Membrane Tether Formation from Blebbing Cells

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ABSTRACT Membrane tension has been proposed to be important in regulating cell functions such as endocytosis and cell motility. The apparent membrane tension has been calculated from tether forces measured with laser tweezers. Both membrane-cytoskeleton adhesion and membrane tension contribute to the tether force. Separation of the plasma membrane from the cytoskeleton occurs in membrane blebs, which could remove the membrane-cytoskeleton adhesion term. In renal epithelial cells, tether forces are significantly lower on blebs than on membranes that are supported by cytoskeleton. Furthermore, the tether forces are equal on apical and basolateral blebs. In contrast, tether forces from membranes supported by the cytoskeleton are greater in apical than in basolateral regions, which is consistent with the greater apparent cytoskeletal density in the apical region. We suggest that the tether force on blebs primarily contains only the membrane tension term and that the membrane tension may be uniform over the cell surface. Additional support for this hypothesis comes from observations of melanoma cells that spontaneously bleb. In melanoma cells, tether forces on blebs are proportional to the radius of the bleb, and as large blebs form, there are spikes in the tether force in other cell regions. We suggest that an internal osmotic pressure inflates the blebs, and the pressure calculated from the Law of Laplace is similar to independent measurements of intracellular pressures. When the membrane tension term is subtracted from the apparent membrane tension over the cytoskeleton, the membrane-cytoskeleton adhesion term can be estimated. In both cell systems, membrane-cytoskeleton adhesion was the major factor in generating the tether force.

INTRODUCTION

There are several intensive variables of plasma membranes that can affect the whole cell, including transmembrane electrical potential, bilayer couple imbalance (Sheetz and Singer, 1974), and membrane tension (Sheetz and Dai, 1996). The tension in the plasma membrane is hypothesized to be important in many cellular functions, including membrane trafficking and cell motility (Sheetz and Dai, 1996). Tether formation with a micropipette has been used to measure membrane tensions of lipid vesicles or red cells. The mathematical relationships between tether force and tension have been derived for several vesicle shapes (Evans and Yeung, 1994; Hochmuth et al., 1973; Waugh et al., 1992). However, cells are much more complicated than lipid vesicles, because membrane-cytoskeleton adhesion also contributes to the tether force. Energy is needed to separate the tether membrane from the cytoskeleton, which has been described as a membrane osmotic pressure (Dai and Sheetz, 1995: Waugh and Bauserman, 1995). Previously, we developed a flexible method to form membrane tethers and measure tether forces with laser tweezers (Dai and Sheetz, 1995). The apparent membrane tension (T) was calculated from tether forces, and T combines membrane tension (T_m) with membrane-cytoskeleton adhesion (γ), because the two are tightly coupled (Hochmuth et al., 1996; Sheetz and Dai, 1996). Methods are needed to separate the two components of apparent membrane tension to better understand how apparent membrane tension affects cell functions.

The tension in plasma membranes of cells in culture has not been measured. Lipid vesicles and erythrocytes under isotonic conditions have very small membrane tensions, but micropipette suction has been used to create tension in the bilayer. In irregularly shaped cells with a rigid cytoskeleton, several factors could generate a tension in the membrane plane. Under certain circumstances, the binding of the plasma membrane to the cytoskeleton can create a tension in the bilayer, particularly when additional cytoskeleton binding sites are present (Dai et al., 1998). In cases where the plasma membrane has been expanded by hypotonic swelling, the rapid return to isotonic conditions results in an excess of membrane. That excess membrane still conforms to the cytoskeleton through the formation of deep invaginations of the plasma membrane (Dai et al., 1998). A second factor is the ratio of plasma membrane area to cell volume for a given cell shape. In many cells, there is rapid membrane exchange, and endocytosis is coupled with exocytosis to maintain the correct surface area. In separate studies we have shown that the endocytosis rate is inversely dependent upon apparent membrane tension, suggesting that membrane area is regulated by apparent membrane tension (Dai et al., 1997). Thus the endocytic machinery of the cell could create tension in the bilayer by drawing in membrane until the tension is too great to allow further endocytosis. A third factor affecting membrane tension is intracellular pressure (Kelly and Macklem, 1991). The measured pressures correspond to an osmotic pressure created by a few micromolar greater salt within the cell (this corresponds to approximately a million molecules per cell volume). Membrane tensions and changes in membrane tension along the cell

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surface may be affected by cytoskeletal associations, pressure differences between the cell interior and environment, and the balance of endo- and exocytosis. It is unknown how these factors affect the apparent tension of plasma membranes as measured by tether formation with the laser tweezers.

Membrane-cytoskeleton adhesion is critical for many cell functions and cell viability. The linkage between plasma membrane and cytoskeleton is complex and includes both membrane protein-cytoskeleton and lipid-cytoskeleton interactions. A number of membrane-cytoskeleton linkage proteins have been found and characterized. The results indicate that the interaction between membrane and cytoskeleton not only stabilizes the plasma membrane, but also affects many cell functions. Human melanoma cells lacking an actin-binding protein (ABP-280), which cross-links actin filaments and connects these filaments to plasma membrane glycoproteins, bleb extensively and have defects in locomotion (Cunningham et al., 1992). Spectrin and ankyrin defects also appear to affect a number of important aspects of neural membranes (Bennett et al., 1997). A role for lipid-cytoskeletal protein interactions has been indicated by the presence of multiple lipid-binding domains in many cytoskeletal proteins found at the plasma membrane.

