Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching

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SUMMARY

The actin-dependent sensory and response elements of stromal cells that are involved in mechanical signal transduction are poorly understood. To study mechanotransduction we have described previously a collagen-magnetic bead model in which application of well-defined forces to integrins induces an immediate (<1 second) calcium influx. In this report we used the model to determine the role of calcium ions and tyrosine-phosphorylation in the regulation of force-mediated actin assembly and the resulting change in membrane rigidity. The collagen-beads were bound to cells through the focal adhesion-associated proteins talin, vinculin, α2-integrin and β-actin, indicating that force application was mediated through cytoskeletal elements. When force (2 N/m²) was applied to collagen beads, confocal microscopy showed a marked vertical extension of the cell which was counteracted by an actin-mediated retraction. Immunoblotting showed that force application induced F-actin accumulation at the bead-membrane complex but vinculin, talin and α2-integrin remained unchanged. Atomic force microscopy showed that membrane rigidity increased 6-fold in the vicinity of beads which had been exposed to force. Force also induced tyrosine phosphorylation of several cytoplasmic proteins including paxillin. The force-induced actin accumulation was blocked in cells loaded with BAPTA/AM or in cells pre-incubated with genistein, an inhibitor of tyrosine phosphorylation. Repeated force application progressively inhibited the amplitude of force-induced calcium ion flux. As force-induced actin reorganization was dependent on calcium and tyrosine phosphorylation, and as progressive increases of filamentous actin in the submembrane cortex were correlated with increased membrane rigidity and dampened calcium influx, we suggest that cortical actin regulates stretch-activated cation permeable channel activity and provides a desensitization mechanism for cells exposed to repeated long-term mechanical stimuli. The actin response may be cytoprotective since it counteracts the initial force-mediated membrane extension and potentially strengthens cytoskeletal integrity at force-transfer points.

Key words: Actin, Mechanotransduction, Force

INTRODUCTION

The stromal cells of load-bearing tissues are subjected to physical forces which play an important role in regulating tissue remodelling and repair. The mechanisms by which these cells sense physical forces, transduce the forces into signals and generate appropriate responses leading to physiological remodelling of extracellular matrices have yet to be determined. Further, it is not understood how stromal cells maintain their shape or their signalling systems after application of periodic, high amplitude applied forces. Conceivably, mechanotransduction involves a coordinated system of molecules including extracellular matrix receptors, cytoskeletal proteins and membrane channels (Wang et al., 1993; Sachs, 1989). For example cells may communicate with the extracellular matrix by changing the physical properties of their membranes (Sheetz and Dia, 1996) which in turn could regulate the sensitivity of mechanosensitive ion channels (Morris, 1990).

Forces acting on the cell membrane are likely distributed to the underlying cytoskeletal network which may help determine not only the biophysical properties of the membrane but also regulate calcium influx from the extracellular environment into the cytoplasm (Ghosh and Greenberg, 1995; Sachs, 1988). In this study we asked whether actin, calcium and tyrosine kinases act coordinately to regulate cellular responses to applied forces. As a coordinated, cytoprotective system is also likely to involve the physical properties of the cell, we also examined membrane rigidity. To address these questions we developed a collagen-magnetic bead model based on that of Wang et al. (1993). The new model applies linear, quantified forces to integrins and the associated actin cytoskeleton and permits real-time monitoring of cell shape and calcium. Subsequent processing allows quantification of tyrosine phosphorylation and actin recruitment to force transfer sites in focal adhesions. As we sought to model forces which more closely approximated those encountered by force-loaded fibroblasts in vivo, the direction of applied force was altered to a vertically directed pull, perpendicular to the dorsal membrane, as opposed to the twisting force (torque) used by Wang et al. (1993). Our results suggest that cortical actin assembly...
interacts with calcium and tyrosine phosphorylation to coordinate a cytoprotective system during and after mechanical force application.

MATERIALS AND METHODS

Reagents

Mouse anti-talin (clone # 8D4), anti-vinculin (clone # HVIN-1), anti-β-actin (clone # AC-15), FITC-goat anti-mouse antibodies and TRITC-phalloidin were purchased from Sigma Chemical Co. (St Louis, MO). α2-integrin was purchased from Calbiochem (San Diego, CA). Anti-phosphotyrosine (clone # 4G10) was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Antipaxillin (clone # Z035) was purchased from ZYMED (San Francisco, CA). Calcein AM was purchased from Molecular Probes (Eugene, OR). All antibodies were against human antigens. Verification of collagen coating on beads was performed as described (Glogauer et al., 1995).

Cell culture

Human gingival fibroblasts (HGF) were derived from primary explant cultures as described (Pender and McCulloch, 1991). Cells from passages 6 to 19 were grown as monolayers in T-75 flasks (Glogauer et al., 1995). Twenty-four hours before each experiment cells were harvested with 0.01% trypsin and 200,000 cells were plated into 60 mm diameter culture dishes (Falcon, Becton Dickinson, Mississauga, ON). The cells were sub-confluent prior to all experiments.

