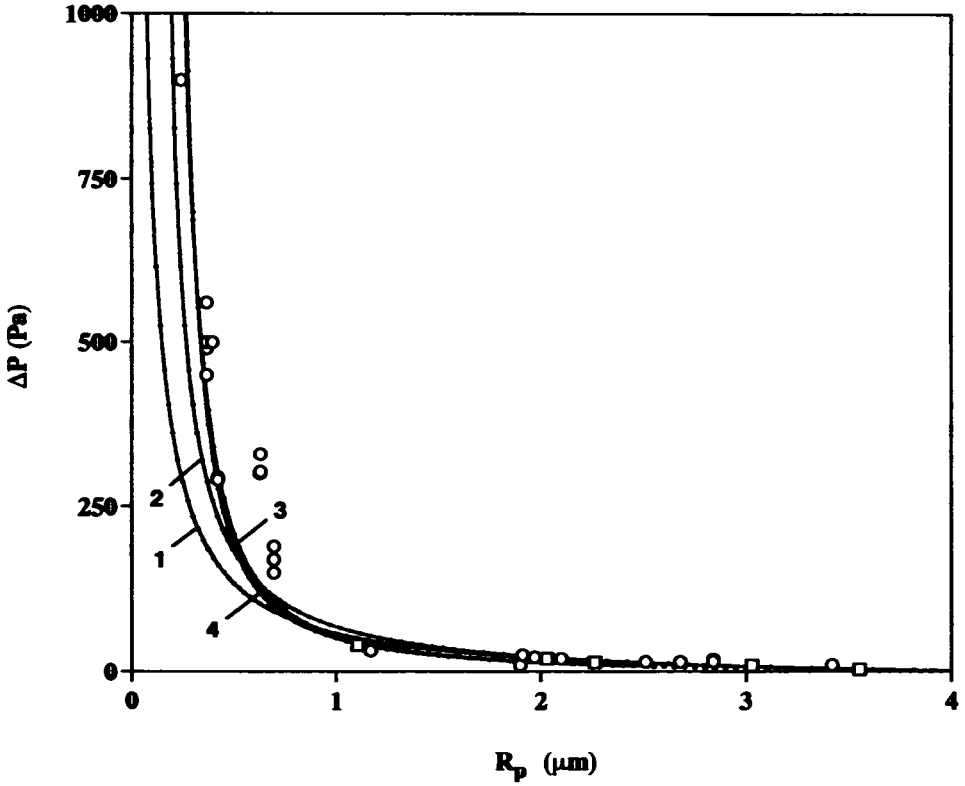

FIGURE 5 Critical suction pressures for cell flow into a cylindrical pipet verses pipet radius. Open circles are the individual measurements from this study; the squares are the critical suction pressures reported in Evans and Yeung (1989). Curves 1 and 2 are found from Eq. 2 for cortical tension $T = 0.038$ mN/m and distances of the neutral surface from the pipet wall $d = 0$ and $d = 0.12$ μm, respectively. Curves 3 and 4 are found from Eq. 5 for $T = 0.024$ mN/m (Needham and Hochmuth, 1992) and for $r = 0.2$ μm. The apparent bending modulus and the distances of the neutral surface from the pipet wall for the two curves are: $k_c = 2 \times 10^{-18}$ J and $d = 0$ for curve 3, and $k_c = 1 \times 10^{-18}$ J and $d = 0.05$ μm for curve 4. The average radius of the spherical neutrophils in the experiments is $R_c = 4.25$ μm.

Next, it is assumed that the membrane-cortex complex shows some elastic rigidity. The elastic rigidity of such a thin region could be defined in a similar way as it is for red cell membranes (Evans and Skalak, 1980), and in this case it comes from two sources: 1) the free energy change caused by in-plane deformations (area dilation and shear), and 2) the free energy change caused by changes in curvature (bending). For these small area changes, the elastic response to area dilation is negligible (Needham and Hochmuth, 1992), and as it was already shown in the preceding paragraph, there is no experimental evidence for the existence of a shear elasticity. The hypothesis is that the bending resistance of the membrane-cortex complex is the reason for the observed larger critical suction pressures than those predicted by Eqs. 1 and 2. To account for this, a bending energy per unit area of the neutral surface g_b is introduced, which is similar to that for lipid bilayers (Helfrich, 1973):

$$g_b = \frac{k_c}{2}(c_1 + c_2)^2, \tag{4}$$

where k_c is the apparent bending modulus and c_1 and c_2 are the two principal curvatures. The spontaneous curvature is not introduced because it is not essential for the following analysis. The Gaussian curvature term is also omitted, because the cell surface maintains its liquid behavior and the saddle bending modulus, if it exists, is expected to be very small.