Cell blebs may provide an opportunity to determine the membrane tension. In the phenomenon of bleb formation, there is evidence of a complete separation of membrane from cytoskeleton. At the light and electron microscopic levels, the plasma membrane lacks most cytoskeleton proteins in blebs. Membrane glycoprotein diffusion is dramatically increased to the level seen in pure lipid bilayers (Tank et al., 1982). Membrane blebs are spherical membrane extensions that are commonly seen at the periphery of eukaryotic cells as they spread on a substrate, during mitosis, or at the leading edge of migrating cells (Cunningham, 1995). Alternatively, large, nonretracting blebs form on cells injured by physical or chemical stress (Trump et al., 1971). The rabbit renal proximal tubule cells are highly polarized epithelial cells that bleb extensively after hypoxic injury (Chen et al., 1997). Study of blebs and the physical strength of the membrane-cytoskeleton association in renal tubules could provide clues about the mechanism of formation of special membrane domains. Because of the transient nature of blebs in active cells, i.e., the rapidity with which they transform into other types of protrusions, studies of bleb properties have been limited until the discovery of a cell line that blebs extensively (Cunningham, 1995).

In this study, we examined tether forces from bleb membranes and from adjacent membrane regions, which were supported by the cytoskeleton. We then estimated the membrane tension and the membrane-cytoskeleton adhesion components of tether forces. This was done for the highly polarized rabbit renal proximal tubule epithelial cells after anoxic injury and for the human melanoma cells that normally display circumferential blebs of the plasma membrane. From these studies, the membrane tension appears to be a continuum property of the whole cell surface, and membrane-cytoskeleton adhesion is responsible for a major portion of the tether force.

MATERIALS AND METHODS

Cell culture and preparation

The human melanoma lines M2 and A7 are cultured at 37°C and 5% CO_2 in minimum essential medium (MEM) supplemented with 8% newborn calf serum and 2% fetal calf serum. The M2 cell line is from human melanoma cells that lack the expression of actin-binding protein 280 (ABP-280). M2 cells show extensive bleb formation. A7 cells are produced from M2 cells that are transfected with wild-type ABP-280 gene, and A7 cells show decreased bleb formation. The A7 medium also contains G418 (1000 μg/ml).

Rabbit proximal tubules (PTs) are isolated and purified as previously described (Dickman and Mandel, 1989). Briefly, female New Zealand White rabbits (1–2 kg; Robinson, Winston-Salem, NC) are injected with heparin and anesthetized. The cortices are trimmed from the excised kidneys; minced; digested for 60 min at 37°C in DME-F12 culture medium containing 150 U/ml collagenase, 2 mg/ml bovine serum albumin (BSA), and 1 U/ml DNase; and gassed with 95% O_2/5% CO_2. The resulting isolated tubules are washed free of collagenase. The PTs are separated from other segments by centrifugation on a self-generating 50% Percoll gradient for 30 min at 36,000 $\times$ g. The PTs are then washed and resuspended at 2 mg protein/ml. For the anoxia treatment, the PTs are subjected to anoxia in the absence or presence of 3 mM glycine for 30 min by gassing with 95% N_2/5% CO_2 after the preincubation period.

Bead preparation

To form a membrane tether, a "handle" is required for the laser tweezers to hold a part of the membrane. The handle is a bead that tightly binds to the cell surface. We used rat IgG-coated beads for melanoma cell experiments and ConA-coated beads for renal cell experiments. To prepare IgG-coated beads, rat IgG (Sigma, St. Louis, MO) is solubilized at a concentration of 10 mg/ml in phosphate-buffered saline. Then 50 μl of covaspheres (0.5 μm; Duke Scientific, Palo Alto, CA) is added to 50 μl of the above IgG solution and incubated at 4°C overnight. The beads are pelleted by centrifugation at 2000 $\times$ g and 4°C for 10 min. Then the beads are resuspended in 1 mg/ml bovine serum albumin–phosphate-buffered saline solution, rinsed by pelleting and resuspension with MEM three times, and resuspended in 100 μl MEM. Similarly, ConA (Sigma Chemical Company, St. Louis, MO) was used to coat beads. For the experiments, the bead solutions were diluted 3:100 in cell medium.

Calibration of laser tweezers

At present there is no theory that can be used to directly calculate the trapping force for beads. All of the forces must be determined empirically, and the forces are commonly calibrated against viscous drag exerted by fluid flow. For a bead of radius of r, the drag force can be obtained from Stokes' Law: $F = 6\pi\eta rv$. Here η is the fluid viscosity and v is the flow rate. The laser optical trap is calibrated by measuring the trap's stiffness as described before (Dai and Sheetz, 1995). Briefly, a viscous force was generated by moving the specimen at a constant velocity with a piezoceramic-driven stage. The position of the bead in the trap was tracked using the nanometer-level tracking program (Gelles et al., 1988) to analyze video records of the experiments. The viscous force on the bead was calculated through Stokes' Law. The calibration showed a linear force-displacement relationship for the optical tweezers, and the slope of the linear fit gave the trap stiffness. After the trap stiffness was determined, the force on the beads was calculated from the displacement of the beads from the trap center.

