Flexural Rigidity of Marginal Bands Isolated from Erythrocytes of the Newt

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Abstract. The marginal band is a bundle of microtubules residing at the periphery of nucleated erythrocytes of nonmammalian vertebrates and some invertebrates. Marginal bands from erythrocytes of the newt (*Notopthalmus viridescens*) were isolated from the cells as intact structures by treatment with detergent and either mild protease or high salt. Isolated bands were subjected to mechanical testing by stretching the band between a glass microhook and a calibrated glass fiber. The deflection of the fiber provided a measure of the force on the band. The flexural rigidity of the band was determined from measurements of the band deformation as a function of applied force. Bands isolated with either of two proteases (pepsin or elastase) or with high salt exhibited elastic behavior with a flexural rigidity of $\sim 9.0 \times 10^{-12}$ dyn·cm^2. Treatment of bands with chymopapain caused an increase in band rigidity and inelastic behavior. Estimates of the contribution of the band to cellular rigidity are made based on the measurements of the structural properties of the isolated band. The band provides the cell with a large resistance to indentations at the rim and to large extensions, while maintaining a high degree of flexibility in small extensions or flexure.

THE marginal band is a major cytoskeletal element in erythrocytes of nonmammalian vertebrates, in erythrocytes of some invertebrates, and in mammalian platelets. It consists of a bundle of microtubules located at the periphery of the cell just beneath the plasma membrane. Its function appears to be mechanical. It has been implicated in the determination of cell shape during morphogenesis (1) and in stabilizing the shape of the mature cell (10). Unlike other microtubular structures, some marginal bands remain stable after cell lysis and, under proper conditions, they can be isolated intact from the rest of the cytoskeleton. This feature makes them an attractive model system for studying the structure, composition, and mechanical properties of a microtubular assembly.

Evidence that the marginal band plays a role in stabilizing the shape of the mature erythrocyte was first obtained by Joseph-Silverstein and Cohen (10). They observed that cells subjected to mechanical stresses are more grossly deformed when the marginal band is absent than when it is present. Because marginal bands can be isolated as intact structures it is possible to study their properties directly and quantitatively. In a previous report from this laboratory (17), a method was described for determining the resistance of the band to flexure (a change in curvature) and extension (a change in the length of its contour). These structural properties are characterized by two coefficients: the flexural rigidity (*EI*) and the extensional rigidity (*EA*). In that previous study accurate determinations of the extensional rigidity were made, but, because the applied forces were large, the determination of the flexural rigidity was only approximate. In the present study we apply smaller forces to isolated bands to make accurate measurements of the flexural rigidity. To control against possible artifactual effects of the isolation procedure on the structural properties of the band, we have tested bands isolated by four different methods. Three of the methods, one using pepsin, one using elastase, and one using high salt in the presence of protease inhibitors, produced bands with nearly identical properties. Treatment of the bands with chymopapain was found to produce a large increase in band rigidity.

Materials and Methods

Band Isolations

Protease Isolations. These procedures were adapted from the original work of Cohen (4) according to methods described by Cohen and Ginsberg (5). A drop of blood was obtained from a newt (*Notopthalmus viridescens*) by tail snip. Elastase, chymopapain, and pepsin were obtained from Sigma Chemical Co. (St. Louis, MO). The basic band buffer consisted of 100 mM Na_2-Pipes, 1.0 mM $MgCl_2$, and 5.0 mM EGTA (pH 6.8). A drop of whole blood was suspended directly into 10.0 ml of band buffer containing 0.4% (vol/vol) Triton X-100 and 100 μM PMSF (stock solubilized in dimethylsulfoxide at 17.42 mg/ml). The resulting "Triton skeletons" were treated differently depending on the particular isolation procedure used.