Then the dependence of the suction pressure on the pipet radius is found by calculating the free energy for a small change of the projection length inside the pipet and equating it to the corresponding work (see Appendix). Then the critical suction pressure ΔP is

$$\Delta P = f_0\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right) \cdot \frac{k_c}{R_p'^3} + f_1\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right) \cdot \frac{T}{R_p'}, \tag{5}$$

where the functional coefficients are defined in the Appendix. Briefly, $R_p' = R_p - d$ is the pipet radius corrected for the

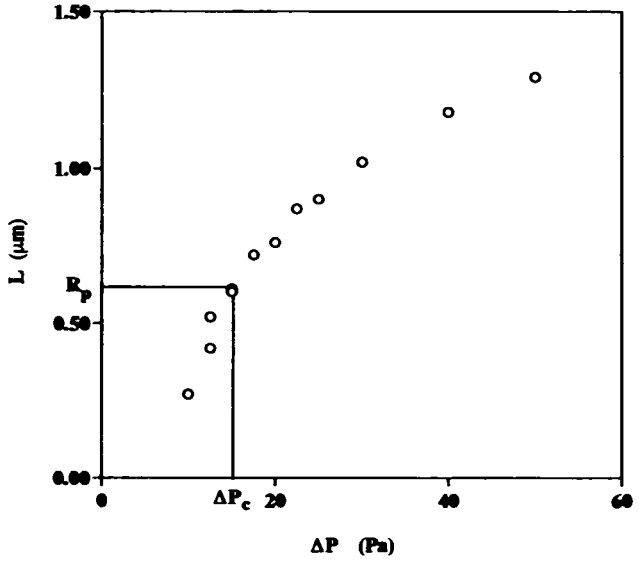

FIGURE 6 Projection length L versus the applied suction pressure ΔP for deforming a red blood cell into a pipet with radius $R_p = 0.62$ μm. The suction pressure ΔP_c, corresponding to the projection length equal to the pipet radius, is the "critical" suction pressure.

actual position of the neutral surface, R_{out} is the outside cell radius, and r is the small torus radius.

Eq. 5 gives Eq. 1 when the bending resistance is small ($k_c \rightarrow 0$), and d and r are much less than R_p. These conditions are met for large pipet radii. Curves 1 and 2 in Fig. 5 are found from Eq. 2 for $T = 0.038$ mN/m and $d = 0$ (curve 1) and $d = 0.12$ μm (*curve* 2), respectively. Curves 3 and 4 are found from Eq. 5 for $T = 0.024$ mN/m (the cortical tension measured with large pipets (Needham and Hochmuth, 1992)) and $r = 0.2$ μm. The apparent bending modulus and the distance of the middle plane from the cell surface for curve 3 are $k_c = 2 \times 10^{-18}$ J and $d = 0$, and for curve 4 they are $k_c = 1 \times 10^{-18}$ J and $d = 0.05$ μm. The apparent bending modulus 2×10^{-18} for a cortex with negligible thickness gives the maximum bending resistance. It is seen from the figure that curves 1, 2, 3, and 4 are very close for pipet radii larger than 1 μm. The dominant term in Eq. 5 for large pipet radii is the cortical tension term. So, the cortical tension can be determined from the measured critical suction pressures for large pipets.

For a given measurement of ΔP_s, R_{out}, and R_p and for a given value of T, there are three parameters in Eq. 5 to be determined: the distance to the neutral surface d, the bending modulus k_c, and the small torus radius r. Fig. 7 shows the relatively weak dependence of the estimated bending modulus on the small torus radius r. The values for the apparent bending modulus in this figure are calculated for $d = 0$. The corrected values of the small torus radius for $d > 0$ are larger, and there will be a weak dependence of the apparent bending modulus on the small torus radius in this case. The distance to the neutral surface d and the apparent bending modulus k_c cannot be determined simultaneously. If one of these characteristics is known, the other can be found from Eq. 5 and the experimental data in Figs. 5 and 6.

