Characteristics of a Membrane Reservoir Buffering Membrane Tension

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ABSTRACT When membrane-attached beads are pulled vertically by a laser tweezers, a membrane tube of constant diameter (tether) is formed. We found that the force on the bead (tether force) did not depend on tether length over a wide range of tether lengths, which indicates that a previously unidentified reservoir of membrane and not stretch of the plasma membrane provides the tether membrane. Plots of tether force vs. tether length have an initial phase, an elongation phase, and an exponential phase. During the major elongation phase, tether force is constant, buffered by the "membrane reservoir." Finally, there is an abrupt exponential rise in force that brings the tether out of the trap, indicating depletion of the membrane reservoir. In chick embryo fibroblasts and 3T3 fibroblasts, the maximum tether lengths that can be pulled at a velocity of 4 μm/s are 5.1 $\pm$ 0.3 and 5.0 $\pm$ 0.2 μm, respectively. To examine the importance of the actin cytoskeleton, we treated cells with cytochalasin B or D and found that the tether lengths increased dramatically to 13.8 $\pm$ 0.8 and 12.0 $\pm$ 0.7 μm, respectively. Similarly, treatment of the cells with colchicine and nocodazole results in more than a twofold increase in tether length. We found that elevation of membrane tension (through osmotic pressure, a long-term elevation of tether force, or a number of transitory increases) increased reservoir size over the whole cell. Using a tracking system to hold tether force on the bead constant near its maximal length in the exponential phase, the rate of elongation of the tethers was measured as a function of tether force (membrane tension). The rate of elongation of tethers was linearly dependent on the tether force and reflected an increase in size of the reservoir. Increases in the reservoir caused by tension increases on one side of the cell caused increases in reservoir size on the other side of the cell. Thus, we suggest that cells maintain a plasma membrane reservoir to buffer against changes in membrane tension and that the reservoir is increased with membrane tension or disruption of the cytoskeleton.

INTRODUCTION

One of the most important properties of the plasma membrane is to serve as a flexible continuous barrier between the cell constituents and the external environment. Active cells undergo rapid morphological changes and can withstand changes in medium osmolarity. It is clear that the plasma membrane is very active, since dramatic changes in cell morphology often require large changes in membrane area. The plasma membrane cannot stretch to accommodate the changes, since the maximum elastic stretching of a membrane is about 4% (Evans and Skalak, 1979; Waugh, 1983) at lytic tensions, which are 100- to 1000-fold greater than normal (Dai and Sheetz, 1995, 1997). Therefore, additional membrane must be drawn from internal compartments of the cell. In a similar manner, the cell must accommodate changes in medium osmolarity that would cause cell swelling. In our previous studies, we have found that the cell plasma membrane tension is constant under normal conditions and even major osmotic perturbations cause relatively minor changes in tension (Dai et al., 1998). Thus, the cell must have mechanisms to regulate membrane tension. A working hypothesis maintains that tension is tied to membrane traffic and membrane-cytoskeleton adhesion and serves to regulate several important cell functions, including endocytosis rate and motility. Control of membrane traffic through membrane tension could help to maintain the correct plasma membrane area.

The traffic of membrane to and from the plasma membrane is rapid in most cells and membrane could be added in response to morphological or osmotic changes. However, membrane tensions have a variation of 10%, whereas to increase the plasma membrane area by only 1 μm^2 in a typical cell (1000 μm^2 of plasma membrane) would cause a 500% increase in membrane tension. A membrane reservoir could buffer variations in membrane tension. The concept of a membrane reservoir has been considered for platelets and phagocytic cells. In platelets, there are many invaginations of the plasma membrane that contain the membrane needed for the extension of long filopodia following platelet activation. Excess membrane must be available in macrophages to provide the plasma membrane that is endocytosed during phagocytosis. Several previous studies of the membrane reservoir have focused on measuring variations in the plasma membrane surface area of macrophages (Burwen and Satir, 1977; Petty et al., 1981). Using scanning electron microscopy, Petty et al. (1981) have shown that the macrophage surface folds decrease dramatically after phagocytosis, suggesting that the membrane reservoir in macrophages is contained in surface folds. A reservoir of membrane in either invaginations or surface folds could buffer changes in membrane tension. Therefore, in order to elucidate the nature and to characterize the dynamics of the membrane reservoir, it is necessary to find a method for probing the relative size of the membrane reservoir during chemical or mechanical perturbations.

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There is evidence of a membrane reservoir that does serve as a buffer of membrane tension from studies of tethers in a variety of cells. When a latex bead attached to the cell plasma membrane is moved away from the cell, a thin cylindrical strand (tether) of membrane material is extended that links the bead and cell. During this process, membrane flows from the cell plasma membrane into the growing tether (Dai and Sheetz, 1995). The force on the tether does not increase with increasing tether length over many micrometers. In neurons, tethers hundreds of micrometers in length can be formed with no measurable change in tether force. A logical explanation for the constant force with length is that the membrane is being drawn from a reservoir that buffers membrane tension. Therefore, the tether phenomenon presents an excellent opportunity to probe the size of the membrane reservoir that buffers changes in membrane tension.

Membrane tension has been measured with membrane tethers in a variety of cellular and model membrane situations. Although in pure lipid vesicles the force on the tether is directly related to the in-plane tension and the bending stiffness of the lipid bilayer (Waugh et al., 1992; Evans and Yeung, 1994), in biological membranes there is an additional term that arises from membrane-cytoskeleton adhesion. Because the membrane and the cytoskeleton adhere to one another and the cytoskeleton does not move into the tethers, there is a separation of membrane components from their cytoskeleton binding sites in the tether. Membrane components in the tether will have a higher free energy because of the absence of cytoskeleton and will tend to leave the tether for the membrane in contact with the cytoskeleton. This results in a membrane osmotic pressure in the bilayer between the tether and the rest of the plasma membrane that increases the tether force (Dai and Sheetz, 1995; Waugh, 1983). In cases where blebs have formed on cells, we have found that the force on tethers formed on blebs is less than half the tether force of tethers formed on cytoskeleton-containing regions (Dai and Sheetz, unpublished results). Thus, the membrane-cytoskeleton adhesion contributes the major portion of the tether force.

Using tether length as a method for characterization of the membrane reservoir, we examined the effect of cytoskeleton disruption, osmotic pressure, and increased membrane tension on the reservoir size. Fibroblasts were used as the experimental cells because they have a relatively smooth surface, and we found experimentally that the apparent reservoir was small. An interesting observation of these studies is that the reservoir is accessible throughout the whole cell. The behavior of the cells has important implications for the organization of the plasma membrane and the cell cytoskeleton.