Laser tweezer manipulations

The cells were viewed with a video-enhanced differential interference contrast (DIC) microscope (IM-35 microscope; Zeiss, Oberkochen, Germany) with a fiber optic illuminator. The stage was maintained at 38°C with an air current incubator. The laser trap consisted of a polarized beam from an 11-W TEM00-mode near-infrared (1064 nm) Nd:YAG laser (model 116Fn; Quantronix Corp., Smithtown, NY) that was expanded by a 3× beam expander (CVI Corporation) and then focused through an 80-mm focal length achromatic lens (Melles Griot, Irvine, CA) into the epifluorescence port of a Zeiss IM-35 microscope.

To prepare a melanoma cell sample, the coverslip containing cells was mounted on an aluminum coverslip holder with silicone grease, and then a second cleaned coverslip was mounted on top to form a flow cell. IgG-coated latex beads in medium were exchanged for the normal medium. To form a tether from the melanoma cell, an IgG-coated latex bead was held on the cell surface for several seconds. Then the bead was pulled away from the cell surface by moving the sample with a piezoceramic-driven stage (Wye Creek Instruments, Frederick, MD) at a constant velocity. All of the experiments were recorded on videotape for later analysis. A relatively short tether (2–5 μm in length) was formed from the bleb membrane to avoid affecting the bleb size.

To prepare renal cell samples, PTs were mounted on a coverslip with Cell-Tak (Collaborative Biomedical Products, Bedford, MA) after 30 min of incubation or 30 min of anoxia. Then a second cleaned coverslip was mounted on top to form a flow chamber. The ConA-covered latex beads were added in the flow chamber. Tethers were formed on the PT renal cell apical region, the basal region, and blebs from the anoxic cells. Because the size of these blebs is larger than that of the melanoma cell, tethers from cells and blebs are relatively the same in length (~5–10 μm in length). The tether force was independent of tether length over this range.

Tether force measurement and the calculation of T_m and F_{ad}

To measure the static tether force, a tether was formed and kept at a constant length. Then the bead position in the trap during tether formation was tracked using the nanometer-scale tracking program (Gelles et al., 1988). The tether force on the bead was calculated from the calibration of the laser trap. The static tether force only contained the contributions from membrane tension, bending stiffness of the bilayer, and membrane-cytoskeleton adhesion. According to Hochmuth et al. (1996),

$$T = T_m + \gamma = F_0^2/8B\pi^2, \quad (1)$$

where T_m is the membrane tension, T is the apparent membrane tension, F_0 is the static tether force from the cell surface, γ is the membrane-cytoskeleton adhesion term, and B is the bending stiffness, and the value is assumed to be 2.7×10^{-19} N·m (a value typical for lipid bilayer, red cell, and neutrophil membranes). Because the bleb membrane lacked cytoskeleton support, the membrane tension calculated from bleb membrane static tether force (F_b) was assumed to be the membrane tension, and Eq. 1 becomes

$$T_m = F_b^2/8B\pi^2. \quad (2)$$

We think that it is helpful to calculate the force component of the tether force generated by membrane-cytoskeleton adhesion (F_{ad}). If we assume that the bleb membrane does not contain cytoskeleton support and that the membrane tension is a constant over the whole cell surface, then the adhesion term is simply the difference between the tether force over the cytoskeleton and the tether force over a bleb:

$$F_{ad} = F_0 - F_b. \quad (3)$$

Calculation of the intracellular pressure in melanoma cells

We define intracellular pressure as the pressure difference (ΔP) between the inside pressure (P_i) and the outside pressure (P_o) ($\Delta P = P_i - P_o$), which presumably is the atmospheric pressure for the cultured cells. Fig. 1 illustrates the balance of forces on a membrane bleb. By analyzing the force balance on the bleb, we can obtain the following equation:

$$\Delta P = 2T_m/R_b \quad (4)$$

where R_b is the radius and T_m is the bleb membrane tension. In fact, Eq. 4 is an alternative form of the law of Laplace. When Eqs. 2 and 4 are combined, the following is obtained:

$$\Delta P = F_b^2/4\pi^2 B R_b \quad (5)$$

B is a constant parameter (it is assumed that $B = 2.7 \times 10^{-19}$ N·m). With the measured static tether force (F_b) and bleb radius (R_b), ΔP can then be calculated from Eq. 5. We have assumed that the contribution of the tether formation to the pressure is insignificant, because the tether force does not change with tether length.