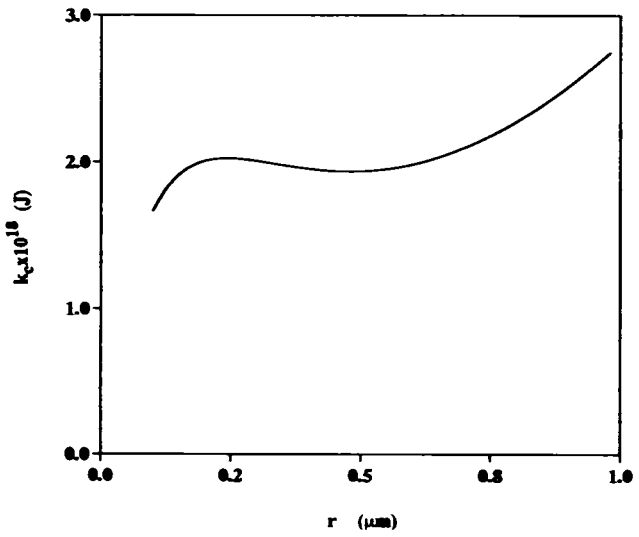

FIGURE 7 Dependence of the apparent bending modulus on the radius of curvature at the pipet orifice (the radius r in Fig. A1). The apparent bending modulus is calculated for $\Delta P = 209$ Pa, $R_p = 0.5$ μm, $R_{out} = 4.25$ μm, $T = 0.024$ mN/m, and $d = 0$.

DISCUSSION

The surface of a resting neutrophil has a spherical shape. This shape is a result of an isometric contraction characterized by the cortical tension. Its nature is unknown; it possibly is a result of active processes that involve the cell membrane and eventually the underlying polymerized actin, which forms the supporting structure of the membrane. The actin filaments, which are 0.1–0.5 μm in length (Hartwig et al., 1985), are cross-linked by different proteins (Stossel et al., 1981; Hartwig and Kwiatkowski, 1991) and together with the microtubule-associated structures (Loor, 1981) form a three-dimensional cortex. The cortex plays a crucial role in active cell movements (Hartwig et al., 1985; Loor, 1981; Oliver and Berlin, 1982; Stossel et al., 1981). During neutrophil locomotion, the cortex is far from thermodynamic equilibrium and the rates of polymerization and depolymerization of actin filaments are much larger than their values in the resting state (Stossel et al., 1981). In the resting state, the complex of neutrophil membrane and the underlying F-actin meshwork is also far from thermodynamic equilibrium, which allows fast shape changes of the cell after chemical or physical stimulation. It is expected that in the resting state the density, thickness, and cross-linking of F-actin structures will be minimum. Thus, the resistance of the cell surface to deformation will be minimum in the resting state, and the measured minimum critical suction pressure for a given pipet radius can be used for its indication.

In the experiments of neutrophil deformation into pipets with decreasing diameters, the excess suction pressure from Fig. 5, above the one predicted by Eq. 1, characterizes the additional mechanical resistance of the neutrophil surface beyond the resistance caused by the surface cortical tension. There is a possibility of an elevated suction pressure because of the presence of persistent wrinkles on the neutrophil surface. The existence of persistent wrinkles will lead either to a poor seal at the pipet orifice or will decrease the effective radius of the pipet. In the first case, if the wrinkles persist, there will be a significant gap at the pipet orifice. Through this gap, there will be a flow of media from the chamber into the pipet. The possible existence of a hydrodynamic flow was checked in the experiments, because of the concern that there will be a pressure drop at the pipet orifice related to the hydrodynamic flow. Such a flow was not observed. In the second case, if the wrinkles can be deformed, but have a finite thickness, they will alter the pipet radius and at the same time will successfully seal the pipet. Because the wrinkles are highly flexible (a good seal exists at the pipet orifice), they could add a thickness of about 0.2 μm to the outer surface of the neutrophil if the folded wrinkle is everywhere dense. The effective density of the wrinkles, obtained from an examination of scanning electron micrographs, is probably less than one-half or even one-fourth of their maximum density. Then the wrinkles could only increase the effective thickness of the membrane and the cortex by about 0.05 to 0.1 μm at the most. Effectively, this is equivalent to the decrease of the pipet radius of the same amount.