MATERIALS AND METHODS

Cell culture

Chick embryo fibroblasts and 3T3 mouse fibroblasts were grown in monolayers at 37°C in 5% CO_2. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, New York) containing 10% fetal bovine serum, 1 mM L-glutamine, and 50 IU/ml penicillin/streptomycin (Gibco). One day before the experiments, the cells were removed from culture with trypsin/EDTA and seeded into Petri dishes that contained several 24 × 50 mm glass coverslips.

Bead preparation

To prepare IgG-coated beads 40 μl of 2.5% carboxylated polystyrene microparticles (1 μm diameter, Polysciences, Warrington, PA) were placed into an Eppendorf centrifuge tube (1.5 ml capacity). The Eppendorf tube was then filled with 0.1 M carbonate buffer prepared by adding 0.1 M Na_2CO_3 to 0.1 M $NaHCO_3$ until the pH was 9.6. The beads were then pelleted by centrifugation at 2000 × g at 4°C for 10 min. The supernatant was removed using a Pasteur pipette and the pellet was resuspended in carbonate buffer and centrifuged one more time. Using the same procedure, the beads were then washed twice in 0.02 M sodium phosphate buffer, prepared by adding 0.02 M Na_2HPO_4 to 0.02 M NaH_2PO_4 until the pH was adjusted to 4.5. The pellet was then resuspended in 0.625 ml of sodium phosphate buffer and 0.625 ml of 2% carbodiimide solution (1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride dissolved in phosphate buffer) was added dropwise. The beads were then mixed for 3–4 h at room temperature using a rotary shaker, washed twice with phosphate solution, and washed two more times with Dulbecco's phosphate buffered saline (DPBS, Gibco BRL, Grand Island, NY). Then the beads were resuspended in 100 μl of PBS and incubated overnight at 4°C with 20 μl of 1 mg/ml mouse IgG (Sigma, St. Louis, MO). Unbound IgG was washed out with PBS, and the beads were then resuspended in 1 ml of 1 mg/ml bovine serum albumin-PBS to block unreacted sites, rinsed by pelleting, and resuspended in DMEM three times. For the experiments, the bead solution was diluted 30 times.

Laser tweezers manipulation

Cells were observed with a video-enhanced differential interference contrast (DIC) microscope (IM-35, Zeiss, Oberkochen, Germany). The laser trap consisted of a polarized beam from a near-infrared (1064 nm) Nd:YAG laser (model 116Fn, Quantronix Corp., Smithtown, NY) which was expanded by a 3X beam expander (Newport Corporation, Irvine, CA) and then focused through an 80 mm focal achromatic lens (Melles Griot, Irvine, CA).

For the tether length experiments, beads were held for 4–5 s on the cell surface with the laser tweezers and then the membrane tether was pulled out with a constant velocity of 4 μm/s by a piezoceramic-driven stage (Wye Creek Instruments, Frederick, MD). The force of the tether on the bead was estimated from the measured displacement of the bead in the trap. The position of the bead in the trap was analyzed from video records of the experiments using the nanometer-level tracking program (Gelles et al., 1988). Calibration of force was performed by flowing solution past a trapped bead at a known velocity and calculating the force from Stokes' law (Kuo and Sheetz, 1993).

Feedback tracking of particle position

To keep the particle at a defined distance from the center of the trap, we have modified the tracking algorithm that was originally applied by Choquet et al. (1996) to the output of a quadrant detector (see under Membrane Dynamics and Fig. 4.).

RESULTS

Membrane tether force during tether elongation

To characterize the dependence of the tether force on tether length, IgG-coated beads were held on the cell surface

approximately 4–5 s (Fig. 1 *a*) and then pulled with optical tweezers forming thread-like membrane tethers (Fig. 1 *b*). The membrane tethers were pulled at a constant rate until the beads escaped from the laser tweezers. After the beads escaped from the laser tweezers, the membrane tethers rapidly retracted back (Fig. 1 *c*). As shown in Fig. 1 *d*, the profile of the force vs. tether length during tether elongation consisted of three parts. After the initial tether formation, there was a small increase in force over the first micrometer. Force then reached a plateau and further elongation did not affect tether force, suggesting that membrane was drawn from a reservoir. Finally, there was an abrupt exponential rise in force that brought the tether out of the trap, indicating depletion of the membrane reservoir. If the tether elongation was dependent upon membrane stretching, then tether force should increase with tether length. However, the presence of a plateau in the force vs. tether length profile implies that additional membrane is available for tether elongation from a buffered reservoir. Under control conditions, the plateau phase in chick embryo fibroblasts and 3T3 cells was 5 μm (range, 3–7 μm), when pulled at a velocity of 4 μm/s. We have also measured length of the plateau phase at different pull rates (Fig. 1 *e*). From Fig. 1 *e* it is clear that tethers are longer with a slower pull rate. The slower rate may allow more time for vesicles underlying the plasma membrane to incorporate into the membrane and therefore produce longer

