Cell Spreading and Lamellipodial Extension Rate Is Regulated by Membrane Tension

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Abstract. Cell spreading and motility require the extension of the plasma membrane in association with the assembly of actin. In vitro, extension must overcome resistance from tension within the plasma membrane. We report here that the addition of either amphiphilic compounds or fluorescent lipids that expanded the plasma membrane increased the rate of cell spreading and lamellipodial extension, stimulated new lamellipodial extensions, and caused a decrease in the apparent membrane tension. Further, in PDGF-stimulated motility, the increase in the lamellipodial extension rate was associated with a decrease in the apparent membrane tension and decreased membrane–cytoskeleton adhesion through phosphatidylinositol diphosphate hydrolysis. Conversely, when membrane tension was increased by osmotically swelling cells, the extension rate decreased. Therefore, we suggest that the lamellipodial extension process can be activated by a physical signal (perhaps secondarily), and the rate of extension is directly dependent upon the tension in the plasma membrane. Quantitative analysis shows that the lamellipodial extension rate is inversely correlated with the apparent membrane tension. These studies describe a physical chemical mechanism involving changes in membrane–cytoskeleton adhesion through phosphatidylinositol 4,5-biphosphate–protein interactions for modulating and stimulating the biochemical processes that power lamellipodial extension.

Key words: membrane–cytoskeleton adhesion • membrane expansion • laser optical tweezers • cell spreading • membrane tether

Introduction

Cell adhesion and motility play central roles in a variety of biological phenomena, including embryonic development, inflammation, and wound healing. Both cell adhesion and motility involve the extension of the plasma membrane, and alterations in the rate of extension would be expected to have dramatic effects on many processes. Extension occurs through the formation of either thin actin-rich veils called lamellipodia or fingerlike projections called filopodia. Both types of extension involve the polymerization of actin filaments coupled with displacement of the plasma membrane. The load against which this process must work is provided by external objects and tension within the plasma membrane (Oster, 1988; Peskin et al., 1993; Mogilner and Oster, 1996). There are very significant tensions in plasma membranes (Dai and Sheetz, 1995a; Dai et al., 1997) and increasing membrane tension in snail neuronal growth cones by hypotonic swelling caused lamellipodial and filopodial retraction (Dai et al., 1998). The quantitative relationship between the extension rate and the tension is unknown. Therefore, we have measured lamellipodial extension and membrane tension in parallel under a variety of conditions.

Several different systems are postulated to drive extension of the leading edges of cells (Theriot, 1997; Condeelis, 1993; Stossel, 1993; Heidemann and Buxbaum, 1998). The role of the extension system is to create a gap at the growing end of the actin filaments to allow further assembly. Whether the mechanism involves the creation of an osmotic pressure pushing the membrane forward or a mechanical displacement of the membrane coupled with filament assembly, the velocity of the extension should be inversely dependent upon the resistive force at the membrane surface. For cells moving through tissues, the resistive force is largely from the surrounding extracellular matrix. However, for cells in vitro, the major force resisting extension arises from tension in the plasma membrane. Models of the extension process at the biophysical level have predicted that the velocity of the lamellipodial extension would decrease with the increasing tension, although the exact relationship depends on the model (Oster, 1988; Mogilner and Oster, 1996).

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In many instances, the initiation of extension is stimulated, perhaps mechanically but definitely biochemically. The release of a cell tail and the addition of chemoattractants and migratory hormones all caused the cell to initiate new extensions (Chen, 1981; Weber et al., 1995; Heldin, 1997). From the measurements of tail retraction coupled with leading edge extension, the change in the cell shape was seen as a release of the tension that prevented forward extension. One possibility is that a decrease in membrane tension after tail release would result in increased membrane extension. Chemoattractants and migratory hormones activate the extension process at the biochemical level, but the downstream signaling pathways could initiate changes in membrane tension as well. The extension process could be enhanced by a physical change such as a decrease in the resistance to extension, which was coupled with activation of the extension process.

Apparent membrane tension can be measured from the force exerted on membrane tethers. Because of conformity of the membrane to the cytoskeleton, the apparent membrane tension contains components of membrane–cytoskeleton adhesion as well as tension in the bilayer plane. For animal cells with nonspherical shapes, membrane tension is largely determined by the membrane cytoskeleton adhesion (Sheetz and Dai, 1996). Recent comparisons of forces on tethers formed from normal plasma membranes and from adjacent membranous blebs, in which the membrane has separated from the cytoskeleton, show that in-plane bilayer tension accounts for less than one quarter of the apparent membrane tension (Dai and Sheetz, 1999). For many processes including endocytosis and extension, the membrane–cytoskeleton adhesion component of the tether force could be important. In the case of endocytosis, clathrin must displace the normal membrane-associated cytoskeleton to cause endocytosis, and there is an inverse correlation between the endocytosis rate and tether force (Dai et al., 1997; Raucher and Sheetz, 1999). In the case of membrane extension, the attachment of the protein complex that drives extension of the plasma membrane, such as Arp2/3 (Mullins et al., 1998, Welch et al., 1998), requires displacement of the normal cytoskeletal components and, thus, could be sensitive to changes in membrane–cytoskeleton adhesion as well as the tension in the bilayer plane.

There are several ways to nonspecifically alter membrane tension. Detergents, organic solvents, or local anesthetics cause expansion of the plasma membrane (for review see Seeman, 1972) and, consequently, cause a decrease in apparent membrane tension. Conversely, osmotic swelling of a cell causes an increase in membrane tension. Thus, the tension can be modulated in predictable ways to determine if the cellular functions are modulated in a corresponding fashion. In other studies, high membrane tension and low endocytosis rates were reversed by the addition of detergents (Dai et al., 1997; Raucher and Sheetz, 1999). We have now measured the relationship between tether force and the rate of lamellipodial extension under a variety of conditions. Interestingly, an inverse relationship between tension and extension rate was observed in the quantitative analysis. Further, we found that the process of lamellipodial extension was dramatically stimulated by a nonspecific decrease in membrane tension.

Materials and Methods

Cells

NIH 3T3 cells (a mouse fibroblast line) were grown in DME (Gibco Laboratories) supplemented with 10% FBS (Gibco Laboratories), 100 units/ml penicillin, 100 mg/ml streptomycin, and 7.5 mM Hepes at 37°C in 5% CO_2. Cells were removed from tissue culture flasks by brief treatment with trypsin-EDTA (Gibco Laboratories), and plated onto glass coverslips. Cells were used for experiments 1–2 d after plating.