An elevated suction pressure will also exist if there is a structure (cortex) associated with the cell membrane and having a finite thickness. In this case, as in the case of deformable wrinkles, the radii of curvature in the surface of the cortical tension (here the neutral surface) will be smaller than the pipet radius. However, only a reduction of the pipet radius as large as 0.3 μm would allow a successful interpolation of the experimental data in Fig. 4 with the law of Laplace (Eq. 2). In addition, in the experiments of this study, the neutrophils flowed into a pipet having a radius of 0.24 μm. This indicates that the reduction of the pipet radius should not exceed 0.12 μm. In this case, there is still an excess suction pressure, which for a 0.6 μm pipet is on the order of 90 Pa. The predicted thicknesses of the cortex from 0.3 to 0.7 μm by the law of Laplace are larger than the values (from 0.05 to 0.1 μm) measured by electron microscopy (Esaguy et al., 1989; Sheterline and Rickard, 1989). A layer with a thickness of this magnitude can be seen with the light microscope as the refractive index in this region changes. A region with a different refractive index was not observed except in the case of activated cells. The conclusion is that the experimental data cannot be explained by the law of Laplace alone.

Two other sources are considered for the measured additional resistance to deformation of the neutrophil surface: the shear elasticity and the bending elasticity. A red cell membrane used as a test surface has a small bending rigidity ($0.3–0.7 \times 10^{-19}$ J, (see Table 1) and a high shear elasticity ($6–7 \times 10^{-3}$ mN/m, Markle et al., 1983). The neutrophil surface compared with the red cell membrane has a very small (if any) shear elasticity (Evans and Yeung (1989) and the experiments of this study). The critical suction pressure for red cell membrane in a 0.62 μm pipet is on the order of 20 Pa. Its component related to the thermal fluctuations is 10 Pa, and the component related to the shear elasticity is 10 Pa. The suction pressure necessary to pull the neutrophil into a 0.6 μm pipet is 230 Pa. The maximum predicted critical suction pressure from the law of Laplace (for a 0.24 μm thick cortex) is 140 Pa. There is still an excess suction pressure of 90 Pa. Thus, even if the neutrophil surface were to have a shear elasticity as large as that for red cell membrane, only one-ninth of the excess suction pressure could be explained by such a shear elasticity. In addition, such a shear elasticity would prevent the neutrophil from flowing smoothly into larger diameter pipets. Because this is not observed, the measured excess suction pressures for neutrophils is caused primarily if not completely by a bending rigidity.

There is a different dependence of the suction pressure on the pipet radius when the membrane-cortex complex has zero or nonzero bending rigidity (compare Eq. 1 and Eq. 5). For a surface with a cortical tension and a negligible bending rigidity, the estimates for the cortical tension found from the law of Laplace (Eq. 1 or 2) for pipets with different radii will be the same. For a surface with bending rigidity, these estimates will be smaller for large pipets and larger for small pipets. The apparent cortical tension for large pipets (with radii in the range from 2.5 to 3.5 μm) is 0.024 mN/m (Needham and Hochmuth, 1992), for pipets in the range from 1 to 3.5 μm it is 0.035 mN/m (Evans and Yeung, 1989), for the range from 0.3 to 2.5 μm it is 0.038 mN/m, and from 0.3 to 0.8 μm, it is 0.051 mN/m. It is seen that the estimates of the apparent cortical tension do depend on the pipet size and are larger for small pipets compared to their values for large pipets. This is another indication for the existence of a bending rigidity of the membrane-cortex. The bending resistance is characterized by the apparent bending modulus. Its values are from 1×10^{-18} J (for 0.1 μm thick cortex) to 2×10^{-18} J (for cortex having a thickness much less than the radius of the smallest pipet (0.24 μm) used in this study). These values for the apparent bending modulus are 5–50 times the values for the bending modulus of red cell membrane or lipid bilayer membranes (see Table 1).