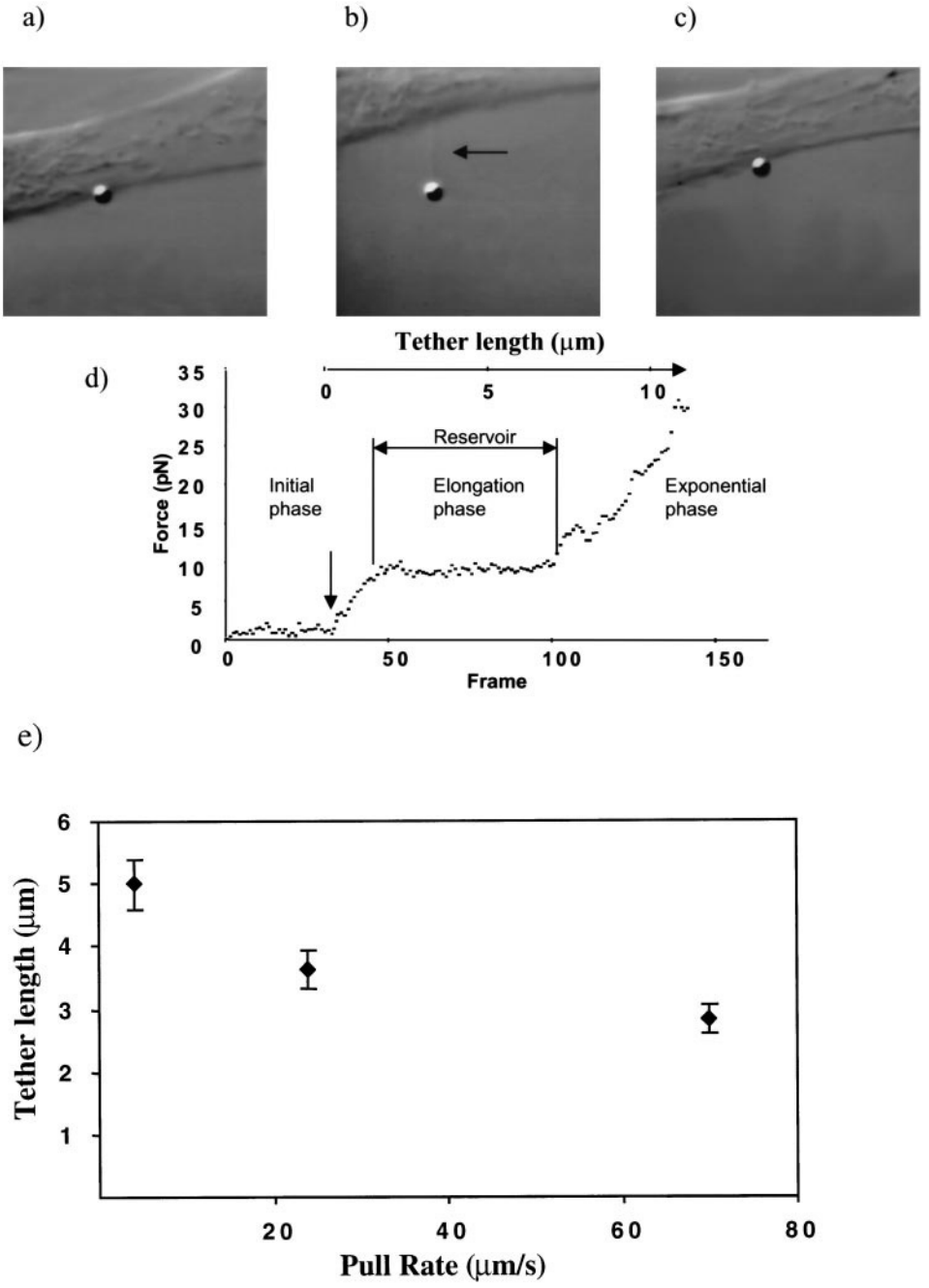

FIGURE 1 A bead trapped with laser optical tweezers was held on the cell surface for 4–5 s (*a*) and a membrane tether (*arrow*) was formed by pulling out the bead with constant velocity of 4 μm/s (*b*). (*c*) After the bead escaped the trap the tether rapidly retracted. (*d*) Tether force with time during the tether formation. (*e*) Tether elongation with response to different pull rates.

tethers. Alternatively, cytoskeleton could be rearranging under the high tension, which should be time-dependent as well. In the following experiments membrane tethers were pulled at a constant rate of 4 μm/s until the bead escaped from the laser tweezers, indicating depletion of the membrane reservoir.

Role of the cytoskeleton

The cytoskeleton, which is associated with the plasma membrane, determines the shape of the cell and it is intimately involved in mechanical functions of the plasma membrane. In the cytoskeletal network, microtubules and microfilaments are important structural components. To understand the influence of these filamentous proteins on the membrane reservoir, we modified their polymerization state with drugs. When actin polymerization was inhibited with cytochalasin B, the tether length increased almost threefold with respect to the tether length of untreated cells (Fig. 2). Similarly, 2.5-fold longer tethers were formed when cells were treated with cytochalasin D. Disruption of microtubules by nocodazole and colchicine resulted in 1.7- and 2.3-fold longer tethers (Fig. 2), respectively, when compared to untreated cells. In contrast, when microtubules were stabilized with taxol, tethers became 27% shorter than the tethers of untreated cells. Therefore, alteration in the organization in the actin or microtubule cytoskeleton results in a dramatic change in tether length, suggesting that the cytoskeleton plays an important role in limiting the size of the available membrane reservoir.

Membrane reservoir during osmotic swelling

When cells are exposed to a hypotonic solution, osmotic imbalance induces water influx through the plasma membrane and consequently causes cell swelling. In order to prevent cell lysis under swelling pressure, it is necessary to increase the membrane area of the cell. To quantify changes in the size of the membrane reservoir associated with osmotic swelling, we measured the tether length of 3T3 fibroblasts before, during, and immediately after incubation in hypotonic solutions. As shown in Fig. 3, when cells were exposed to a solution containing 90% medium, tether length decreased only 7%. Immediately after the solution was exchanged for normal medium, the tether length increased by 15% with respect to control cells. Similarly, for cells exposed to 70% and 50% medium the tether length decreased by 18% and 25%, respectively, during expansion. After exchange of hypotonic solutions for an isotonic medium, tether length increased by 25% for cells exposed to 70% medium and 35% for cells previously exposed to 50% medium. These results indicate that an increase in membrane tension due to osmotic swelling reduces the size of the available membrane reservoir. However, immediately after recovery from osmotic swelling there is an increase in tether lengths, suggesting that additional membrane has been