Triton skeletons were infused into a perfusable chamber on the microscope stage. Band buffer containing the appropriate protease was infused into the chamber until bands began to become free of the nuclei. Then the appropriate protease inhibitors were introduced into the chamber as the protease was washed out. Freed bands were then manipulated for mechanical measurements. Three different proteases were used: pepsin (20.0 mg/ml) at pH 6.8 in the presence of PMSF followed by pepstatin (1 μM, solubilized at 1.1 mg/ml in methanol); elastase (0.5 IU/ml) followed by PMSF (100

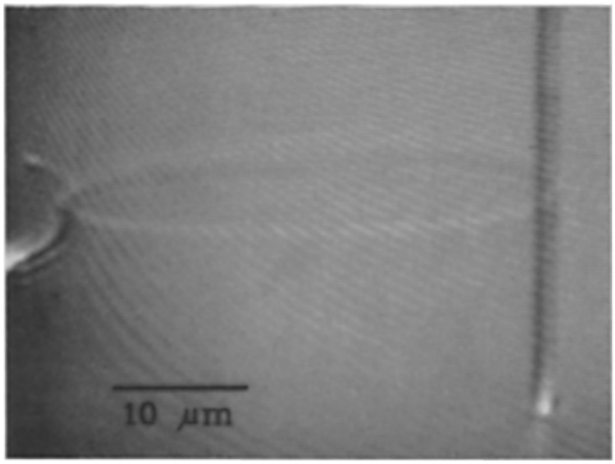

Figure 1. Photograph of the mechanical testing of an isolated band. The band is stretched between the glass fiber (at right) and a glass hook (at left). As the band is stretched, the fiber is displaced, providing a measure of the force on the band.

μM); and chymopapain (4.5 mg/ml) followed by leupeptin (2.5 μg/ml) in the presence of PMSF (100 μM).

High Salt Isolations. This procedure was adapted from the methods of Monaco et al. (13). A drop of whole blood was added directly to 3.0 ml band buffer containing PMSF (130 μM), DNase I (0.1 mg/ml), 0.4% (vol/vol) Triton X-100, KCl (0.25 M), iodoacetamide (1.0 mM), and pepstatin (1.0 μm solubilized in methanol at 2 mg/ml). (Note, in two experiments the pepstatin and iodoacetamide were not included in the buffer.) The skeletons remained suspended in the buffer for 25 min at room temperature, after which they were spun at 500 *g* for 5 min to remove the cell nuclei. The pellet was discarded and the supernatant was spun again at 500 *g* for 5 min onto a sucrose (50% wt/vol) cushion. Isolated bands were collected from the suspension above the sucrose cushion, and infused into the microchamber for mechanical measurements.

Mechanical Measurements

A free band was manipulated with a glass microhook and looped over the end of a calibrated glass fiber (see below). The hook was withdrawn slowly and the band was stretched between the hook and fiber. Each band was stretched three or four times. The procedure was observed through a microscope and recorded on videotape for subsequent analysis. A photograph of a band being tested is shown in Fig. 1.

Calibration of the fibers. Fibers were made by cementing glass wool fibers into the tips of tapered capillary tubes. The fibers were stretched on a microelectrode puller (David Kopf Instruments, Tujunga, CA) to produce a long, thinned tip. To calibrate the fiber, a short length (0.2–0.5 mm) of thin-walled plastic tubing was slipped over the fiber tip and the resulting deflection was observed on a horizontally mounted microscope. The weight per unit length of the plastic tubing was determined by weighing a long section (~30 cm) of the tubing on a precision balance. Thus, from the length of the section placed on the tip of the fiber the force corresponding to the observed deflection could be determined. Within the precision of the measurements and over the range of deflections observed, the relationship between force and deflection was linear. Four different fibers were used in these experiments. The beam constants were 4.29, 4.06, 4.95, and 11.5 × 10^{-6} dyn/μm.