Bead Preparation

To prepare IgG-coated beads, mouse IgG (Sigma Chemical Co.) was solubilized at a concentration of 1 mg/ml in PBS (Gibco Laboratories). 50 ml of carboxylated polystyrene microparticles (1 mm diam, Polysciences Inc.) was added to 100 ml of the above IgG solution and incubated at 4°C overnight. The beads were rinsed by pelleting and resuspension in PBS and finally resuspended in 100 ml of DME. For the experiments, the bead solution was diluted 1:100 NIH 3T3 medium.

Laser Optical Trap Manipulations

The NIH 3T3 fibroblasts were viewed by a video-enhanced differential interference contrast (DIC)[1] microscope equipped with laser optical tweezers (Choquet et al., 1997). Beads were trapped with 600 mW of laser power, attached to the cell membrane, and pulled away from the cell surface, forming a membrane tether. The tether force was calculated from the displacement of the bead from the center of the laser trap during tether formation and the trap calibration (Dai and Sheetz, 1995b).

Cell Spreading Measurements

We measured the spreading of NIH 3T3 cells on uncoated glass coverslips in an assay similar to that described earlier (Felsenfeld et al., 1999). Cells in confluent cultures were briefly trypsinized (1–2 min), and then plated on acid-washed glass coverslips at a density of 10^6 cells/ml in DME with 10% FBS. Cells were incubated at 37°C either in the presence or in absence of 0.4 mM deoxycholate for 15 or 30 min. Nonadherent cells were washed out and adherent cells were fixed by 4% paraformaldehyde. Adherent cells were scored by visual examination in the DIC microscope. The number of adherent cells that were spread in the presence of deoxycholate was normalized to the number of adherent cells in the control solution. Results represent the average of three independent experiments (for each experimental point >500 cells were counted).

Measurement of Lamellipodial Extension Rate

The morphology of cultures of NIH 3T3 fibroblasts growing under the conditions employed in this study is very similar to that described previously (Lombardi et al., 1990; Symons and Mitchison, 1991). The fibroblasts are spread and move relatively slowly across the substratum. The motility of individual fibroblasts caused changes in morphology with time. Typical fibroblasts have a trigonal shape with a broad lamellipodium at the leading edge and a pointed trailing tail. The characteristic size and cell shape is preserved for long periods, provided that no major changes occur in external conditions. Here, we define lamellipodia as those thin regions 3–10 microns wide located at the cell periphery where the actin meshwork is much more dense than in the remainder of the cell (Vasiliev, 1991). To measure the lamellipodial extension rate, we have used the following criteria. We selected fibroblasts that had a trigonal shape with a broad lamellipodium (at least 6 microns wide) at the leading edge. We selected events where the period of extension was greater than 30 s. Successive positions of the tip of leading lamella were measured every 3 s. When the edge is ex-

[1]*Abbreviations used in this paper:* β-DPH-HPC, 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; C_5-DMB-SM, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-indacene-3-pentanoyl) sphingosyl phosphocholine (BODIPY FL C_5-sphyngomyelin); DiA, 4-4(4-(didecylamino)styryl)-*N*-methyl-pyridiniumiodide (4-Di-10-ASP); DIC, differential interference contrast; FITC-PL; L-α-phosphatidylethanolamine, dipalmitoyl, *N*-(5-fluoresceinthiocarbamoyl); PIP_2, phosphatidylinositol 4,5-biphosphate.

tending, it moves forward continuously as the slope of the edge position versus time plot shows (see Fig. 2 d). The distance moved was defined as the distance from the initial position of the lamellipodial edge at the beginning of extension. To measure the lamellipodial extension, a line was positioned at several locations on the actively spreading lamellipodia, and the position of the edge was recorded. We only considered the cells whose lamellipodial extension rate remained roughly constant over the period of observation ($r^2 > 0.96$ for every fitted line), which occurred in about half of the cases recorded. Results were the same when we compared the relative extension rate within individual cells before and after cell exposure to membrane expanding drugs. Therefore, although the determination of the edge position for a given time point may be subjective, a constant rate of extension was consistently observed.

Cell Labeling with Fluorescent Phospholipids

Cells were incubated with 2 mM L-α-phosphatidylethanolamine, dipalmitoyl, *N*-(5-fluoresceinthiocarbamoyl) (FITC-PL; Sigma Chemical Co.) or 2 mM 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (β-DPH-HPC), or 5 mM *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-indacene-3-pentanoyl) sphingosyl phosphocholine (BODIPY FL C_5-sphyngomyelin) (C_5-DMB-SM; Molecular Probes, Inc.) for 10 min to label plasma membrane, and then washed with medium.

Results

Effect of Membrane Expansion on Cell Spreading

In response to contact with the substrate, NIH 3T3 fibroblasts adhere to the surface, and then spread out to acquire a flattened morphology (Fig. 1 a). This process of cell adhesion and spreading is mediated by integrins and involves complex dynamic rearrangements of the actin cytoskeleton. This dynamic process is coordinated and controlled by the Arp2/3 protein complex and intracellular signaling pathways (for reviews see Hartwig et al., 1996; Welch et al., 1997). How specific signaling pathways regulate the cytoskeleton during cell spreading is, however, poorly understood. To test whether perturbation of the membrane bilayer affects cell spreading, we expanded the membrane bilayer by addition of the detergent, deoxycholic acid, and measured the cell spreading rate. Fig. 1 (a and b) show video-enhanced DIC images of cells spread in the absence and presence of deoxycholic acid. Quantification of cell spreading after 15 and 30 min shows more than a twofold increase in cell spreading after deoxycholic acid treatment (Fig. 1 c). These results suggest that the cell spreading rate is increased by the membrane bilayer expansion, i.e., by reduction in the membrane tension.

Cells spread by putting out extensions that contact the surface, forming adhesions, and then exert tension to further draw cytoplasm outward. This process is reminiscent of the lamellipodial extensions, and this similarity prompted us to investigate the role of membrane tension in lamellipodial extension of spread cells.

Characterization of Lamellipodial Extension

Lamellipodial extension in NIH 3T3 fibroblasts is relatively uniform in rate and localized to a small fraction of the cell edge. These fibroblasts are spread and move relatively slowly across the substratum. Many cells have a trigonal shape with a broad lamellipodium at the leading edge and a pointed trailing tail. The leading edge normally undergoes cycles of extension and retraction. When the edge is extending, it moves forward continuously as the slope of the edge position versus time plot shows (Fig. 2 d). The lamellipodial extension rate is determined from the slope of the plot (5 μm/min in this case) and it is in quantitative agreement with actin polymerization rate in lamellipodia and the rate measured in similar fibroblasts (Felder and Elson, 1990). The average interval for extension is 1–2 min and, during that period, the edge moves continuously forward for a distance of 5–10 microns. We selected events where the period of extension was >30 s. These observations are consistent with the idea that the process of lamellipodial extension is a stochastic process presumably catalyzed by an activated protein complex in the lamellipodial region.