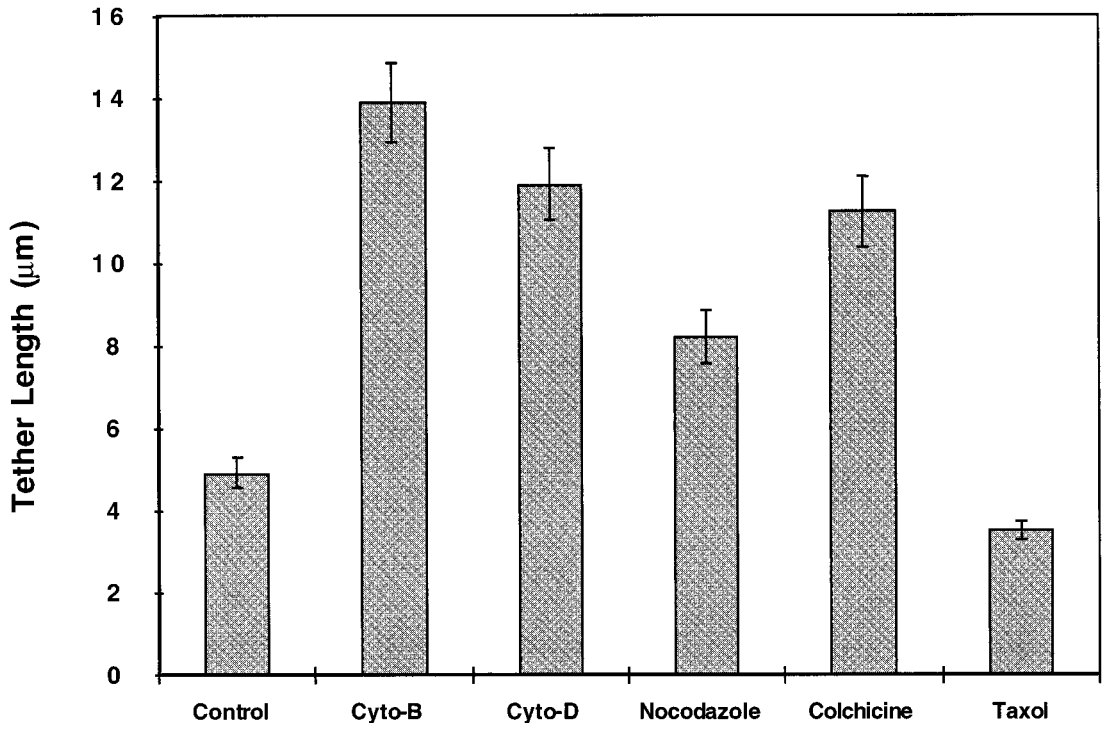

FIGURE 2 Influence of the cytoskeleton on the membrane reservoir. Tether length before and after the treatments with cytochalasin B (10 μM), cytochalasin D (10 μM), nocodazole (10 μg/ml), colchicine (1 μg/ml), and taxol (0.5 μM). Control solution was exchanged with treatment solutions and cells were incubated 10 min before starting the measurements. *Error bars* indicate S.E.M. for 3–5 tether length measurements on 15–20 cells.

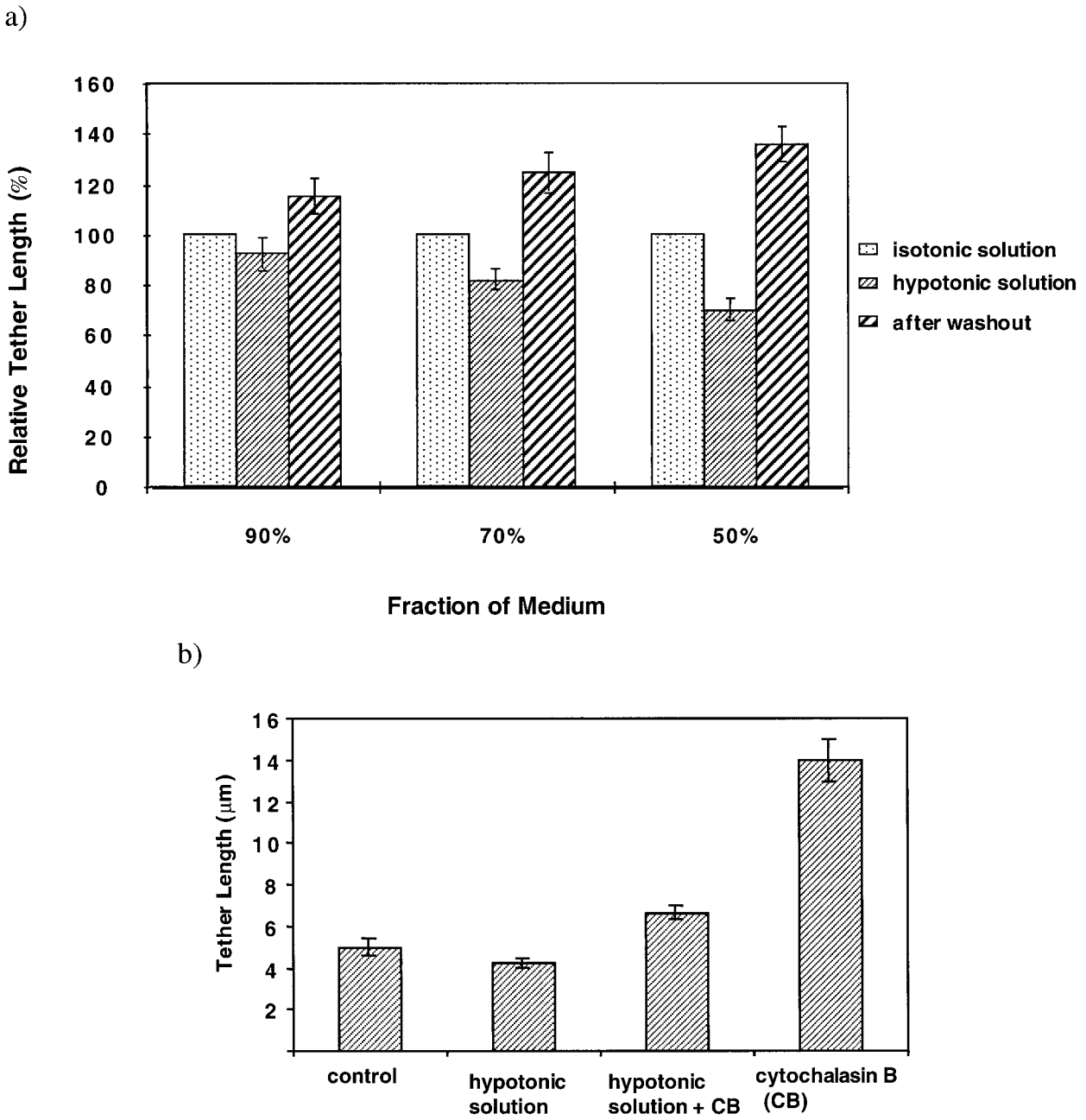

FIGURE 3 (*a*) Tether lengths during osmotic swelling and recovery. Tether lengths were normalized to tether lengths measured in isotonic solution (*coarse dots*). *Light hatching*, cells were incubated 10 min in hypotonic solution containing 90%, 70%, or 50% media. *Heavy hatching*, tether lengths immediately after cells were returned to isotonic solution. (*b*) Tether length in presence of 10 μM cytochalasin B and hypotonic solution (70% medium). Measurements were done within 3–10 min after addition of hypotonic solution and cytochalasin B or hypotonic solution alone. *Error bars* indicate S.E.M. for 3–5 tether length measurements on 12–15 cells.

added to the plasma membrane reservoir in response to increased osmotic pressure and perhaps increased membrane tension. We also measured tether length in presence of cytochalasin B and hypotonic solutions. As shown in Fig. 3 *b* there is about 20% increase in tether length in presence of cytochalasin B with respect to cells treated with hypotonic solution alone. These measurements were done within 3–10 min after addition of hypotonic solution and cytochalasin B or hypotonic solution alone. This suggests that rearrangement of underlying cytoskeleton during osmotic swelling may increase the size of membrane reservoir.