Analysis of Data

The videotape of the experiments was played back and the elongated dimension of the band (D) was measured as a function of the fiber deflection, which provided a measure of the force on the band ($2P$). The mechanical properties of the band were calculated from the force, diameter data pairs by least-squares regression to the theoretical prediction for the behavior of an elastic ring. (For details see reference 17.) The ring is assumed to exhibit elastic behavior in both flexure and extension. The curvature of the band ($1/\rho$) is related to the local bending moment on the band (M) by the flexural rigidity, which is the product of the elastic modulus (E) and the moment of inertia of the band cross section (I): $M = EI/\rho$. In extension, the fractional change in the length of the band contour ($\Delta\ell/\ell_o$) is related to the axial force within the band (F) by the extensional rigidity, which is the product of the elastic modulus (E) and the cross-sectional area of the band (A): $F = EA \cdot \Delta\ell/\ell_o$.

During the initial deflection of the band, the primary change in band geometry is a change in curvature. Therefore, for small forces the flexural rigidity determines the extent of deflection that occurs in response to the applied force. For large forces, when the opposite sides of the band are nearly parallel, further deflection (increase in D) requires an elongation of the band contour, and the extensional rigidity determines the response of the band to the applied force. Thus, for small forces, the measured force–deflection data pairs provide information about the flexural rigidity of the band but little information about the extensional rigidity. Conversely, for large forces, the force–deflection data pairs can be used to determine the extensional rigidity, but provide little information about the flexural rigidity. In the present study the fibers used to measure force were very sensitive and provided good resolution at low forces. However, at high forces, the fibers deflected out of the field of view and no data could be obtained. Thus the present data can be used to make accurate determinations of the flexural rigidities of the bands, but provide little information about the extensional rigidity. Because of the insensitivity of the data to the value of EA, it was necessary to fix EA at a value consistent with those obtained in an earlier study (0.017 dyn). The value of EI calculated from the present data was insensitive to the value selected for EA for values of EA between 0.001 and 10.0 dyn. With EA fixed at a value of 0.01 dyn, EI and the unstressed band diameter, D_o, were determined by least-squares regression using a simplex algorithm (3). The functional relationship between force and deflection that was used in the calculations was obtained from the work of Libai and Simmonds (11, 12), as is outlined in the Appendix.

Results

Type I Bands

Bands prepared using pepsin, elastase, and high salt plus DNase I exhibited similar behavior and had similar properties. Because of their similar behavior, we will refer to these as type I bands. Bands isolated using chymopapain (type II) were considerably more rigid and exhibited qualitatively different behavior during deflection, as is described below. (Note, the designation type I or type II is an operational definition based on the mechanical behavior of the band. As is described below, a band exhibiting type I behavior can be made to exhibit type II behavior when exposed to chymopapain.) The properties of the type I bands are summarized in Table I. After isolation, the resting shape of the band was a flat circular ring. When stretched, the band behaved elastically, exhibiting identical behavior in the loading and unloading phases of the deformation (Fig. 2). For some bands, multiple tests were performed, and separate determinations of EI were made for each loading and unloading data set. Standard deviations in the calculated value of EI for these multiple determinations on the same band were typically 15.0% (4.0–36.0%). Properties of the isolated bands were stable for periods of >2 h, during which time the measurements were made. Generally, bands isolated by a particular method had similar properties from preparation to preparation. For example, the three pepsin preparations had mean rigidities of 9.7 ± 2.3 × 10^{-12} dyn·cm², 8.8 ± 2.1 × 10^{-12} dyn·cm², and 8.4 ± 2.5 × 10^{-12} dyn·cm². However, this was not always the case. Of the two sets of measurements performed on elastase-isolated bands, one had a mean value for EI of 13.2 × 10^{-12} dyn·cm² (SD = 2.2 × 10^{-12}, n = 14) and the other had a mean value of 5.7 × 10^{-12} dyn·cm² (SD = 1.9 × 10^{-12}, n = 20). We also observed a significant increase in rigidity for salt-isolated bands when two of the four