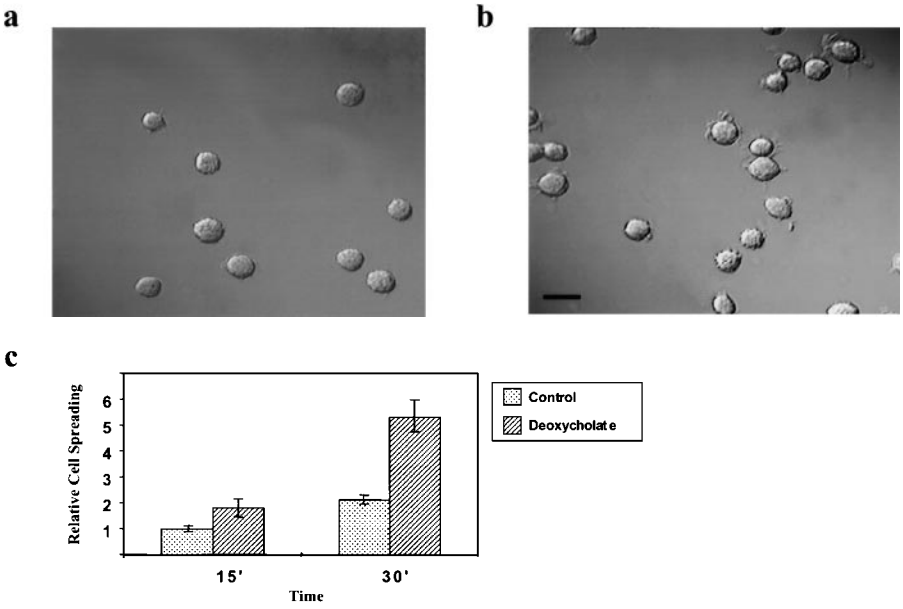

Figure 1. Effect of membrane expanding reagent, deoxycholate, on cell spreading. After brief trypsinization, NIH 3T3 cells were plated on acid-washed glass coverslips either without or with 0.4 mM of deoxycholate added to the medium. Coverslips were washed and fixed with 4% paraformaldehyde after 15 or 30 min. Adherent cells were counted by visual examination with DIC microscopy (see micrographs of cell spreading without [a] or with [b] deoxycholate), and the relative number of spread cells with respect to the control cells was calculated (c) represent the average of three independent experiments, in each of which >500 cells were counted). Error bars are SEM.