Membrane dynamics

Since increased membrane tension with osmotic swelling is found to increase membrane area (Dai et al., 1998), a tether under high force in the exponential phase should increase in

length by causing membrane addition. In order to characterize the rate of membrane addition as a function of tension, we have measured the rate of tether elongation while a constant force was applied to the bead holding the tether in the exponential phase (Fig. 1). The force of the tether on the bead was estimated from the measured displacement of the bead in the trap. To keep a particle at a defined position from the center of the trap we have used a modified tracking algorithm, which relies upon output of a quadrant detector Choquet et al. (1997).

A diagram of the tracking system used to hold the bead at a position of known force in the tweezers is shown in Fig. 4 *a*. When the particle was at the desired position in the tweezers, the feedback system was activated to hold the bead's position in the trap constant. As an external force is applied to the trapped bead and the position of the bead in the trap changes, a voltage from the computer is sent directly to the power supply for the piezoelectric element of the x-y stage. The stage then moves to keep the bead at the defined position from the center of the trap, thereby maintaining a constant force on the bead.

Fig. 4 *b* shows the tether force during the tether pulling sequence. A bead was held on the surface of the cell by the laser trap. A tether was pulled with constant velocity and after the reservoir was depleted, the bead was held at a constant force in the tweezers. Application of high constant force to the tether resulted in a linear increase in reservoir length as shown in Fig. 4 *c*. Tether elongation was measured from the change in the position of a reference bead, which was attached to the coverslip surface. Similarly, we have measured rates of reservoir elongation for tethers over the range of 5–32 pN. As shown in Fig. 4 *d*, we have found that tether length increased at a rate directly proportional to the tether force. When the tethers were released, they always retracted rapidly and immediate reformation of tethers showed that the reservoir size had been increased.

A number of tether pulls expand a continuous membrane reservoir

If the membrane reservoir is accessible from the whole cell surface, then expansion of the reservoir from increased tension on one side of the cell should be detected on the other side of the cell. A simple mechanism to expand the membrane reservoir is to pull a bead out of the trap a number of times. The high force transient at the end of the pull causes a rapid increase in the reservoir size. When a membrane tether was repeatedly formed using the same bead and the tether length was measured (Fig. 5 *a*), the tether length increased with subsequent pulls. Fig. 5 *b* shows the tether length during five successive tether pulls normalized to the tether length measured during the first pull. Tether length increased linearly with the number of pulls; after five pulls, membrane tethers were 37% longer than tethers formed during the first pull.

The increases in tether length with number of tether pulls could represent increases in reservoir size over the whole cell surface or a local phenomenon. We designed the following experiment to determine the nature of the increase in reservoir size. First, we measured the tether length on one side of the cell on position 1 (Fig. 6, *inset*). Then by using five successive tether pulls, we recruited additional membrane on the other side of the cell at position 2. Finally, we measured the tether length at position 1 again. As shown in Fig. 6, the average tether length at position 1 increased by 34% when we recruited additional membrane at position 2 of the cell. It is important to note that this 34% increase in tether length compares very well to the 37% increase in tether lengths after five successive pulls (Fig. 5), suggesting that the same amount of membrane which is recruited on one side of the cell is also available on the other side of the cell. There was no difference in tether length at position 1, when the tether length was measured without successive pulls at position 2. To check for the reversibility of the membrane recruitment seen with successive tether formation, we formed a membrane tether with the same bead approximately 20 s after completing a succession of pulls. As shown in Fig. 5 *b*, there is no difference in tether length between the first pull and the post-sequential pull, indicating that the membrane recruited into a membrane tether was absorbed after the tether was relaxed. These results indicate that membrane, which is recruited at a localized area of the cell, is available throughout the whole cell.

DISCUSSION

We have reported here experiments designed to measure the size of the plasma membrane reservoir and its dynamic properties. Membrane tethers were extracted at a constant rate until the bead escaped from the laser tweezers, indicating depletion of the membrane reservoir, and therefore its relative size. The actual area of membrane drawn into the tethers was 3–10 μm^2, which is a relatively small fraction of the total membrane area (approximately 1000 μm^2/cell). We have shown that during tether formation the initial rise in tether force is followed by a plateau where tether force does not change with the tether length. At the end of a pull, there is an exponential rise in tether force that brings the bead out of the laser trap, defining the tether length. Decreasing the cell cytoskeleton structural integrity with the cytochalasins, nocodazole, or colchicine caused an increase in tether length whereas increasing microtubule density with taxol decreased the size of the membrane reservoir. Thus, we suggest that cytoskeleton rigidity plays a role in limiting the size of the membrane reservoir. We found that an increase in membrane tension due to osmotic swelling reduces the size of the membrane reservoir while the cell is under the higher tension. However, the increase in tether lengths immediately after recovery from osmotic swelling indicates an increase in the size of the membrane reservoir in response to increased osmotic pressure. We have also measured the rate of tether elongation when a constant force was applied to the bead holding the tether. By measuring the