RESULTS

Bead binding and tether formation

We have tested the bead binding affinities for the membranes by trapping a bead and holding the bead on the membrane for 1–3 s before trying to move the bead away with the laser tweezers. For the melanoma cells, ~50% of the IgG-coated beads bound to either the cell membrane or the bleb membrane. The IgG-coated beads rarely bound to the rabbit renal cell surface; therefore, ConA-coated beads were used to increase the binding affinity. The same tether force was observed when either IgG- or ConA-coated beads were used to form tethers. About 40% of the ConA-coated beads bound to both the cell membrane and the bleb membrane. These observations suggest that the membrane binding component is at the same concentration in the blebs and the membrane. When we first formed the tether, we could move the tether laterally on the bleb membrane, but in only rare cases could it be moved on the nonblebbing region. The free lateral movement of the tether on the blebs indicates that the bleb membranes lack cytoskeleton support. In addition, the probability of tether formation for the bleb membrane-bound beads is ~95%, while it is ~30% for the beads bound to the cell surface, further suggesting that the cytoskeleton support is lost in the bleb membrane.

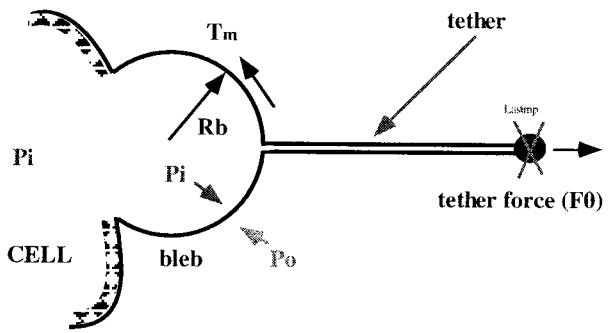

FIGURE 1 Diagram shows the balance of forces on the bleb membrane. P_i is pressure inside the cell, and P_o is pressure outside the cell. In our case, P_o equals the atmospheric pressure.

Membrane tether forces for the polarized renal cells

We have measured the static tether forces of tethers from the renal epithelial cell membrane at apical and basolateral surfaces and from the membrane blebs under anoxic conditions. Blebs induced by anoxia are not self-healing. Many smaller blebs eventually fuse and form larger blebs, as would be expected, because the membrane tension in the larger blebs is greater with the same internal pressure. The plasma membrane of renal cells still keeps its integrity under our experimental conditions. However, during prolonged renal anoxia (>45 min), the plasma membrane ruptures, causing the release of intracellular contents, such as lactate dehydrogenase (LDH), into the extracellular space (Chen et al., 1997). Tether forces from the basal region and basal blebs were ~14 pN and ~8 pN (Chen et al., 1997; also shown in Fig. 2), respectively. The tether forces from the apical region were larger than those from the basal region, whereas for the blebs on either the basal or apical regions, the tether forces were the same (Fig. 2 *A*). Thus, the loss of the cytoskeleton-membrane adhesion resulted in a major decrease in the tether force, and regional differences were seen in the membrane-cytoskeleton adhesion component and not in the membrane tension component.

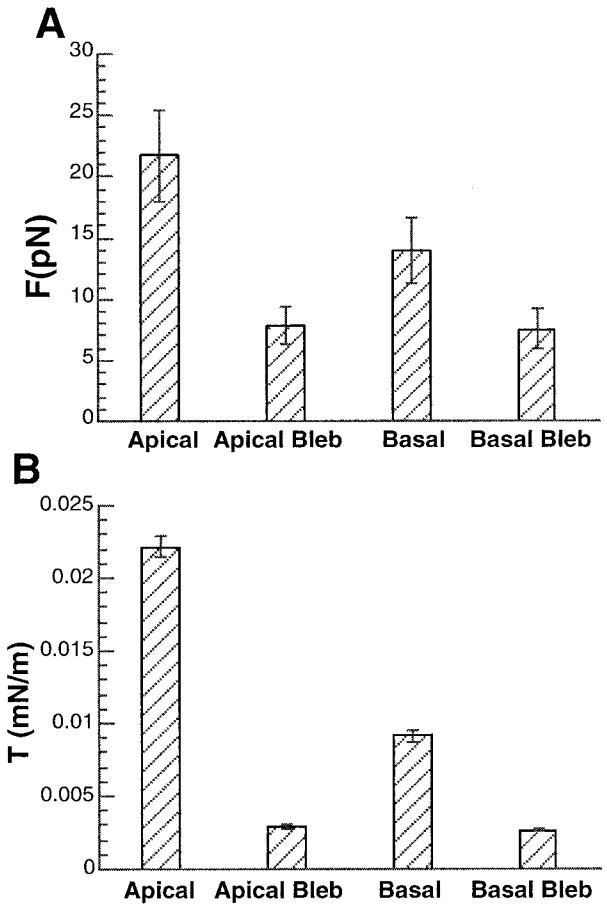

FIGURE 2 Static tether forces (*A*) and calculated membrane tensions (*B*) from renal cells after anoxia. The average tether force over the cytoskeleton at the apical region is higher than that at the basal region. However, when cells bleb under anoxic conditions, there is no difference in tether forces for tethers formed on basal or apical blebs (the data on tether forces from bleb and nonbleb membranes at the basal region have been published; Chen et al., 1997). This indicates that tensions on blebs (assumed to be the membrane tension) at the apical and basal areas are the same.