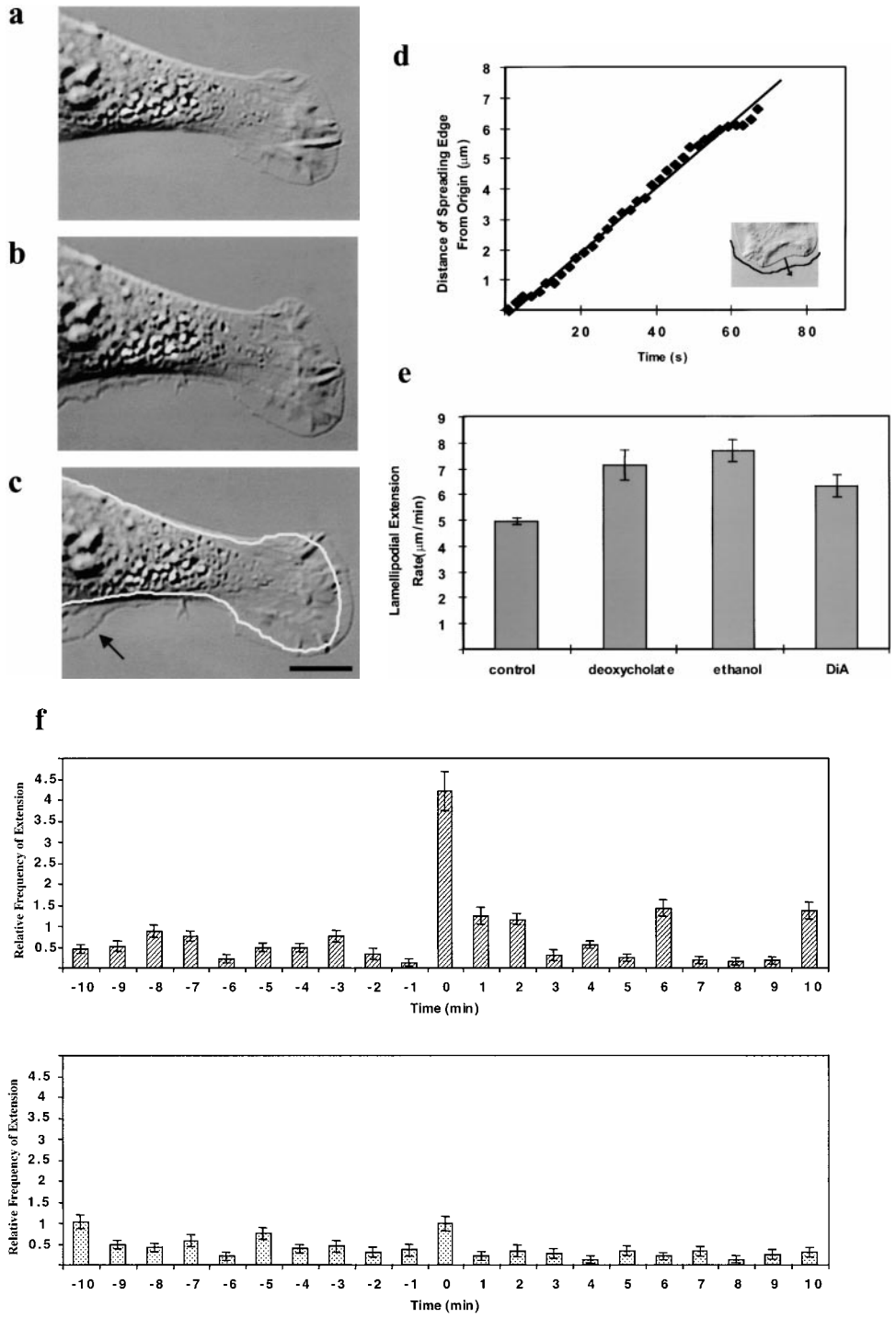

Figure 2. Lamellipodial extension in NIH 3T3 cells. (a) Photomicrograph of an NIH 3T3 cell observed during the initial formation of a lamellipodial protrusion. (b) The same cell 1 min after solution was rapidly exchanged with a solution containing 0.4 mM deoxycholic acid. (c) The cell after 2 min in the deoxycholate solution. For comparison, the outline represents the initial cell contour from a. Bar, 10 μm. (d) Lamellipodial extension rates were averaged over 40–60 s by fitting a straight line to a plot of distance moved versus time, with the position measured every 3 s. The distance moved was defined as the distance from the initial position of the lamellipodial edge at the beginning of extension (inset). For all cells shown, the rate remained roughly constant over the period of observation ($r^2 >$ 0.96 for every fitted line). (e) Average lamellipodial extension rate in control medium, the presence of 0.4 mM deoxycholate, 1% ethanol, or 0.1 mM DiA-treated cells. Error bars show SEM for three to five measurements for 10–12 cells. (f) The number of lamellipodial extensions was monitored for 10 min before the addition (at time zero) and 10 min after the addition of deoxycholate (top). In each minute, the number of lamellipodial extensions (extending for longer than 25 s) was counted, normalized to total number of lamellipodial extensions during the 10 min before the addition of deoxycholate, and averaged for at least 20 cells. The bottom represents the control experiment, where at time zero only fresh medium was added. Error bars represent SEM.

Reagents that Increase Membrane Area Increase Lamellipodial Extension

In other studies, we noted that the addition of sublytic concentrations of detergent to cells caused the dramatic extension of lamellipodia. When 0.4 mM of deoxycholate was added to NIH 3T3 cells, the rate of extension of lamellipodia increased from 5.0 to 7.2 μm/min. Although the rate of extension was increased, the frequency and duration of the extension process were not changed in the leading edge of the cell. However, in regions away from the leading edge there was a threefold higher probability of extension in the presence of detergent (Fig. 2 f). Extension was observed at many new sites (Fig. 2 c, arrow), indicating that the addition of detergent initiated extension. To quantify this phenomenon, we measured the frequency of lamellipodial extension from 10 min before until 10 min after addition of deoxycholate, (Fig. 2 f, top). In each minute, the number of extending lamellae of the cell was counted, normalized to the total number of extending lamellae before addition of deoxycholate, and averaged

for at least 20 cells. As shown in Fig. 2 f, there is a dramatic increase in the number of lamellipodial extensions immediately after addition of deoxycholate. To assure that this increase is not an artifact caused simply by the addition of fresh medium, we quantified extension before and after buffer addition. As shown in Fig. 2 f (bottom), the addition of fresh media without deoxycholate did not cause any significant change in the number of lamellipodial extensions. These results indicate that in the presence of the membrane expansion reagent, deoxycholate, both the rate of extension of membranes and the probability of extension were increased.

Because deoxycholate was an anionic detergent and may have had unusual properties, we tested the effect of a neutral alcohol and a hydrophobic lipid label on motility as well. Addition of ethanol (1%) or the fluorescent membrane marker DiA, 4-4(4-(didecylamino)styryl)-*N*-methylpyridiniumiodide (4-Di-10-ASP) (DiA) (0.1 mM) increased the lamellipodial extension rate to 7.7 μm/min and 6.3 μm/min, respectively (Fig. 2 e). In both of those cases, there was also a dramatic increase in the frequency of membrane extension in the cells. Thus, both the initiation of extension and the rate of extension are increased by the addition of a variety of compounds that intercalate into the plasma membrane.

Membrane Tension Measurements

One explanation for the increase in the extension rate is that the amphiphilic compounds decreased the resistance to extension by decreasing membrane tension. We have estimated membrane tension by measuring the tether force with the laser tweezers. Latex beads coated with rat IgG (Dai and Sheetz, 1995b) were attached to the plasma membrane by holding them on the membrane surface and tethers were formed by pulling on the beads with the laser tweezers (Fig. 3 a). Tethers were always fluid, and when the beads were released, the tethers retracted in <0.1 s. The force needed to hold the tether at a constant length was measured from the displacement of the bead in the laser trap (Dai and Sheetz, 1995b) (Fig. 3 b). We found that tether force was constant in the population of NIH 3T3 cells at 7.0 ± 0.5 pN (Fig. 3 c). When 0.4 mM deoxycholate was added, tether force was decreased to 3.5 ± 0.5 pN. In the presence of 1% ethanol or 0.1 mM DiA, tether force decreased to 5.1 ± 0.4 pN and 5.5 ± 0.5 pN, respectively. Thus, the amphiphilic compounds decrease membrane tension, as estimated from tether force measurements.

Lamellipodial Extension and Membrane Tension after Incubation with Fluorescent Phospholipids

Detergents and solvents intercalate into a membrane bilayer, alter the membrane's stable composition, expand the membrane area and, consequently, decrease membrane tension. A similar approach to expand the membrane area and decrease membrane tension involves the use of fluorescent lipid analogues. Fluorescent lipid analogues can be readily integrated into the outer leaflet of the plasma membrane bilayer of intact cells by spontaneous monomeric lipid transfer. Thus, to further test whether the lamellipodial extension rate is dependent on membrane tension, we have also used fluorescent lipid analogues to expand the membrane area and decrease the membrane tension. When NIH 3T3 fibroblasts are incubated with 5 mM of a Bodipy-labeled sphingomyelin analogue, C_5-DMB-SM, and washed, a continuous plasma membrane fluorescence was observed (Fig. 4 a). A similar plasma membrane staining pattern was observed after cells were incubated with an FITC-phosphatidylethanolamine analogue, FITC-PL (Fig. 4 b), or a phosphatidylcholine analogue, β-DPH-HPC (Fig. 4 c). Whereas incorporation of C_5-DMB-SM into membrane bilayers resulted in a 15% decrease in tether force with respect to control cells,