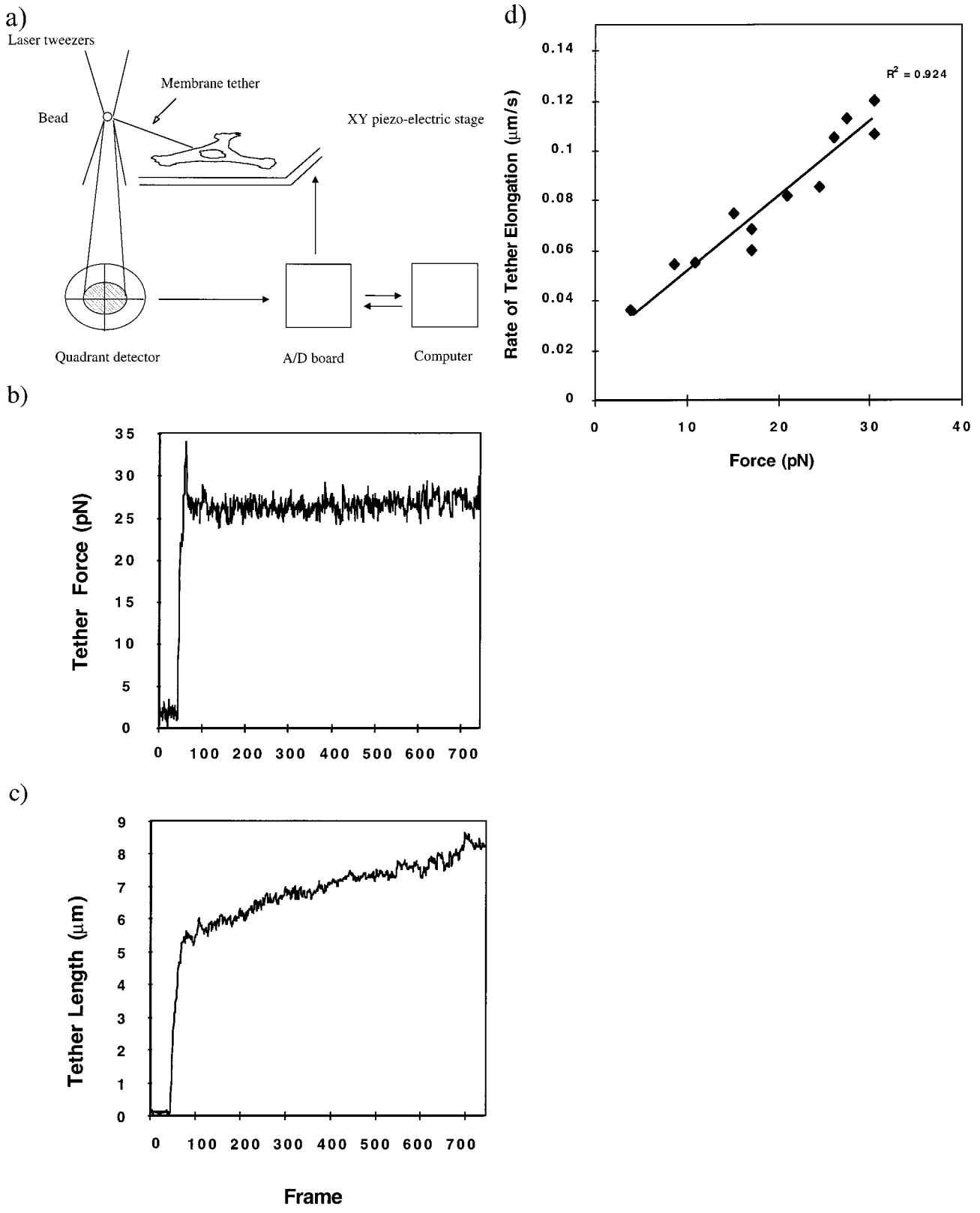

FIGURE 4 (*a*) Diagram of the tracking system that holds a bead at a constant position in the laser optical trap so that constant force is applied to the bead. (*b*) Force applied to the bead during a typical constant-force experiment and corresponding tether elongation. (*c*) Tether elongation was measured by simultaneously monitoring the position of a stationary bead firmly attached to the glass coverslip. (*d*) Different constant force measurements and corresponding rates of tether elongation.

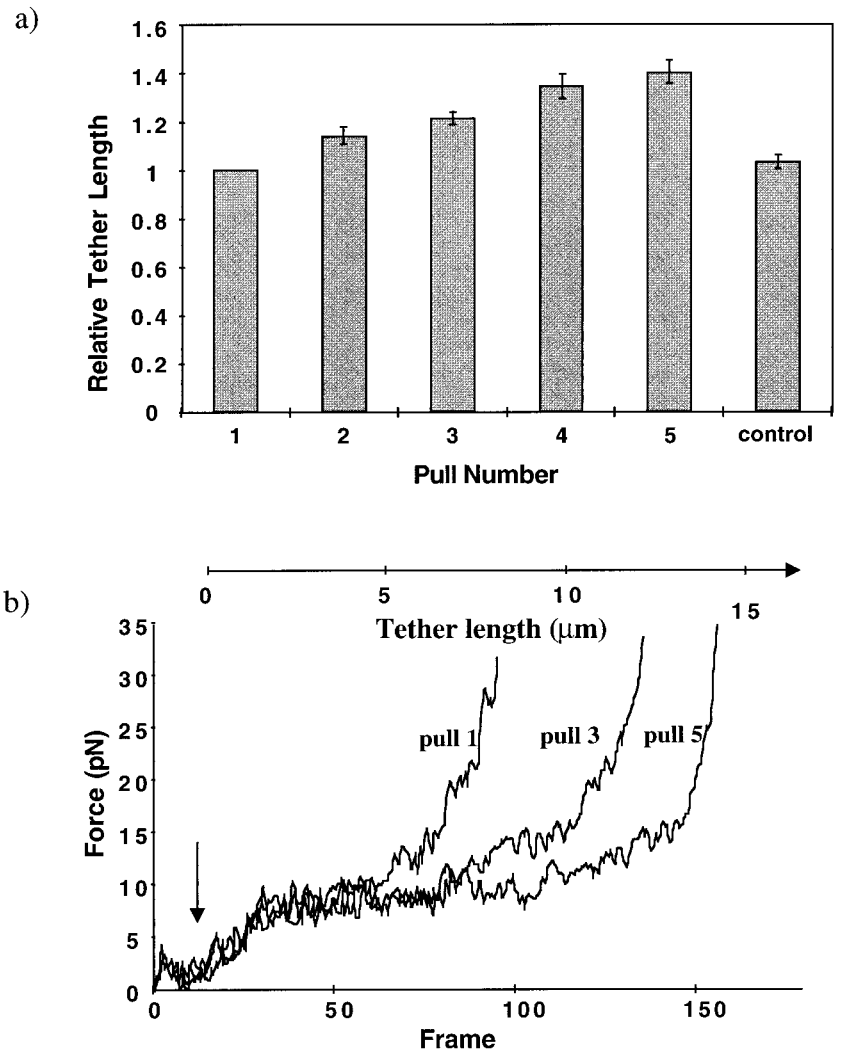

FIGURE 5 Effect of a number of tether pulls on tether length. Membrane tethers were pulled with constant velocity of 4 μm/s and tether lengths at which the bead escaped from the trap was measured (*a*). After the bead escaped the trap the tether rapidly retracted. A number of membrane tethers were extracted with the same bead. The time between tether pulls was less than 1 s. As a control for reversibility of the tether elongation, a post-sequential pull was performed 20 s after five successive pulls. *b* shows the tether force during the multiple tether formation.

rates of tether elongation for various forces we have found that the rate of tether elongation is directly proportional to the applied tether force. Increased membrane tension caused an increase in reservoir area over time indicating that additional membrane was recruited into the reservoir. The increase in membrane reservoir on one side of the cell resulted in an increase on the other side of the cell, indicating that the membrane reservoir is continuously distributed throughout the cell.