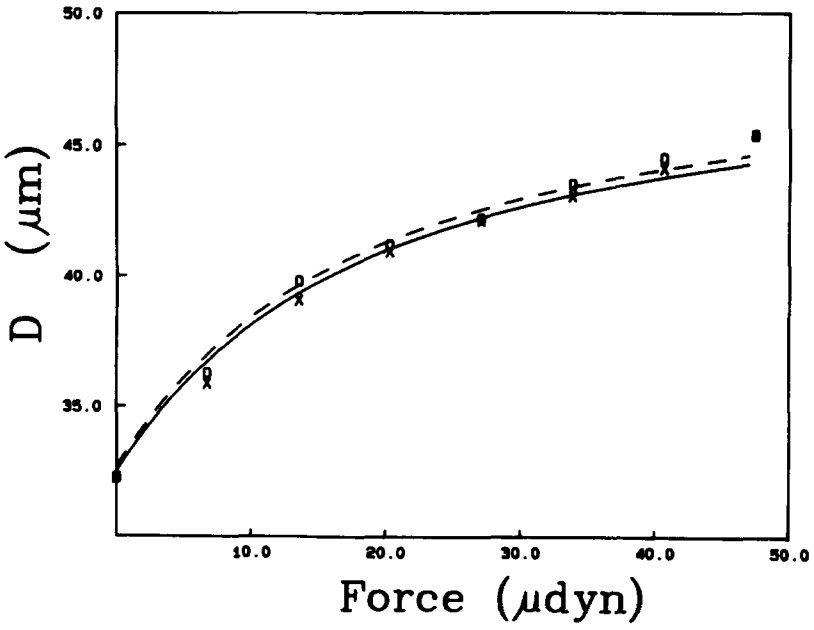

Figure 2. The deformed length of the band, *D*, as a function of the force, *2P*. ×'s represent data pairs taken in extension. Rectangles represent data taken in recovery. Solid and dashed curves are the theoretical curves for the extension and recovery data, respectively, fit to the data with two free parameters, the unstressed band diameter and the flexural rigidity (*EI*). The values for *EI* for these two data sets are 8.0×10^{-12} dyn·cm² in extension and 7.9×10^{-12} dyn·cm² in recovery. The band was isolated by the high salt method.

protease inhibitors (pepstatin and iodoacetamide) were omitted during the isolation. Bands isolated under these conditions showed much more variability in properties, one preparation exhibiting a mean flexural rigidity of 45.0×10^{-12} dyn·cm² (SD = 20.5×10^{-12}, $n = 9$) and the other having a mean flexural rigidity of 15.0×10^{-12} dyn·cm² (SD = 4.9×10^{-12}, $n = 8$). However, when all four protease inhibitors were included, consistent behavior was observed for different preparations: $8.3 \pm 2.6 \times 10^{-12}$ dyn·cm² and $7.4 \pm 1.8 \times 10^{-12}$ dyn·cm².

Chymopapain-isolated Bands

Bands isolated with chymopapain (type II bands) exhibited markedly different behavior than type I bands. After isolation, they kept the flat elliptical shape of the intact cell and Triton skeleton. They were significantly more rigid and had a flexural rigidity approximately ten times greater than type I bands (see Table I). They also exhibited helical anisotropy in their properties, as evidenced by the fact that they twisted into nonplanar rings when stretched, often appearing as figure "8's" as viewed in projection under the microscope (see Fig. 3). Their behavior was also inelastic; the force–deflection curves were different in loading and unloading, and successive measurements on the same band often gave different results (Fig. 4).

Table I. Flexural Rigidity of Marginal Bands Isolated by Different Methods

	EI (10^{-12} dyn·cm²)				
Isolation method	Average	SD	*n*		Different?
Pepsin	8.9	2.3	35	NS	
Elastase	8.8	4.3	34	NS	p = 0.03
Salt (+4)*	7.6	1.9	23		
Salt (+2)‡	36.4	27.3	17		p < 0.001§
Chymopapain	83.3	56.3	44		p < 0.001§

* Isolated in the presence of PMSF, EGTA, iodoacetamide, and pepstatin.
‡ Isolated in the presence of PMSF and EGTA.
§ Significantly different from all other samples.