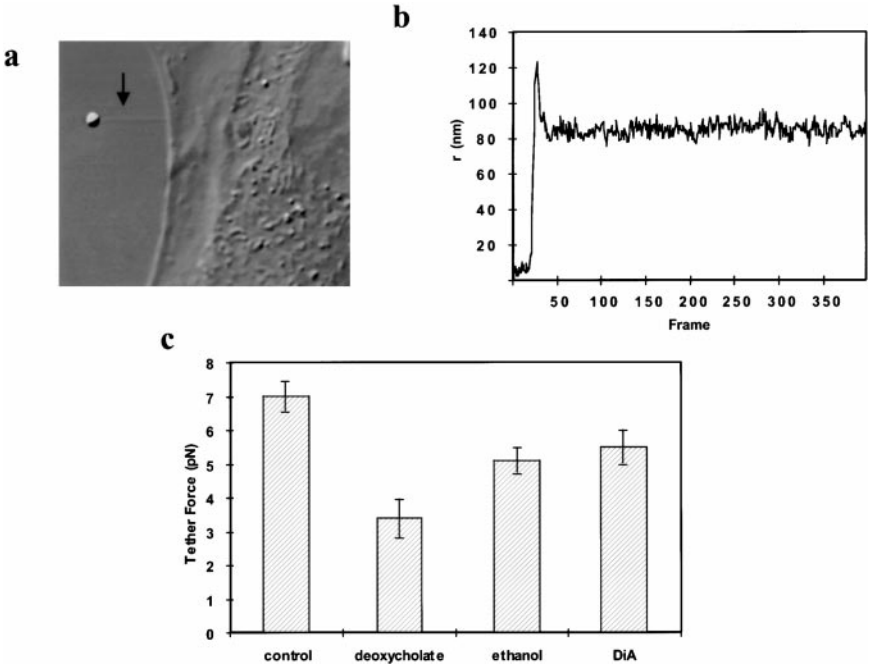

Figure 3. Tether force measurements. (a) Video-enhanced DIC micrograph of membrane tension measurement. Polystyrene beads were coated with mouse IgG and attached to the plasma membrane by holding them on the membrane surface, and tethers (denoted by arrow) were formed by pulling on the beads with the laser tweezers. (b) Displacement of the bead from the center of the laser trap was measured during tether formation. From calibration of the tweezers (Kuo and Sheetz, 1993) displacement may be converted to the tether force (force needed to hold the tether at a constant length). (c) Average tether force in control cells, 0.4 mM deoxycholate, 1% ethanol, or 0.1 mM DiA-treated cells. Error bars show SEM for 12–16 cells from two to five measurements.

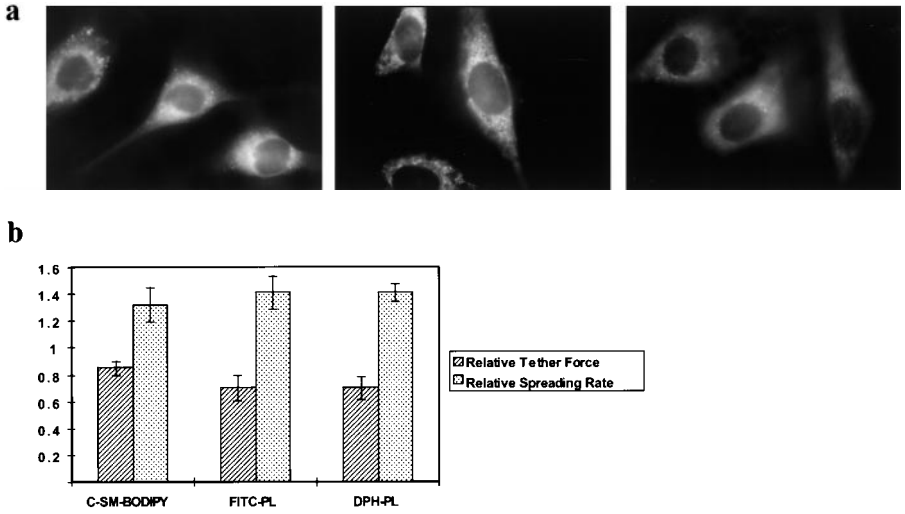

Figure 4. Tether force and lamellipodial extension rate after incorporation of fluorescent phospholipids into the plasma membrane. Fluorescence micrographs of NIH 3T3 fibroblasts plasma membrane fluorescence after incubation with the following: (a) C_5-DMB-SM; (b) FITC-PL; and (c) Phosphatidylcholine analogue (β-DPH-HPC). (d) Average relative tether force and average relative lamellipodial extension rate in NIH 3T3 fibroblasts after the incubation with various fluorescent phospholipids.

FITC-PL or β-DPH-HPC phospholipids reduced the tether force ~25% (Fig. 4 d). As shown in Fig. 4 d, the lamellipodial extension rate increased by 30% in C_5-DMB-SM–treated cells and >40% in FITC-PL or β-DPH-HPC–labeled cells, with respect to control cells. In summary, fluorescent lipid incorporation induced a decrease in membrane tension, which was accompanied by an increase in the lamellipodial extension rate. The percentage of change in both the extension rate and membrane tension was proportionally the same for all phospholipids and deoxycholate. With ethanol, there was a greater change in the extension rate than expected for the change in tension. Of the compounds used, ethanol was unusual because it was an uncharged alcohol that would readily cross the membrane. With all of the charged compounds that slowly cross the membrane, there was a proportional change in the tension and extension rate.

Deoxycholic Acid Titration

To further explore the correlation between tether force and extension rate, we measured the changes in both parameters as a function of deoxycholic acid concentration (Fig. 5). There was an almost linear decrease in the tether force with increasing deoxycholic acid concentration, and (Fig. 5 b) in parallel, an increase in the lamellipodial extension rate (Fig. 5 a).

If there is an inverse dependence of the lamellipodial extension rate on the membrane tension, then the lamellipodial extension rate should continuously increase with a decrease in the membrane tension. Indeed, when the lamellipodial extension rate was plotted versus the tether force for different concentrations of deoxycholate acid, 0.1 mM fluorescent dye DiA, fluorescent phospholipids from Fig. 4 and hypotonic solution represented in Fig. 6, an inverse relationship was readily apparent (Fig. 5 c). Thus, different membrane expanding reagents cause a proportional change in both tension and extension rate.

Hypotonic Swelling Increases Membrane Tension and Decreases the Lamellipodial Extension Rate

In the experiments above, we measured the effect of membrane tension reduction on the lamellipodial extension rate. We wanted to test if increasing membrane tension by hypotonic swelling would decrease the lamellipodial extension rate. In snail neurons, hypotonic swelling caused filopodia and lamellipodia to retract (Dai et al., 1998).