Previous studies of the membrane reservoir did not attempt to quantify its size, but rather they only confirmed its existence. In immunolocalization electron microscopy studies Nielsen et al. (1993) observed that in rat kidney cells the membrane reservoir consists of water channel-laden vesicles from the apical cytosol. Using the same method Escolar et al. (1989) found that peripheral and deep channels of the open canalicular system in platelets represents a membrane reservoir that can be evaginated onto the platelet surface during interaction with other surfaces. However, to characterize the membrane reservoir it is necessary to have a method to measure its relative size.

One of the first quantitative studies of the membrane reservoir was based on monitoring changes in cell surface morphology, implying that the membrane reservoir is contained in surface folds, microvilli or in membrane ruffles. That was accomplished by Burwen and Satir (1977) who followed changes in surface morphology of secreting mast cells by scanning electron microscopy. To assess the possible relationship between secretory activity and surface folding they measured surface fold lengths per unit of smooth sphere surface area. This method was employed by Petty et al. (1981) to measure the variations in size of the membrane reservoir in macrophages during antibody-dependent phagocytosis. However, the size of the membrane reservoir is estimated only from surface folds, and therefore this method may not be appropriate to some other cell types.

Our understanding of the structural interaction between the plasma membrane and the cytoskeleton has changed as a result of many recent studies of membrane mechanical properties including membrane tension measurements (Waugh et al., 1992; Evans and Yeung, 1994; Waugh and Hochmuth, 1987). Of particular relevance here is the con-

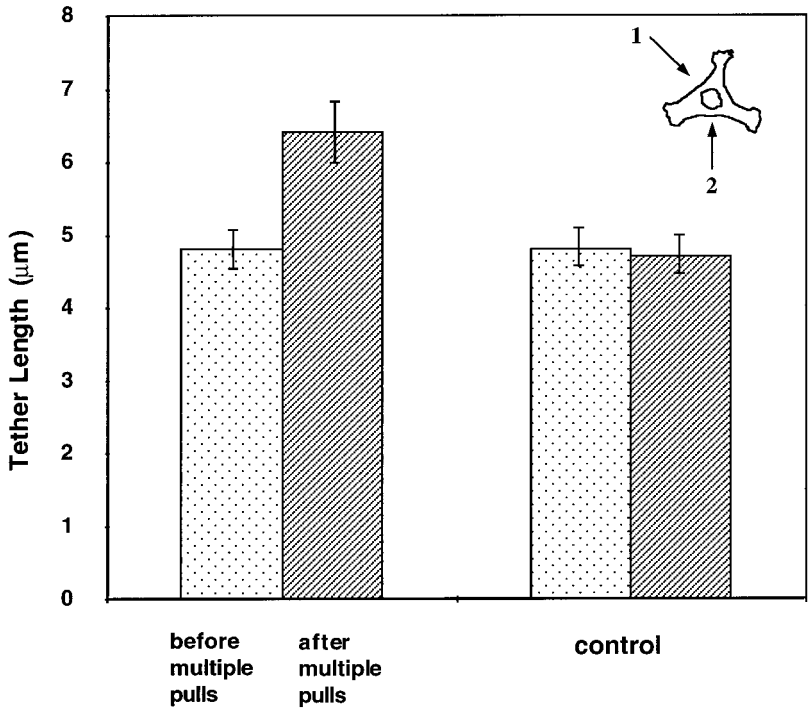

FIGURE 6 Tether length on one side of the cell before and after a number of tether pulls on the other side of the cell. Tether length was measured at position 1 of the cell (*inset*) before and after a number of tether pulls at position 2. The time between the tether pulls on position 1 before and after a number of tether pulls at position 2 was less than 5 s. As a control experiment we measured tether the length at position 1, paused approximately 5 s, and then measured the tether length at the same position.

cept that the membrane and the cytoskeleton adhere to each other through many weak interactions, perhaps lipid-protein bonds. This leads to a continuum behavior in the interaction such that in neuronal growth cones, a 10–15% increase in the plasma membrane area does not cause separation of cytoskeleton and membrane. Further, upon restoration of isotonicity to hypotonically swollen cells, the excess membrane conforms to the cytoskeleton in deep invaginations, which rapidly are resorbed into the cell (Dai et al., 1998). In this work we have used laser tweezers to pull on membrane-attached latex beads and form membrane tethers. Since the membrane in a tether is drawn from the plasma membrane, the maximum tether length which can be pulled reflects the relative size of a membrane reservoir. Besides introducing a new method for quantification of the membrane reservoir, we have also demonstrated that this method may be applied during various conditions opening the way for further studies of the membrane reservoir.

Role of the cytoskeleton

The cortical cytoskeleton determines cellular shape, coordinates cell locomotion, and may present a regulative barrier to endocytosis and exocytosis (Aunis and Bader, 1988). Clearly, other studies have shown that the cytoskeleton plays an important structural and regulatory role in a variety of membrane processes (reviewed in Bretscher, 1991). Both the tubulin containing microtubules and the actin containing microfilaments form distinct structures within the cell which are associated with the plasma membrane itself. In this work we have demonstrated that disruption of the cytoskeleton network increases the size of the membrane reservoir, while stabilization of microtubules reduces the size of the available membrane reservoir.