Treatment of Type I Bands with Chymopapain

Experiments were performed to determine if the different properties of the chymopapain bands were due to differences in the structural components of the band isolated from the cell by the different methods or by a direct effect of chymopapain on the band properties. Bands were isolated by the high salt method and the properties were tested. After testing several of the bands to confirm that they exhibited type I behavior, the bands were exposed to chymopapain. After chymopapain treatment, the band rigidity was increased approximately tenfold, and bands twisted into nonplanar shapes when stretched.

Discussion

A detailed understanding of the mechanical function of a cytoskeletal system requires knowledge of the mechanical characteristics of the individual components as well as the interactions among those components. Determination of the characteristics of a particular cytoskeletal element is usually confounded by the complexity of the cytoskeleton in situ. Thus, the ability to separate marginal bands structurally intact from the rest of the cellular structures provides an unusual opportunity to study the properties of a particular cytoskeletal element and so assess its contribution to the overall cellular behavior.

A concern of studying an isolated structure is whether or not the properties of the structure have been altered by the isolation procedure. Our ability to produce bands of similar behavior and properties by three different methods is a strong indication that the band properties we have observed are not a function of the isolation process but in fact reflect the mechanical contribution of the band in situ. Clearly this is not

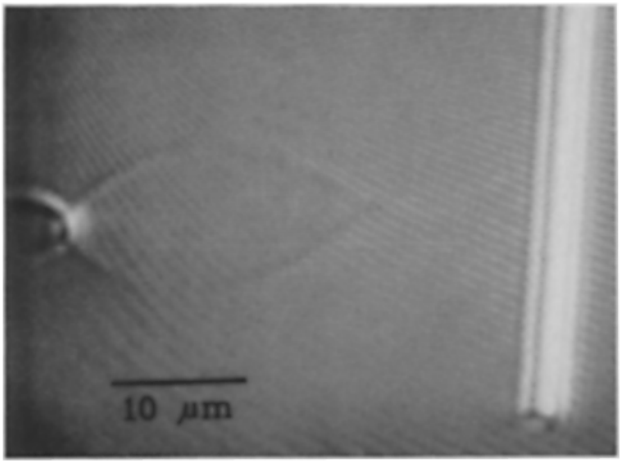

Figure 3. Photograph from a television monitor showing deformation of a band isolated with chymopapain. Chymopapain-isolated bands were more rigid than bands isolated by other methods and usually buckled and twisted into a figure "8" as they were extended.

always the case, as is indicated by our observations of bands isolated with chymopapain. It is important to emphasize, however, that the increased rigidity of chymopapain-isolated bands is a direct result of the action of chymopapain on the band and does not reflect a difference in structural preservation by the different methods. This conclusion is based on our observation that bands isolated with high salt and exhibiting normal type I behavior are made more rigid by subsequent treatment with chymopapain.

Contribution of the Band to Cellular Rigidity

Our knowledge of the structural properties of isolated marginal bands enables us to make quantitative estimates of the contribution of the band to cellular rigidity. In the following examples we assess the relative importance of the mechanical rigidity of the band compared with the mechanical rigidity of the cell membrane. In assessing the membrane contribution we are hampered by a lack of direct knowledge about the membrane properties. Cohen et al. (6) have shown that there is a spectrin-actin submembranous skeleton in nucleated erythrocytes similar to that found in mammalian erythrocytes, suggesting that the behavior of the amphibian membrane is at least qualitatively similar to that of the mammalian membrane. Micropipet measurements of surface deformations of amphibian erythrocytes (15) indicate that the surface-shear modulus of amphibian erythrocytes is not more than ten times greater than the modulus of mammalian red cells, but the values obtained in that study probably overestimate the membrane rigidity because the contribution of the marginal band to the cellular rigidity was not considered. To account for this uncertainty we use two different shear moduli in our calculations, one that is roughly equal to the mammalian modulus (0.007 dyn/cm) and one which is ten times greater (0.07 dyn/cm).