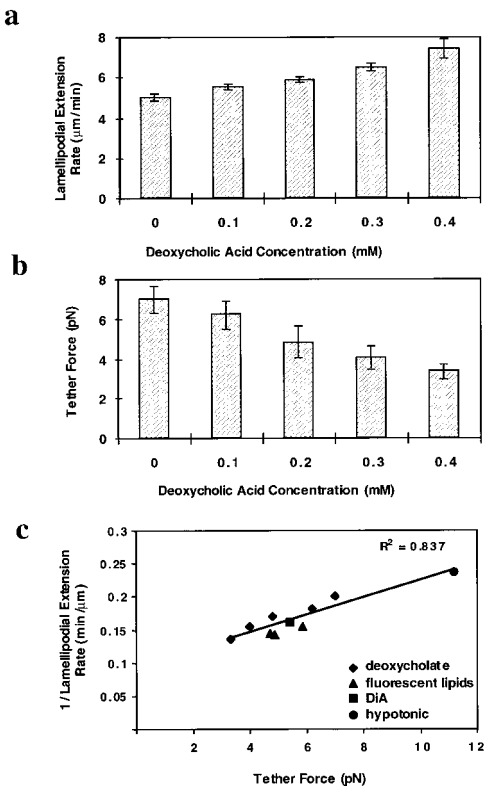

Figure 5. Lamellipodial extension rates and tether force are inversely proportional. (a) Tether force after application of increasing concentration of deoxycholate. (b) Lamellipodial extension rates in increasing concentrations of deoxycholate. (c) Lamellipodial extension rates versus tether force for various concentrations of deoxycholate, 0.1 mM fluorescent dye DiA, fluorescent phospholipids from Fig. 4, and hypotonic solution represented in Fig. 6.

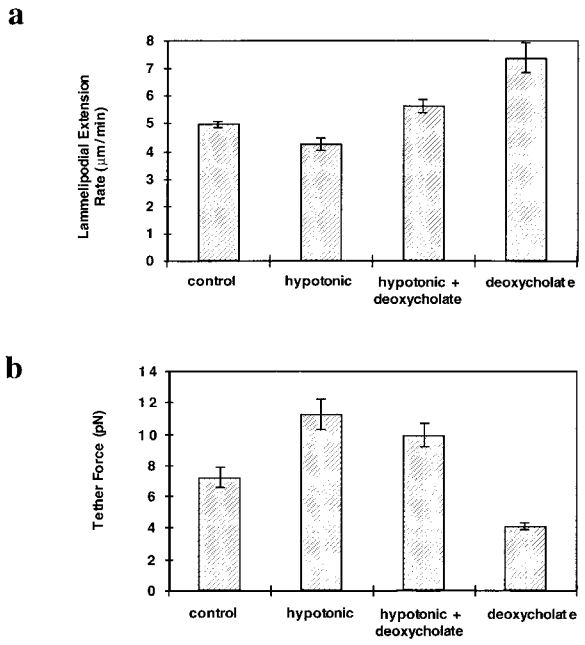

Figure 6. Lamellipodial extension rates and tether force measurements in hypotonic media. (a) Tether force measured after 3 min in hypotonic solution (30% dH_2O), after the hypotonic solution was combined with 0.4 mM deoxycholic acid, or after 0.4 mM deoxycholic acid alone. (b) Lamellipodial extension rates measured under same conditions as in a.

When 3T3 cells were placed in hypotonic media, there was a dramatic increase in the tether force (Fig. 6 a) and about a 15% decrease in the lamellipodial extension rate (Fig. 6 b). Interestingly, as shown in Fig. 6, this decrease in the lamellipodial extension rate may be reversed by reducing the membrane tension with 0.4 mM deoxycholate. In summary, whereas a decrease in membrane tension by amphiphilic compounds and fluorescent phospholipids is accompanied by an increase in the lamellipodial extension rate, an increase in membrane tension in hypotonic solution is accompanied by a decrease in the lamellipodial extension rate. These results indicate that there is an inverse relationship between the membrane tension and the lamellipodial extension rate.

Lamellipodial Extension Rate and Tether Force in PDGF-activated Cells

Dramatic changes in surface morphology such as outgrowth of new membrane lamellae and filopodia are often seen in response to growth hormones. These effects seem to reflect changes in the actin cytoskeleton. From our previous studies, we found that apparent membrane tension is dependent on membrane–cytoskeleton attachment through phosphatidylinositol 4,5-biphosphate (PIP_2)–cytoskeleton interaction (Raucher et al., 1999). Thus, to further examine the role of membrane tension in lamellipodial protrusion, we measured the tether force and the lamellipodial extension rate during PDGF activation. As shown in Fig. 7, 5 min after addition of PDGF, the lamellipodial extension rate increased 40% with respect to control cells, whereas the membrane tension decreased 15% with respect to controls. The increase in extension rate was greater than expected for the level of decrease in membrane tension based upon the changes with lipids or detergents. The lamellipodial extension rate further increased when PDGF was combined with deoxycholic acid (~65% with respect to the control cells). Since deoxycholic acid itself caused only a 50% increase in the lamellipodial extension rate, it is very likely that the underlying mechanisms of lamellipodial extension mediated by deoxycholic acid and PDGF are independent. One possible explanation for the disproportionately high activation of motility compared with the change in apparent membrane tension is that the effect of PDGF is localized to regions of lamellipodial extension, where it is much more pronounced than the global effect on membrane tension. If the PDGF effect was mediated by a membrane-associated enzyme, then the change could be localized.

Binding of PDGF to the PDGF receptor activates phospholipase C. Therefore, to further explore if signal transduction processes can directly modulate membrane tension and, consequently, changes in lamellipodial extension rate, we have used U73122, an inhibitor of phospholipase C. As shown in Fig. 7 c, the increase in the lamellipodial extension rate caused by PDGF is blocked by U73122. Inhibition of phospholipase C during PDGF stimuli also increases membrane tension (Fig. 7 d), suggesting that the activation of phospholipase C is an important step in the control of membrane tension and, consequently, lamellipodial extension rate.

Discussion

In previous models of the extension process, the plasma membrane was assumed to inhibit actin polymerization. How that inhibition could be quantified was not clear. We provide here evidence that the membrane tension is inhibiting extension and there is an inverse relationship between tension and extension rate. Further and perhaps surprisingly, a rapid drop in membrane tension initiates the generation of new lamellipodial extensions, as schematically represented in Fig. 8 a. Hormonal activation of motility is accompanied by a decrease in membrane tension, and motility can be further increased by expansion of the plasma membrane by amphiphilic compounds (Fig. 8 b). These findings provide a means for quantitating the inhibition of lamellipodial extension by membrane tension, and indicate that tension changes can stimulate the biochemical process underlying extension.