In conclusion, we studied the deformation of human neutrophils in pipets with increasingly small radii (down to 0.24 μm). The results show that there is a structure beneath the membrane (cortex) with finite thickness. The membrane-cortex complex defines the shape and determines the resistance of the cell surface for deformation. The resistance for deformation depends on the apparent cortical tension and the apparent bending modulus. The cortical tension can be found by measuring the critical suction pressure for pipets having radii larger than 1 μm. It has a value on the order of 0.024 mN/m (Needham and Hochmuth, 1990). The apparent bending modulus has values on the order of 1×10^{-18} to 2×10^{-18} J for a membrane-cortex thickness from 0.1 μm down to the thickness of the plasma membrane.

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APPENDIX

A simplified model is used for the neutrophil geometry (see Fig. A1). The two spherical surfaces with different radii and the torus surface between the two regions represent the average shape of the aspirated neutrophil observed experimentally. The cell is divided into three regions. The first region consists of the spherically shaped cap inside the pipet. The second region is the curved portion of the cell surface at the pipet orifice, which is taken as a segment of a torus. The third region is the outside spherically shaped part of the cell. The final position is when the inside cap forms a hemisphere.

The apparent cortical tension and the apparent bending modulus are defined at the neutral (or "middle") surface of the membrane-cortex complex. The relationship between the cortical tension and the bending elasticity of the cell and the forces acting on its surface is obtained by equating the free energy change during small cell deformations inside and outside the pipet and the work of deformation done by the external forces. The mechanical free energy for the closed cell surface is calculated using the geometric simplifications and Eqs. 3 and 4.

Let the cell be in a position near the final one. Then the total mechanical free energy G_{total} is

$$G_{total} = G_s + G_{ts} + G_{out} \tag{A1}$$

where G_s, G_{ts}, and G_{out} are the free energies of the first, second, and the outside region, respectively. The free energy for the first region is

$$G_s = \pi T(R_p'^2 + h^2) + \frac{\pi k_c}{2}(R_p'^2 + h^2)\left(\frac{4h}{R_p'^2 + h^2}\right)^2, \tag{A2}$$

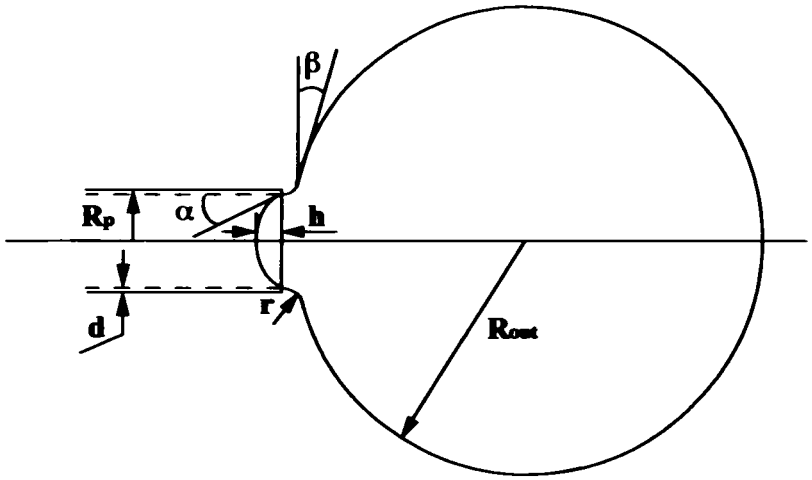

FIGURE A1 A Schematic of the geometry of a cell aspirated into a cylindrical pipet. Only the neutral surface is shown.

where, $h < R_p'$ is the maximum projection length of the cell inside the pipet (see Fig. A1), $R_p' = (R_p - d)$ is the radius of curvature of the middle surface inside the pipet, R_p is the pipet radius, d is the distance of the middle surface from the cell surface, and T and k_c are the apparent cortical tension and the apparent bending modulus of the middle surface, respectively.

The free energy of the second region is

$$\begin{aligned} G_{ts} &= T\int_{(-\pi)}^{(\pi)}\int_{((\pi/2)+\beta)}^{(\pi-\alpha)} r(R_{ts} + r\cos v)\,dv\,d\varphi \\ &\quad + \frac{k_c}{2}\int_{(-\pi)}^{(\pi)}\int_{((\pi/2)+\beta)}^{(\pi-\alpha)} \frac{1}{r}(R_{ts} + r\cos v)\left(2 - \frac{R_{ts}}{R_{ts} + r\cos v}\right)^2 dv\,d\varphi \\ &= 2\pi TrR_{ts}\left(\frac{\pi}{2} - \alpha - \beta\right) + 2\pi Tr^2(\sin\alpha + \cos\beta) \\ &\quad + 4\pi k_c(\sin\alpha + \cos\beta) + \frac{2\pi k_c R_{ts}}{r(1 - (r/R_{ts})^2)^{1/2}} \\ &\quad \times \left(\tan^{-1}\left(\frac{C_{ts}\sin\alpha}{1 - \cos\alpha}\right) - \tan^{-1}\left(\frac{C_{ts}(1 + \sin\beta)}{\cos\beta}\right)\right), \end{aligned} \tag{A3}$$