We have greater confidence in our estimate of the relative resistance of the band and membrane to local changes in curvature (bending). The bending rigidity of biological membranes is attributable to the bending stiffness of the membrane bilayer. Thus, it is expected that bilayer membranes in general will have bending stiffnesses of similar magnitude. For example, the bending stiffness of a pure bilayer membrane, $1.2\text{–}2.5 \times 10^{-12}$ ergs (2, 14) is essentially equal to the bending stiffness of the human erythrocyte membrane, 1.8×10^{-12} ergs (2, 7, 16). Thus, a value of 2.0×10^{-12} ergs is a reasonable estimate for the bending stiffness of biological membranes in general.

One type of deformation in which the band makes a predominant contribution is a local indentation at the cell periphery. This conclusion comes from a comparison of the flexural rigidity of the band to the flexural rigidity of an unsupported strip of bilayer membrane. The bending stiffness of the membrane is $\sim 2.0 \times 10^{-12}$ dyn·cm (2, 7). A strip of

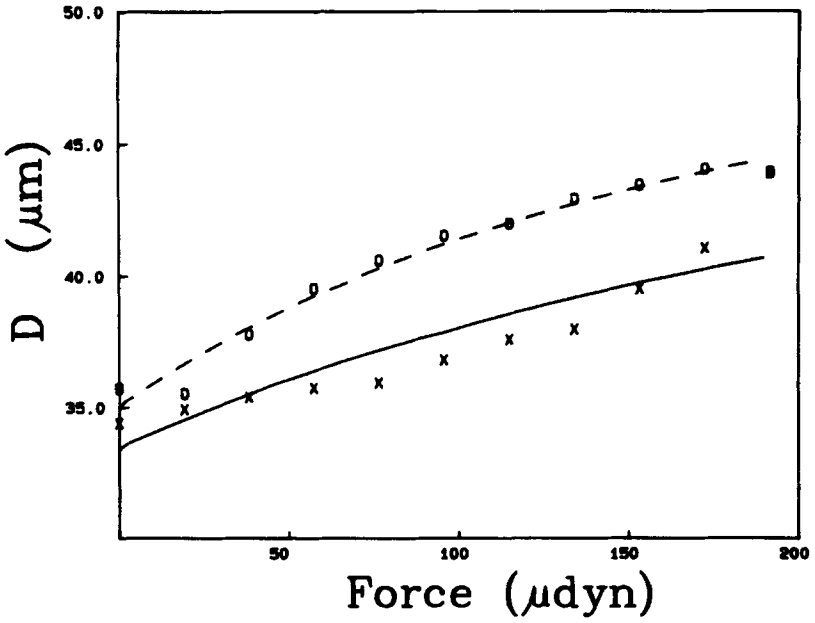

Figure 4. The elongated band dimension, *D*, as a function of the applied force for a band isolated with chymopapain. The extension for a given force is much smaller for this band than for Type I bands (Fig. 2). Also note that there is significant hysteresis between extension (solid curve, ×'s) and recovery (dashed curve, rectangles). Bands isolated with salt and subsequently treated with chymopapain exhibited similar behavior.

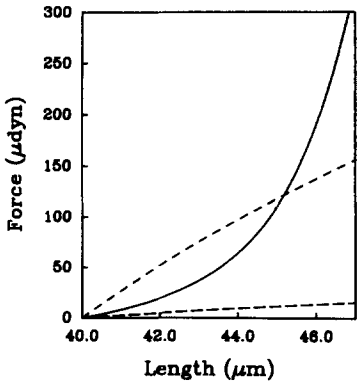

Figure 5. Relative stiffness of the membrane and the marginal band in elongation. The cell is assumed to have a resting length of 40 μm and a resting width of 20 μm. The force needed to stretch two membrane strips (dashed curves) is compared to the force needed to increase the length of the marginal band (solid curve). For the long (lower) dashed curve, a modulus of 0.007 dyn/cm was used; for the short (upper) dashed curve a value of 0.07 dyn/cm was used. Note that for small extensions (<10.0%), the rigidities of the band and the membrane are comparable, but for large extensions (>15.0%) the marginal band dominates the cellular response.

membrane 1.0 μm wide would have a flexural rigidity of 2.0 $\times$ 10^{-16} dyn·cm^2. This is 4,000 times smaller than the rigidity of the marginal band. Clearly, the band makes the predominant contribution to the stability of the cell against indentations or local changes in surface curvature at its rim.