That membrane tension may regulate the actin polymerization rate during lamellipodial extension is consistent with Brownian-ratchet models (Peskin et al., 1993). The Brownian-ratchet model is a simple explanation for lamellipodial protrusion in which the fluid membrane bilayer at the leading edge, once free from constraints, fluctuates as a result of Brownian motion. Each time the membrane is displaced a distance sufficient to permit addition of an actin monomer, the return of the membrane to its original position is prevented. As the filaments polymerize, their Brownian motion impinges on the cytoplasmic surface of the plasma membrane and exerts a pressure, deforming the membrane. Since the extent of deformation depends

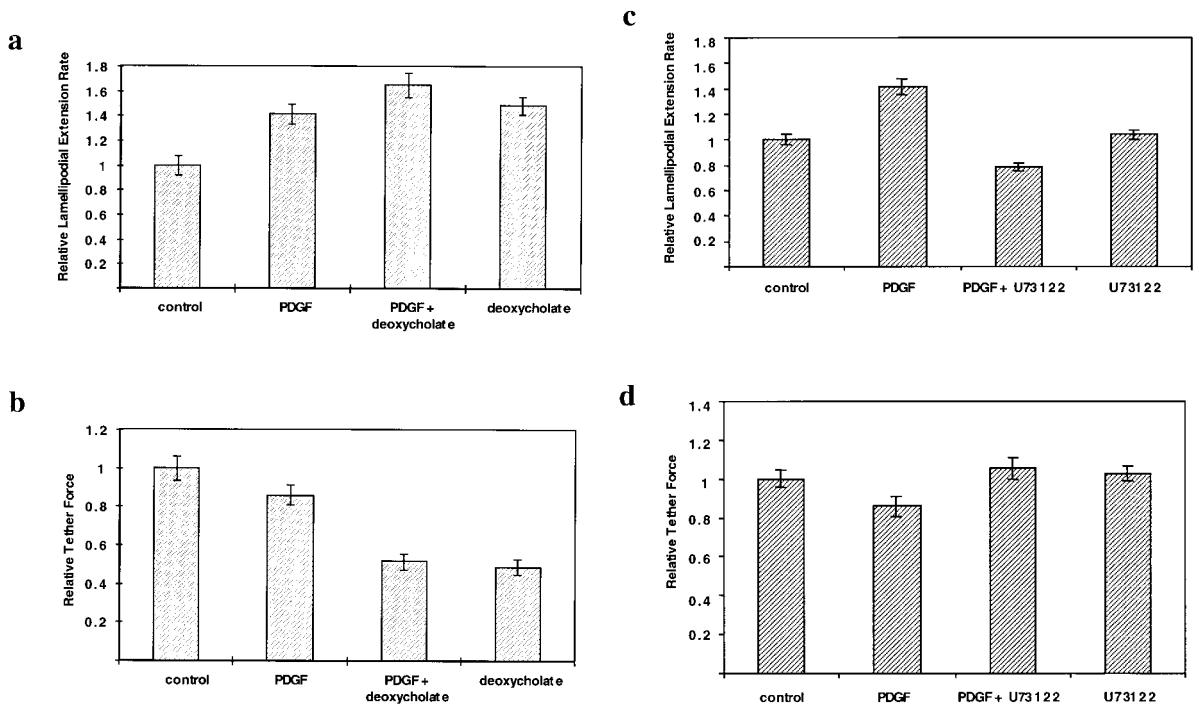

Figure 7. Tether force and lamellipodial extension rate after PDGF activation. (a) Lamellipodial extension rates measured after 5 min in 40 ng/ml PDGF, after PDGF was combined with 0.4 mM deoxycholic acid, and after 0.4 mM deoxycholic acid treatment alone. (b) Tether force measured under same conditions as in a. (c) Lamellipodial extension rates measured after 5 min in 40 ng/ml PDGF, after PDGF was combined with 1 μm U73122, and after 1 μm U73122 treatment alone. (d) Tether force measured under same conditions as in c.

on the resistance tension in the membrane, lowering the membrane tension would enhance the rate of actin polymerization and, consequently, lamellipodial extension rate. This concept is experimentally demonstrated in our study. We have shown that a decrease in membrane tension by amphiphilic compounds and fluorescent phospholipids is accompanied by an increase in the lamellipodial extension rate. Furthermore, a membrane tension increase, induced by a hypotonic solution, was accompanied by a decrease in the lamellipodial extension rate. These data suggest an important role for membrane tension in regulation of the lamellipodial extension rate.

The concept that physical parameters may stimulate alterations in cellular biochemistry and regulate many cellular functions, such as cell motility, has been confirmed experimentally many times (Watson, 1991; Khan and Sheetz, 1997). Kolega (1986) found that stretching an epithelial cell causes it to withdraw its lateral protrusions, and that suppression of spreading activity, at least in the case of fibroblasts and nerve axons, is reversed upon release of tension. Similarly, in certain growth cones and cells, the polymerization of actin into filopodia-like processes can be induced by the physical separation of the membrane from the cytoskeleton (Sheetz et al., 1992). Using the laser tweezers to pull on membrane-attached latex beads, cylinders of membrane were formed that became rigid and behaved like filopodia. This implies that the membrane has an inhibitory role in protrusion formation and that the normal extension process may be limited by membrane tension.

Quantitative Relationship between Tension and Extension Rate

Previous models of the extension process have suggested that the plasma membrane resists actin polymerization,

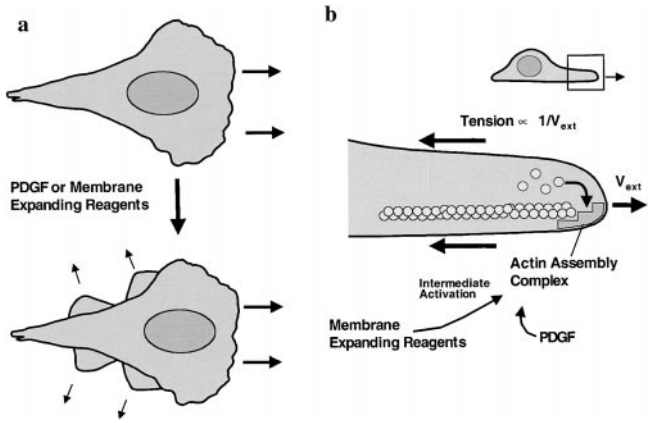

Figure 8. PDGF and membrane expanding reagents initiate the generation of new lamellipodial extensions and stimulate lamellipodial extension rates. (a) A rapid drop in membrane tension caused by PDGF or membrane expanding reagents initiates the generation of new lamellipodial extensions. (b) There is an inverse relationship between tension and extension rate, and tension changes caused by PDGF, or membrane expanding reagents, can stimulate the biochemical processes underlying extension, such as actin polymerization.

implying that membrane tension may have a direct role in regulating the lamellipodial extension rate. These measurements of tether force show that tether force is a tightly regulated parameter in motile cells that is inversely correlated with the lamellipodial extension rate. From the tether force, we can estimate membrane tension using the membrane bending stiffness measured in other systems, and the estimated membrane tension is on the order of 0.03 mN/m. This apparent tension includes the membrane–cytoskeleton adhesion term, which is the major factor in nonspherical cells. This value is on the same order of magnitude as assumed by Mogliner and Oster (1996) and, therefore, allows those values to be used. However, the basis given for choosing that number comes from monolayer surface pressure studies in vitro and not from measurements of cell membrane tension.