T_m and F_{ad} for renal cells

Because the bleb membrane does not have cytoskeleton support, it is suggested that there is no contribution from the membrane-cytoskeleton adhesion in the bleb membrane. When the membrane tensions were calculated from the tether forces using Eq. 2, it was found that the membrane tensions in the blebs were the same in apical and basal regions (Fig. 2 *B*). This suggests that membrane tension was small and continuous over the cell surface.

Using Eq. 3, the membrane-cytoskeleton adhesion component, F_{ad}, of the tether force is calculated for renal cells (Fig. 3). The membrane-cytoskeleton adhesion accounts for a force of ~14 pN in the apical region and ~6 pN in the basolateral region. This suggests that membrane-cytoskeleton adhesion is the major factor in generating the tether force. The lower value for the basolateral region is consistent with the less extensive cytoskeleton support beneath the basolateral membrane.

Membrane tether forces for melanoma cells

Several melanoma cell lines were selected for metastatic potential and were found to have the property that they continuously formed blebs under normal growth conditions (Cunningham et al., 1992). The M2 line was deficient in actin binding protein 280 (ABP 280). Unlike blebs from renal cells under anoxic conditions, blebs on the melanoma cell surface are self-healing. A bleb on a melanoma cell progressively goes from expansion to stasis, and then to the development of ruffling. Bleb expansion is smooth and typically requires 4–7 s to reach full size. After reaching its full size, a bleb remains extended for ~10 s, then retracts

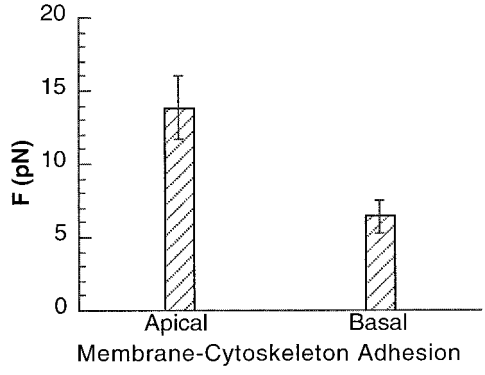

FIGURE 3 The membrane-cytoskeleton adhesion component, F_{ab}, of tether forces for renal cells. The value of F_{ab} at the apical region is larger than that at the basal region.

over ~30 s. The stable bleb gives us ~10 s to measure the tether force. F-actin assembly is directly related to the bleb self-healing (Cunningham, 1995). Fig. 4 is a picture of a tether formed from a bleb on an M2 cell surface.

We have found that the size of small blebs decreased dramatically when long tethers were formed from them, indicating that the tether membrane was preferentially drawn from the bleb. Short tethers (2–5 μm) were formed from relatively small blebs (less than 2 μm in diameter) for tension measurements, and these short tethers did not change the bleb size significantly. As shown in Fig. 5 *A*, the static tether force for the bleb membrane tether was smaller than the tether force over regions of cytoskeletally supported membrane. M2 cells are human melanoma cells that are ABP-280 deficient. In contrast, A7 cells are a subclone of M2 cells wherein the level of ABP-280 has been restored to normal (Cunningham et al., 1992). The normal phenotype has been partially resorted in A7 cells, and blebbing was reduced. Tether forces on cytoskeletally supported areas of A7 cells are larger than those for M2 cells, which is consistent with the hypothesis that ABP-280 is one of the membrane-cytoskeleton linkers and its expression strengthens the membrane-cytoskeleton attachment. The tension on the blebs, which presumably is the membrane tension, is about the same for the two cells.

T_m and F_{ad} for melanoma cells

The apparent membrane tensions are calculated from the tether forces over the cytoskeletally supported membranes, and the value for M2 cells is slightly smaller than that for A7 cells. Compared with the apparent membrane tensions, the membrane tensions calculated using Eq. 2 for M2 and A7 cells are relatively small and about equal at 0.011 mN/m and 0.012 mN/m for M2 and A7 cells, respectively (Fig. 5 *B*).

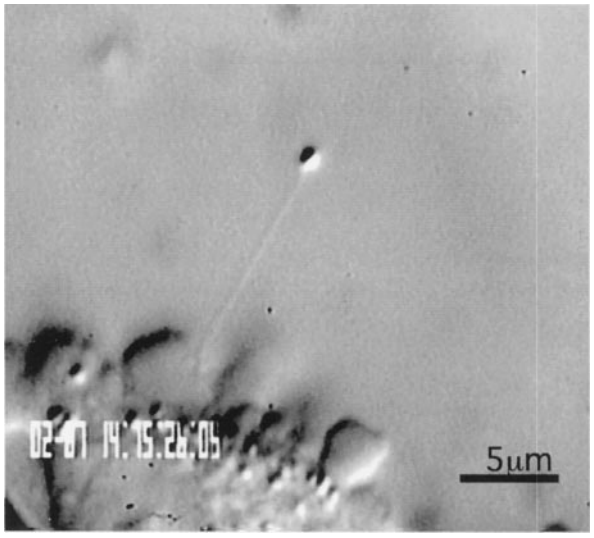

FIGURE 4 Video-enhanced DIC micrograph of a membrane tether formed from a bleb on an M2 cell.