Force generation

A ceramic permanent magnet (Gr. 8, 2.2 cm × 9.6 cm × 11 cm; Jobmaster, Mississauga, ON) was used to generate physical force. For all experiments the pole face was parallel with and 2 cm from the cell/culture dish surface. At this distance the field strength is ~400 Gauss (G) with a vertical gradient of ~100 G/cm as measured by a fluxgate magnetometer (Jobmaster, ON). A constant force of varying durations was used for all experiments, which induced an upward flux of beads since the beads were not removed from the cell membrane during force application (Glogauer et al., 1995).

Immunofluorescence

Cells grown on coverslips were fixed with 3.7% formaldehyde in PBS for 10 minutes, stained with TRITC-phalloidin and examined using a ×63, 1.3 NA oil immersion objective under epifluorescence optics and confocal imaging (Leica CLSM, Heidelberg, Germany). For antibody staining cells were permeabilized in 0.3% Triton X-100 in PBS for 15 minutes at room temperature. Cells were incubated with primary antibody (vinculin or talin; 1:80 dilution) for one hour at 37°C, washed 3 times with PBS containing 0.03% Triton and 0.2% BSA, and incubated with fluorescein-conjugated goat anti-mouse (1:100 dilution) or rhodamine-conjugated donkey anti-rabbit (1:100 dilution). Non-specific control staining was performed on the same slide using secondary antibody only. Coverslips were washed with PBS and mounted with an anti-fade mounting medium (ICN).

Confocal microscopy

The spatial distribution of probes for vinculin, talin and actin in bead/membrane complexes was imaged in single cells by confocal microscopy. For FITC-labeled probes, excitation was set at 488 nm and emission was collected with a 530/20 nm barrier filter. For TRITC, excitation was set at 530 nm and emission was collected at 620/40 nm. Cells were imaged with a ×63 oil immersion lens (NA 1.4) and transverse optical sections were obtained from the level of cell attachment at the substratum to the dorsal surface of the cell.

Intracellular Ca2+

Measurement of intracellular calcium ion concentration ([Ca2+]i) was conducted as described previously (Glogauer et al., 1995). Briefly, cells on coverslips were incubated at 37°C with 3 μM fura-2/AM (Molecular Probes, Eugene, OR) for 20 minutes and then at room temperature for 10 minutes. Whole cell [Ca2+]i measurements were obtained with a dual excitation, microscope-based spectrofluorometer (Photon Technology Int., London, ON). A variable aperture, intra-beam mask was used to restrict measurements to single cells. Estimates of [Ca2+]i, independent of the precise intracellular concentration of fura-2 were calculated from dual-excitation emitted fluorescence as described (Grynkiewicz et al., 1985). Repeated force applications (2 N/m²) were undertaken every 6 minutes. As demonstrated previously (Glogauer et al., 1995) microscopic evaluation
Quantitation of bead binding

The percentage area of cell dorsal membrane in contact with either BSA or collagen-coated beads after a 30 minute force exposure was estimated by image analysis (Bioquant, R&M Biometrics; Nashville, TN). Cells plated on coverslips were incubated with 150 µg of beads for ten minutes and washed three times with PBS to remove unbound beads or loosely attached beads. Equal bead loading per µm² of membrane in the BSA and collagen-coated cell groups was verified before force application. Cells were stained with hematoxylin and eosin prior to analysis to facilitate identification of the cell periphery and area calculations.

F/G actin ratio

Cells exposed to force for various time periods were washed in ice-cold PBS and detached by scraping into actin stabilization buffer (Heacock and Bamberg, 1983), a procedure that stabilizes the G and F actin pools. The cell lysate was ultracentrifuged (100,000 g for 2 hours) and the supernatant (G-actin) was removed from the pellet (F-actin). The two fractions were quantitated by densitometry of western blots (F-actin) and the F/G actin ratio was calculated.

Isolation of focal adhesions

Proteins enriched in bead-associated focal adhesion complexes were assessed using the methods of Plopper and Ingber (1993). Briefly, protein from plated samples was incubated on a rotator with pansorbin (Calbiochem) for 2 hours at 4°C, and incubated with Protein G-Sepharose beads (Amersham). Tyrosine-phosphorylated paxillin was identified by immunoblotting and immunoprecipitation. Isolated protein from BSA and collagen-coated cell groups was verified before force application. Samples were separated by SDS-PAGE (10% acrylamide), and transferred to nitrocellulose. Blots were blocked for 1 hour with 5% skim milk in PBS and incubated in the indicated antibody (diluted 1:1,000 in 0.5% Tween-PBS) for 1 hour at room temperature. Blots were washed with 0.5% Tween-PBS for 10 minutes, incubated with goat anti-mouse HRP or anti-rabbit HRP (Amersham) for 30 minutes, washed 5x in PBS-Tween and developed by chemiluminescence (ECL; Amersham). Tyrosine-phosphorylated paxillin was identified by immunoprecipitation and immunoblotting. Isolated protein from plated samples was incubated on a rotator with pansorbin (Calbiochem) for 2 hours at 4°C, and incubated with Protein G-Sepharose beads which had been incubated with anti-paxillin (50 µg/ml beads) overnight. Beads were collected by centrifugation in a microfuge. The isolation procedure was carried out at 4°C using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, New York). The cell-bead suspension was sonicated for 10 seconds (output setting, 3, power 15%; Sonifier 185, Branson), and homogenized in a 2 ml dounce homogenizer (20 strokes). The magnetic beads were pelleted and washed 3 times with CSKB prior to protein analysis.