Although the alteration of cell shape caused by cytochalasin and nocodazole may cause an initial change in the reservoir, the rapid dynamics of the reservoir suggest these agents cause a stable change in the reservoir. The cytoskeleton determines the shape of cell surface structures like microvilli, microspikes, filopodia, and membrane ruffles. Disruption of the underlying cytoskeletal network may transform a cell with many surface structures into a cell with more planar topology, generating excess membrane; however, recovery of the excess membrane occurred in 20 s in membrane expansion studies. Thus, the tether length after 30 min in the presence of cytochalasin or nocodazole should represent a change to a new steady-state value of the reservoir. The steady-state values of the cell that could affect apparent reservoir size are cytoskeletal rigidity and membrane-cytoskeleton interaction. Because both microtubules and actin filaments contribute significantly to cellular rigidity (Duszyk et al., 1989) but actin filaments are more tightly linked to the membrane-cytoskeleton interaction, we favor the idea that the rigidity change is most important. Since changes in cell shape sometimes may require large changes in membrane area, membrane must be added perhaps initially from the membrane reservoir and then from internal membrane stores. Therefore changes in cell shape and membrane addition must be carefully coordinated to maintain a significant membrane reservoir.

Osmotic swelling

There are many studies and experiments testing the effect of changes in the osmolarity of solutions upon growth of the

cells and upon individual cell structures. In early studies Hogue (1919) observed morphological changes in fibroblasts from embryonic chick heart in hypotonic solution. She found that a hypotonic solution induces water influx through the plasma membrane and the resulting hydrostatic pressure caused an enlargement of the cell cytoplasm and its separation into granular and clear areas. Rapid enlargement of the cell requires an increase in plasma membrane area. Since enlargement is considerably faster than the rate of synthesis of the membrane proteins and lipids, most of the plasma membrane must be drawn from internal membrane stores. The reservoir area is only several square micrometers, whereas the expansion of the surface area can be tens to hundreds of square micrometers. The reservoir can provide a buffer against fluctuations in tension that would otherwise lead to lysis but clearly does not provide all the membrane needed for cellular shape changes.

In this study, we have shown that during an increase in osmotic pressure there is a rapid proportional decrease in the size of the membrane reservoir indicating that the size of the reservoir is inversely proportional to the plasma membrane tension. The notion that cell surface is unchanged, and that the membrane needed to prevent cell lysis is coming from the unfolding of surface folds is supported by cell capacitance measurements. Immediately after addition of hypotonic solution cell capacitance does not change (K. Strange, personal communication). Instead there is a delayed increase in capacitance, indicating that it takes time before there is addition of membrane to the cell surface. Similarly, Graf et al. (1995) have used the whole-cell patch-clamp technique to study changes in membrane conductance and membrane capacitance after osmotic swelling in rat hepatocytes. The rise in conductance was not correlated with changes in capacitance, neither in time after the initiation of cell swelling nor in magnitude. Therefore, they concluded that an osmotically induced increase in conductance is probably a result of the activation of existing channels in the plasmalemma and not a result of the fusion of vesicle membranes containing ionic channels. In addition, using whole cell patch clamp techniques, membrane capacitance was measured in rat astrocytes in primary tissue culture within 9 min of exposure to 220 mOsm, 190 mOsm, and 145 mOsm PBS (Olson and Li, 1997), with each of these hypoosmotic exposures, no change occurred in membrane capacitance. When we measured the size of the membrane reservoir immediately after the osmotic pressure was reduced to normal by returning the cell to isotonic media, we found that the increase in the size of the membrane reservoir was proportional to the applied osmotic pressure. Because the changes in reservoir size that we observe are very small ($<0.5\%$ of the membrane area) they do not correspond to these capacitance changes, but rather could represent much slower membrane addition process or the relaxation of cell shape, e.g., actin depolymerization as after cytochalasin addition.

We have also measured the dynamics of reservoir expansion as a function of membrane tension in the bilayer plane from the force exerted on a particle attached to a membrane tether. When tensions were applied to the cell, the reservoir expanded linearly with time. We found that the rate of increase of the reservoir area as measured by tether elongation was directly proportional to the tension applied through the tether. From these studies we concluded that when tensions were applied to the cell, either by osmotic pressure or through the membrane tether, membrane material is incorporated into the membrane reservoir either from internal membrane stores or cell rounding. This suggests that the rate of addition to the plasma membrane from internal stores or cell rounding is directly proportional to the tension applied to the plasma membrane.

Working hypothesis

These results are consistent with the hypothesis that there is a reservoir of plasma membrane that serves to buffer against fluctuations in the plasma membrane tension for the whole cell. This reservoir could be an invagination of the plasma membrane that was under tension derived from a molecular motor pulling on an inward directed tether or a membrane projections. Under high tension the reservoir would decrease in size but when tension dropped from a high level, the reservoir would pick up the excess membrane and increase in size. We find that when the membrane tension is increased, the reservoir is small in size (at the maximum tether length very small increases in tether length cause very large changes in tether force). Under high tension, the cell adds membrane to the plasma membrane in an apparent attempt to restore the reservoir. Upon release of the high membrane tension, the reservoir takes up some of the added membrane and has an increased size until a new steady state is established. Although we suggest that an invagination could be the anatomical basis for the reservoir, an evagination such as a microspike or filopodium could also serve the same purpose. Further studies are needed to define the nature of the reservoir.

It has been postulated that membrane tension provides a physical mechanism to control the area of the plasma membrane (Sheetz and Dai, 1996). This hypothesis was based in part on observations that an increase in membrane tension, caused by osmotic swelling, increased membrane area as a result of increased membrane secretion (Dai et al., 1998). The increase in reservoir size with high membrane tension could result from an increase in plasma membrane area. Therefore there is evidence for the notion that membrane tension controls the net incorporation of membrane material into the plasma membrane.

Alternatively, the increase in membrane tension could cause cell rounding such as in mitosis (Raucher and Sheetz, 1999). During rounding the membrane reservoir would increase dramatically in size as well. In plant protoplasts there are indications that traffic from an intracellular membrane pool to the plasma membrane depends upon the tension of the plasma membrane (reviewed in Kell and Glaser, 1993).

Wolfe et al. (1985) have shown that increased tension in the plane of the plasma membrane of plant protoplasts leads to an increase in plasma membrane area up to threefold in some cases and that a decrease leads to a decrease in plasma membrane area. During osmotically induced volume contraction protoplast plasma membrane remained smooth and volume contraction was accompanied by endocytic vesiculation (Gordon-Kamm and Steponkus, 1984). Kell and Glaser (1993) postulated that membrane expansion by exocytotic incorporation of vesicle membranes is controlled by the mechanical forces, which are imposed on the plasma membrane. Therefore, it is very likely that the plasma membrane tension represents an important physical mechanism to control incorporation of membrane material into the plasma membranes of all cell types. A reservoir to buffer fluctuations in membrane tension would be an important factor in the physiological control of tension.

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