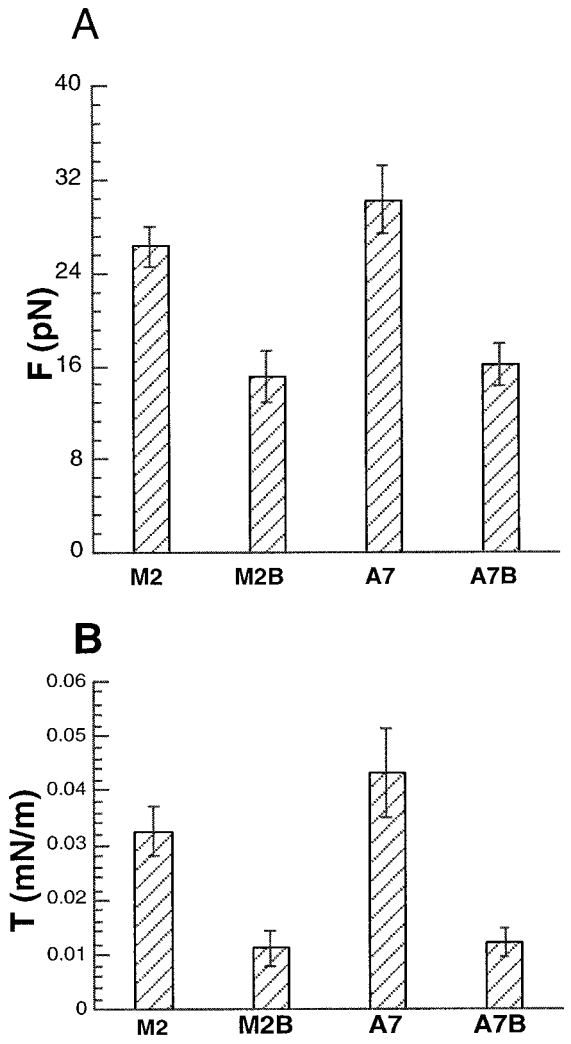

FIGURE 5 Static tether forces (*A*) measured from blebs of both M2 cells (M2B) (n = 94) and A7 cells (A7B) (n = 26), and nonblebbing membranes of M2 cells (n = 40) and A7 cells (n = 26). Tether forces on membrane blebs are about half of those on nonbleb regions. Apparent membrane tensions (*B*) calculated from tether forces measured from human melanoma cells, M2 and A7. Membrane tensions on blebs are very small compared with apparent membrane tensions on nonbleb regions.

From Eq. 3, the values of the membrane-cytoskeleton adhesion force that are obtained for M2 and A7 cells are ~11 pN and 14 pN, respectively (Fig. 6). The greater F_{ad} in A7 cells may be due to the fact that these cells have been transfected with wild-type ABP-280 gene and the membrane-cytoskeleton adhesion is enhanced as expected.

The intracellular pressure of melanoma cells

We noted that the blebs formed by swelling, which indicated that an expansive pressure was present in the cell. As shown in Fig. 7, the tension of tethers from blebs of different size on the same cell increased with the bleb size. The data presented are from a single cell but are consistent with values from other cells. When the line in Fig. 7 is extrap-

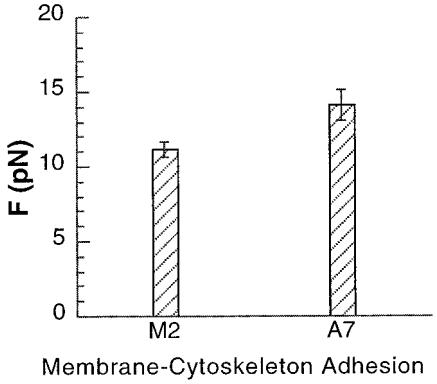

FIGURE 6 The membrane-cytoskeleton adhesion component, F_{ab}, of tether forces for melanoma cells. The value of F_{ad} of A7 cells is larger than that of M2 cells.

olated to a small bleb diameter (1.6 μm), the estimated value of the tether force is ~17 pN, which is in good agreement with the observed value for small blebs (Fig. 5). This is all consistent with the prediction from Eq. 5.

M2 cells display extensive blebbing all the time. On the other hand, when A7 cells were cultured for 4 hours, ~40% of A7 cells stop blebbing. After 8 hours in culture, the majority of A7 cells stop blebbing. We measured the tether force on blebs from both the M2 cells and A7 cells ~6 hours after plating to allow comparisons to be made. The average ΔP is 20.7 $\pm$ 5.9 N/m^2 (STD) for M2 cells and is 24.2 $\pm$ 8.0 N/m^2 (STD) in A7 cells. An explanation for the slightly lower bleb membrane tension and ΔP in M2 cells is that M2 cells bleb more extensively, releasing ΔP and lowering tension on blebs.