It is interesting to note that even with the added structure of the marginal band, cells remain highly flexible for small deformations. Consider the relative resistance of the marginal band and an erythrocyte membrane to extensional deformation. We compare the force required to extend the marginal band to the force required to extend two rectangular strips of membrane that are 20.0 μm wide and 40.0 μm long at rest. The relationship between the shear force resultant within the membrane, T_s, and the membrane extension ratio ($\lambda = L/L_o$) is (reference 8): $T_s = \mu/2\ (\lambda^2 - \lambda^{-2})$, where μ is the elastic modulus of the surface. For a uniaxial extension, the force applied to each membrane, f_t, can be related to the extension λ by: $f_t = \mu W_o (\lambda - \lambda^{-3})$, where W_o is the width of the membrane at rest. The total force on the cell is twice this value. The force needed to produce a given length is plotted in Fig. 5 for two different values of the modulus μ (0.007 and 0.07 dyn/cm). Also shown in Fig. 5 is the force needed to produce comparable deflections of the marginal band. Note that in the undeformed cell, the long axis of the cell is ~30% longer than the circular diameter of the band. The force plotted in Fig. 5 is the additional force required to increase the dimension of the band above 40.0 μm when its resting circular diameter is 31.0 μm (EI = 8 $\times$ 10^{-12} dyn·cm^2). It should be recognized that because both the membrane and the band are elastic structures, Fig. 5 can also be interpreted as the restoring force originating from these structures to return the cell to its resting shape after deformation.

Two features of the curves in Fig. 5 are relevant to the present discussion. At small extensions (<10%) the contributions of the membrane and the band are comparable, indicating that the presence of the band does not cause a dramatic increase in cellular rigidity for small deflections. On the other hand, when extensions are appreciable (>15%) the contribution of the band predominates because such extensions would require an increase in the length of the band contour, and the resistance of the band to changes in its length is large.

Resting Shape of the Cell

Comparison of the rigidity of the band and the membrane can also provide insights about the structures maintaining the elliptical geometry of the intact cell. The fact that the band assumes a nearly circular shape after isolation indicates that the band is stressed in situ. The resting dimension of the cell in the long axis is ~1.3 times the circular diameter of the band. From Fig. 2 we observe that a force of ~12.5 $\times$ 10^{-6} dyn would be needed to produce such an extension. If this force were distributed over the 10.0 μm of membrane in the vicinity of the nucleus it would produce a force resultant of ~1.25 $\times$ 10^{-2} dyn/cm in the membrane. Whether or not the membrane alone could support such a load depends on the intrinsic shear rigidity of the membrane. If the shear modulus of the membrane is similar to the mammalian erythrocyte membrane (0.01 dyn/cm), this force resultant would produce an extension of ~35%. This is a rather large deformation for the membrane to support for extended periods of time, especially in view of the fact that the membrane exhibits stress relaxation, i.e., creep, over long times. On the other hand, if the modulus of the amphibian membrane is significantly greater than the modulus of the mammalian membrane (e.g., μ = 0.07 dyn/cm) the force from the band would produce a surface extension of <5.0% and so could easily be supported by the membrane alone. Thus, either the amphibian membrane must be considerably more rigid than its mammalian counterpart, or there must be additional structures (intermediate filaments?) that are responsible for maintaining an elliptical shape.