Recent studies provide a theoretical basis for modeling the growth of the actin filaments by a simple Brownian-ratchet mechanism versus a motor driven mechanism. Quantitative analysis of the relationship between the extension rate and the force exerted on the filament ends could be used to differentiate between some motor and pure ratchet mechanisms. The linear inverse relationship given by Fig. 5 c is most consistent with the more linear motor models. However, the exact force exerted by the membrane is not known and the effects of membrane–cytoskeleton attachment on the extension process are unknown. Further, multiple assumptions were made in the estimates of the membrane tension. An additional factor that has not been considered is the balance of pressures in the internal and external bilayer halves or the bilayer couple balance (Sheetz and Singer, 1974), which would clearly be different for the permeant ethanol and impermeable phospholipids. More work is needed to define the changes in the membrane–cytoskeleton adhesion and in-plane tension terms with the addition of various agents.

Evidence for Mechanical Stimulation of Extension

These studies provide additional evidence that a mechanical signal can stimulate extension. The initial observations that extension is stimulated by the release of fibroblast tails could have several different interpretations (Chen, 1981). Since many different amphiphilic compounds stimulate extension, it is unlikely that a common biochemical pathway is directly activated by all the compounds and tail release. Thus, we suggest that the drop in membrane tension with tail release and detergent or lipid addition stimulates processes by a physical alteration of the rate-limiting step in lamellipodial extension. Because the tether force is a tightly regulated parameter in a given cell population, there must be enzymatic systems that sense and adjust the tension, and it is only necessary that such a sensor be coupled to motility. A possible reason for increased extension with the lower membrane tension is that extension of lamellipodia would serve to increase the cell surface area for a given volume and would help to increase the membrane tension to the normal value.

While it is evident that membrane tension is an ideal candidate to play a mechanical role in regulating lamellipodial protrusions, the molecular mechanisms involved in regulating membrane tension are, however, unclear. One hypothesis is that membrane tension is regulated by membrane dynamics, i.e., the balance of exocytosis of membrane from an intracellular reservoir and endocytosis of plasma membrane. Another hypothesis is that membrane–cytoskeleton adhesion controls the tension and protrusion locally. A third hypothesis is that the membrane is added locally to stimulate extension. This last hypothesis arose from the observation of rearward movement of small particles attached at the leading edge of the migrating cells over the dorsal surface of the cell (Abercrombie et al., 1970) and was supported by the observation that newly synthesized viral glycoproteins in cells are preferentially inserted into the leading portion of the plasma membrane (Bergman et al., 1983). However, no net flow of membrane has been found in the plasma membranes of rapidly migrating keratocytes or macrophages (Kucik et al., 1989; Lee et al., 1990). In addition, the membrane can be rapidly moved across the cell surface obviating the need for local addition (Dai and Sheetz, 1995a; Hochmuth et al., 1996). Thus, we suggest that local addition of membrane is not a significant factor in lamellipodial extension.

There is evidence that the two other mechanisms can be important. In the case of membrane dynamics, disruption of the Golgi apparatus by Brefeldin A results in inhibition of vesicle transport to the cell surface (Pelham, 1991; Klausner et al., 1992; Bershadsky and Futerman, 1994). If exocytosis is inhibited and endocytosis continues, then there should be an increase in membrane tension as we have observed (preliminary data). The higher tension should inhibit lamellipodial activity as was observed.

We observed here that signal transduction processes modulate membrane tension in parallel with an alteration in the lamellipodial extension rate. PDGF receptor-triggered signal transduction increases the lamellipodial extension rate in correlation with a decrease in membrane tension. This response is blocked by the phospholipase C inhibitor, U73122, suggesting that phospholipase C activation may play an important role in regulating membrane tension and, consequently, lamellipodial extension rate. In the case of local control of membrane–cytoskeleton adhesion, there is evidence that the activation of chemotactic receptors can cause extension locally (Mogilner and Oster, 1996). In many cases, those signals are activating a lipase C to hydrolyze PIP_2 to inositol 1,4,5-triphosphate and diacylglycerol. Loss of PIP_2 decreases membrane–cytoskeleton adhesion (Raucher et al., 1999) and consequently a local decrease in membrane–cytoskeleton adhesion would increase lamellipodial extension rate. This is supported with observation that the plasma membrane of cells conforms to the shape of the cortical cytoskeleton, implying that membrane tension is largely determined by adhesion energy between the plasma membrane and the cytoskeleton. Therefore, cytoskeletal–membrane interactions may be the major factor inhibiting the formation and retraction of filopodia, lamellipodia, neurites, and other membrane processes in response to chemoattractants and other stimuli.

Other Examples of Correlation between Extension and Membrane Tension

There are many other examples where a correlation exists

between extension and membrane tension. Stimulation of secretion of RBL cells results in a stimulation of actin-based motility that appears as the extension of thin veils of actin on the upper surface of the cells. Secretion in RBL cells is coupled with a large decrease in the tether force that is associated with an increase in the endocytosis rate (Dai et al., 1997). The assembly of actin in the veils is analogous to the extension of lamellipodia, and provides evidence that the drop in membrane tension may contribute to the burst of motile activity after secretion. There are clearly other signaling pathways coupled with secretion, but the physical changes could favor such activity. Entry and exit from mitosis have been shown to correlate with an inhibition of and stimulation of motility, respectively. As cells enter mitosis, there is a dramatic increase in membrane tension that will cause cell rounding and will block motility (Raucher and Sheetz, 1999). Blocking actin-based motility may be particularly useful to the cell to enable it to organize the spindle for mitosis and to organize for cytokinesis. During cytokinesis there is a drop in the apparent membrane tension that increases cell motility and enables the cleavage furrow to form.

Regardless of the actual mechanism that is used by the cell to regulate apparent membrane tension, this study clearly demonstrates that the lamellipodial extension rate correlates inversely with membrane tension as measured by tether force. The fact that membrane tension in these cells is determined by the membrane–cytoskeleton adhesion means that factors that alter adhesion can alter motility, such as PIP_2, as we suggest here. Modulation of the lamellipodial extension rate by membrane tension indicates that the cellular plasma membrane is not simply an oil film that conforms to the cytoskeleton. However, with its inelastic physical properties, it also has a mechanical role in controlling cell protrusions and guiding directional growth and the underlying actin network. This model can account for the regulation of the lamellipodial extension rate and suggests simple treatments to modulate the level and rate of extension.

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