Blebs presumably form when the pressure across the plasma membrane exceeds the strength of the bond between the membrane and cytoskeleton. Therefore, increased pressure should increase bleb formation and vice versa. Changing the osmolarity gradient across the plasma membrane altered the intracellular pressure. Cell blebbing was enhanced (both the frequency of bleb formation and bleb size were increased) when the medium osmolarity was reduced by adding 10% H_2O (v/v) to the medium. Conversely, blebbing was suppressed (both the frequency of bleb formation and bleb size were decreased) when the medium osmolarity was increased by adding 5 mM sucrose. If it is assumed that this ΔP is caused by the salt, NaCl, according to the relationship between osmotic pressure and concentration differences, only a ~4 μM difference in NaCl concentration across plasma membrane is needed to generate a ΔP of 20 N/m^2. If the cell diameter is assumed to be 10 μm, then ~10^6 NaCl molecules more in the cytoplasm or the loss of water equivalent to 0.003% of the cell volume is sufficient to generate such an intracellular pressure. This pressure is resisted by the cytoskeleton. The cell appears to tightly regulate both the pressure and the adhesion between the membrane and the cytoskeleton.

In some of the larger blebs, the membrane tension is quite high, and the higher tension should affect membrane tension in other parts of the cell. Interestingly, during a large bleb formation, we often see a spike in the force on a tether formed on the membrane near the bleb (Fig. 8). In 10 observations of the spikes in the tether force off the bleb, we found that the average duration of the spike was less than 1 s. The very small amount of water or salt movement needed to balance the osmolarity difference may explain the short duration of the increase in tether force during blebbing.

DISCUSSION

In pure lipid vesicles, the tension in the membrane bilayer is very low, and the membrane tension of erythrocytes is about zero. Little is known about the tension in the plasma membrane bilayer of normal cells. Here we have calculated the

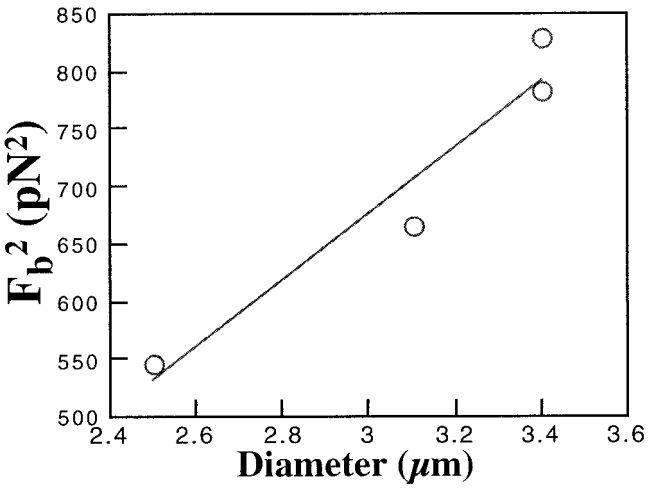

FIGURE 7 Plot of the tether forces squared (F_b^2) versus bleb diameter on M2 cells, which is linear, as predicted by Eq. 5. Larger blebs have tether forces that exceed the tether forces normally found on the supported membrane areas.

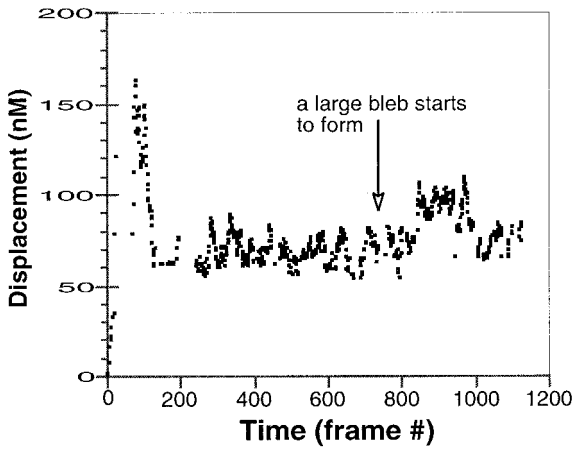

FIGURE 8 Plot of tether force (0.3 pN/nm) versus time (30 frames/s) on a region of the cell off the bleb. About 3 s after the bleb starts to form, the tether force rises from 24 to 28.5 pN and stays high for over 3 s in this case.

membrane tension in both human melanoma cells and renal epithelial cells from tether forces measured on blebs. In both cases, the membrane tension is the same at different places on the cell, indicating that membrane tension is continuous over the cell surface. In addition, we found that membrane tension is a relatively small component of the apparent membrane tension.

In many respects, biological membranes can be treated as two-dimensional materials, because the thickness of the bilayer is only 5 nm. Membrane bilayers are relatively inelastic (~3% stretch at lytic tensions, which are ~100–1000-fold greater than normal membrane tensions; Nichol and Hutter, 1996). Furthermore, the fluid nature of the bilayer ensures that there are no shear forces as the membrane conforms to cytoskeletal projections. An important consequence of these mechanical properties is that tension generated in one portion of the cell membrane is propagated through the membrane to potentially alter the whole cell's state. We indeed find that the apical and basolateral bilayer tensions are the same, although they are separated by the gap junctions. In kidney epithelia under anoxic conditions, the larger blebs absorb smaller blebs, as would be predicted because of the higher tension for the same fluid pressure. In melanoma cells, blebs heal rapidly, and blebs of different sizes are evident on the same cell, because they are reinforced with actin.