Measurements of the mechanical properties of isolated marginal bands demonstrate that the band makes (at least) two important contributions to the rigidity of the intact cell. First, because the bending rigidity of the band is three orders of magnitude greater than the bending rigidity of the membrane, the cell is greatly stabilized against indentations at its rim. Second, because the band has a large resistance to increases in the length of its contour, the cell is constrained to deform with a constant perimeter length. This significantly limits the magnitude of extension that the cell can be made to undergo. Additional contributions to cell rigidity may arise from interactions between the marginal band and other cytoskeletal elements. For example, if the membrane skeleton is tightly adherent to the band, its redistribution from one face of the cell to the other would be prevented. This would increase the resistance of the cell to the formation of protrusions such as those formed in micropipet aspiration studies. It is important to emphasize, however, that in spite of the increased stability that the band provides, our calculations indicate that the band does not limit the flexibility of the cell for small extensions or flexure. Thus, the cell retains sufficient flexibility to pass through the microcirculation with ease.

Appendix

The theoretical prediction for the deflection of an elastic ring

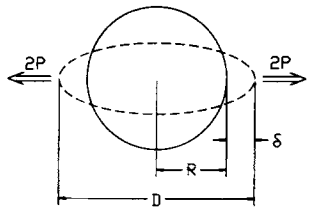

Figure 6. Schematic of the deformation of a marginal band. The initial radius is R, the elongated dimension is D, and the deflection is δ. The total force on the band is $2P$.

was obtained by Libai and Simmonds (11, 12). It is based on a simpler analysis by Frisch-Fay (9) of the deflection of a ring that exhibits elastic deflections in flexure, but is inextensible along the length of its contour (inextensional elastica). The solution for the inextensional elastica is given in terms of the parameters, p and k: $p^2 \cong 2(b/P + 1)^{-1}$; $k^2 \cong 4PR^2/EI$ (A1 and A2). The quantity P is half of the force applied to the ring, R is the unstressed radius of the ring (see Fig. 6), EI is the flexural rigidity of the ring, and b is a constant of integration. The constant b is determined by the condition that the length of the contour is constant. Mathematically, this constraint takes the form: $[K(p) - F(p, \pi/4)] \cdot p/k = \pi/2$ (A3); where $K(p)$ and $F(p, \pi/4)$ are complete and incomplete elliptic integrals of the first kind, respectively. These integrals can be evaluated numerically using standard algorithms. The result for the deflection of the inextensional elastica, δ_{ie} is:

$$\frac{\delta_{ie}}{R} = \pi\left[\frac{1}{p^2} - \frac{1}{2}\right] - 1.0 - \frac{2}{kp}[E(p) - E(p, \pi/4)]; \quad \text{(A4)}$$

where $E(p)$ and $E(p, \pi/4)$ are complete and incomplete elliptic integrals of the second kind, respectively. Note that the solution for δ_{ie} depends on the independent variable P and the parameters EI and R. This functional relationship can be expressed dimensionlessly with δ_{ie}/R as a function of k^2, the dimensionless force. Using equations A1–A4, a tabular function of the corresponding values of δ_{ie}/R, k, and p was created. Throughout this analysis, we have neglected terms of order I/AR^2, which for the marginal band is much <1.0 ($\sim 1.0 \times 10^{-5}$).

The solution for the inextensional elastica is accurate when the deflection of the band is small, but for large deflections, extensions of the band contour may contribute to the total deflection. To account for this Libai and Simmonds (11, 12) developed a modified solution based on a ring that exhibits a linear elastic response to forces along the band contour (semiextensional elastica). The displacement (δ_{se}) for this case is:

$$\delta_{se} = \delta_{ie} + \frac{4PR}{EA}\left[\frac{2}{3p^2}\left(1 + \frac{\delta_{ie}}{R}\right) - \frac{1}{3}\left(1 + \frac{\delta_{ie}}{R}\right) - \frac{\pi}{24}\right]. \quad \text{(A5)}$$

Again, terms of I/AR^2 have been neglected. Note that the semiextensional deflection depends on the extensional rigidity EA as well as EI and R.

In the least-squares regression, the deformed band dimension D was the dependent variable and the load on the band $2P$ was the independent variable. The value of the parameter EA was fixed, and EI and R were determined from the experimental data by least-squares regression to the functional relationship given by equation A5.

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