where $-\pi \le \varphi \le \pi$ and $\pi - \alpha \le v \le (\pi/2) + \beta$, r is the small torus radius of the middle plane (see Fig. A1), which is assumed to remain constant, $R_{ts} = R_p' + 2rhR_p'/(R_p'^2 + h^2)$ and

$$C_{ts} = \left(\frac{1 - r/R_{ts}}{1 + r/R_{ts}}\right)^{1/2}.$$

The free energy of the spherical outside region is

$$\begin{aligned} G_{out} &= 2\pi TR_{out}^2\left(1 + \left(1 - \left(\frac{R_p' + r}{R_{out}}\right)^2\right)^{1/2}\right) \\ &\quad + 4\pi k_c\left(1 + \left(1 - \left(\frac{R_p' + r}{R_{out}}\right)^2\right)^{1/2}\right), \end{aligned} \tag{A4}$$

where R_{out} is the radius of the outside portion of the cell.

When the projection length inside the small pipet changes from h to $(h + dh)$, the area of the cell surface increases while the volume remains constant. Then from the conservation of volume there is a relationship between the projection length change dh and the change of the outside cell radius dR_{out}:

$$dR_{out} = -\frac{R_p'(R_p' + rC_{vt})}{R_{out}^2(2 + C_{vs})}\cdot dh, \tag{A5}$$

where

$$C_{vt} = 1 + \left(\frac{R_p' + r}{R_p'}\right) + \left(\frac{R_p' + r}{R_p'}\right)^2$$

and

$$C_{vs} = \frac{2 - ((R_p' + r)/R_{out})^2}{(1 - ((R_p' + r)/R_{out})^2)^{1/2}}.$$

Then the change of the total free energy when the projection length inside the pipet changes with dh, for the limit $(h \to R_p')$, is

$$\begin{aligned} dG_{total} &= 2\pi T\left(R_p' + r - \frac{R_p'(R_p' + rC_{vt})}{R_{out}}\right)\cdot dh \\ &\quad + \pi k_c\left(\frac{1}{r}\left(1 + \frac{r}{R_p'}\right)^2\left(1 + \frac{2r}{R_p'}\right)^{1/2} - \frac{4R_p'C_{vw}(R_p' + rC_{vt})}{R_{out}^3(2 + C_{vs})}\right)\cdot dh, \end{aligned} \tag{A6}$$

where

$$C_{vw} = \frac{((R_p' + r)/R_{out})^2}{(1 - ((R_p' + r)/R_{out})^2)^{1/2}}.$$

If it is assumed that the displacement of the cell surface is a result only of the normal pressure acting on the surface, then the total work dW_{total} for the displacements of the cell surface in the three regions is

$$dW_{total} = \pi R_p'^2\,\Delta P\cdot dh \tag{A7}$$

where ΔP is the measured pressure difference between the static pressure in the chamber and the static pressure inside the pipet. Then the critical suction pressure as a function of the cortical tension found from Eqs. A6 and A7 is

$$\Delta P = f_0\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right)\cdot\frac{k_c}{R_p'^3} + f_1\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right)\cdot\frac{T}{R_p'},$$

where the functional coefficients are

$$f_0\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right) = \frac{1}{(r/R_p')}\left(1 + \frac{r}{R_p'}\right)^2\left(1 + \frac{2r}{R_p'}\right)^{1/2} - \frac{4C_{vw}(1 + (r/R_p')C_{vt})}{(2 + C_{vs})}$$

and

$$f_1\left(\frac{R_{out}}{R_p'}, \frac{r}{R_p'}\right) = 2\left(1 + \frac{r}{R_p'} - \frac{(1 + (r/R_p')C_{vt})}{R_{out}/R_p'}\right).$$

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