The main concern here is whether it is accurate to assume that the tension on the bleb membrane is equal to the tension in the plasma membrane. Two pieces of evidence may argue that this assumption is reasonable. First, bleb membranes do not have cytoskeleton support. Second, membrane in the tether is mainly from the bleb. We have found that during a long tether formation from a bleb, the bleb size decreases. For a very small bleb, the bleb can be almost completely removed if a relatively long tether is formed (Dai and Sheetz, unpublished observations). We may expect that the actual membrane tension is lower than that measured from bleb tethers if some elements of the cytoskeleton are attached to the bleb or if some membrane proteins do not partition equally into the tether. Thus the calculated value of membrane tension from the bleb tether force provides an upper limit of the actual membrane tension.

Membrane-cytoskeleton adhesion and membrane osmotic pressure

Membrane-cytoskeleton interaction provides stabilization for the plasma membrane and conformity to the cytoskeleton, but more importantly, it can be rapidly adjusted in a variety of cell functions, such as cell signaling, cell migration, and cell polarization. Plasma membranes are supported and confined to domains by the underlying actin cytoskeleton (reviewed in Sheetz, 1995). Membrane lipids are also shown to directly or indirectly bind to actin cytoskeleton (Laliberte and Gicquaud, 1988; Hyvonen et al., 1995). It has been proposed that pleckstrin homology (PH) domains are important in mediating membrane lipid-cytoskeleton adhesion (Hyvonen et al., 1995). Based upon a number of observations it appears that the membrane binds to cytoskeleton continuously, as if supported by a continuum of adhesive interactions. This can explain why the tether force does not jump when a tether can be moved laterally over the cell surface (a rare event in these cells) (Dai and Sheetz, unpublished results).

It is clear that the cytoskeleton does not enter the tether (Berk and Hochmuth, 1992; Hwang and Waugh, 1997). Thus a tether force will arise from breaking the membrane-cytoskeleton interaction. The adhesive interactions between membrane proteins or lipids and the cytoskeleton cause a depletion of those membrane components in the tethers, creating a different chemical environment for lipids in the tethers and in the plasma membrane. Because lipids are fluid and they are in a lower energy state when bound to cytoskeletal proteins, this is analogous to an osmotic pressure resulting from a difference in the activity of water across a semipermeable barrier. Therefore, membrane osmotic pressure is used to explain the tension caused by membrane-cytoskeleton adhesion (Dai and Sheetz, 1995: Waugh and Bauserman, 1995).

Bleb formation and intracellular pressure

Because of their spherical shape and smooth growth, a swelling pressure is believed to inflate blebs. A swelling pressure across the plasma membrane has been inferred in different eukaryotic cells. Some evidence comes from the following observations: large, nonretracting blisters are seen with cell injury by physical or chemical stress. Spherical membrane blebs are also seen with uninjured cells during spreading and mitosis or at the leading edge of moving cells (Cunningham, 1995). The direct measurement of the intracellular pressure has been difficult. By the use of an invasive method, the servo-null technique, the internal pressure in the *Xenopus laevis* oocyte and *Amoeba proteus* has been measured (the values varied from an average of 27 N/m^2 in *Xenopus laevis* oocytes to several hundred N/m^2 in the *Amoeba proteus*; Kelly and Macklem, 1991; Yanai et al., 1996; Fein, 1972). In this study, we estimated the intracellular pressure in a noninvasive manner, and the value we obtained is comparable to the published results. Our findings indicate that a significant pressure drives membrane bleb formation, and tension in the cytoskeleton normally balances the outward pressure.

The significant implication of the intracellular pressure is that because this internal force driving bleb formation always exists in a cell, this process will influence other cell extensions as well. The intracellular pressure has been suggested to be important to different biological functions, particularly in cell motility. It has been suggested that in conjunction with weakening of the cell cortex over a localized area, the intracellular pressure could induce the formation of pseudopods (Dong et al., 1994). The intracellular

pressure also has been shown to provide a motive force for *Amoeba proteus* cell motion (Yanai et al., 1996). In recent studies of Walker carcinosarcoma cell locomotion, membrane protrudes faster (1.2–4.1 μm/s) than known rates of actin elongation (Keller and Bebie, 1996). Thus protrusion could be analogous to bleb formation in certain cases.

SUMMARY

We have calculated the membrane tension from tether forces of cell blebs and suggested that it is continuous over the cell surface. We have also found that membrane-cytoskeleton adhesion is the major component in the apparent membrane tension calculated from tether forces. We postulate that membrane-cytoskeleton adhesion is manifested by a membrane osmotic pressure generated by the separation of membrane from the cytoskeleton in the tether. An intracellular pressure is observed in the cell that is normally supported by contractile tension in the cytoskeleton. Intracellular pressure can contribute to membrane tension and drives some membrane protrusions as well as bleb formation.

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