Immunoblotting and immunoprecipitation

Actin, paxillin, α2-integrin, vinculin, talin and tyrosine phosphorylated proteins were identified by immunoblotting. Isolated proteins were separated by SDS-PAGE (10% acrylamide), and transferred to nitrocellulose. Blots were blocked for 1 hour with 5% skim milk in PBS and incubated in the indicated antibody (diluted 1:1,000 in 0.5% Tween-PBS) for 1 hour at room temperature. Blots were washed with 0.5% Tween-PBS for 10 minutes, incubated with goat anti-mouse HRP or anti-rabbit HRP (Amersham) for 30 minutes, washed 5x in PBS-Tween and developed by chemiluminescence (ECL; Amersham). Tyrosine-phosphorylated paxillin was identified by immunoprecipitation and immunoblotting. Isolated protein from plated samples was incubated on a rotator with pansorbin (Calbiochem) for 2 hours at 4°C, and incubated with Protein G-Sepharose beads which had been incubated with anti-paxillin (10 µg/ml beads) overnight. Beads were collected by centrifugation in a microfuge. The precipitate was washed six times. Protein was separated from beads by heating at 65°C for 10 minutes in 2x Laemmli buffer (Laemmli, 1970).

Atomic force microscopy

Atomic force microscopy (AFM) can provide a highly localized measure of the rigidity of the cell membrane and underlying structures. Measurements were made with an AFM Nanoscope III (Digital Instruments, Santa Barbara, CA) operating in contact mode. The A+B signal of the feedback was 3 V while the A-B signal was set at -1 V and setpoint 1-5 V. Standard silicon nitride integrated pyramidal tips with an estimated radius of curvature of ~100 nm were fixed on 200 µm 'wide leg' cantilevers (spring constant k=0.06 N/m) and used for all measurements. The radii of curvature were estimated by scanning of a rectangular step on a mica surface. The D scan head (maximum area is 12.5 µm²; vertical sensitivity ~9 nM/V) was employed throughout the study. Scanning of live samples was carried out within 20 minutes of completion of magnetic force application.

Section analysis was performed on cell samples to determine relative changes in rigidity at various distances from membrane-bound beads. Section analysis involved the use of two different scanning forces (39 and 42 nN) applied to the cell membrane. From the differences between the two resulting deformations a relative indication of the surface rigidity was determined. As rigidity increases the difference in deformation between the two forces will decrease. To estimate the numerical change in rigidity, Young’s Modulus was used (model case with contact between two rigid bodies): \( E_{h} = \frac{F}{R^2 h} \), where \( h \) is the deformation, \( E \) is Young’s modulus, \( R \) is the AFM-tip radius and \( F \) is the force of scanning. The assumption was made that the rigidity of the tip material (silicon nitride) is much higher than the rigidity of the cell. We measured the changes in deformation, \( \Delta h \), and calculated \( E_{h} = \frac{F_1 - F_2}{R^2 (\Delta h)^2} \), where \( F_1 \) and \( F_2 \) are the two forces used to scan the sample. \( R = 100 \) nm. From the changes in deformation (\( \Delta h \)) at various distances from the bead we were able to calculate the rigidity (E) of the membrane in various samples.

Blockade experiments

To determine the dependence of actin redistribution on tyrosine phosphorylation, samples were pretreated with genistein (100 µM) for 45 minutes at 37°C prior to force application. This protocol is known to inhibit >80% of tyrosine phosphorylation in gingival fibroblasts treated with IL-1 (Arora et al., 1995) and also inhibits volume activated calcium flux (Bibby and McCulloch, 1994). We assessed the dependence of actin rearrangement on calcium ions by incubating cells with BAPTA/AM at 3 µM for 45 minutes at 37°C prior to force application. Previous pilot experiments have shown that this reduces [Ca²⁺], to <50 nM and blocks ligand-induced calcium fluxes.

Statistical analysis

For all assays, 3 or more separate experiments were performed; means ± s.e.m. were calculated for continuous variables and comparisons were made by unpaired t-tests.

RESULTS

Cell extension and contraction

Time-lapse monitoring of cells subjected to 30 minutes of a constant, vertically-directed stretching force was conducted in cells loaded with calcein-AM, a fluorescent space-filling probe which enters and remains in the cytoplasm of the cell. Control experiments assessing leakage of calcein dye during confocal monitoring demonstrated no change in the minimal leakage rate when force was applied to calcein loaded cells (n=3; Fig. 1A) indicating that the force did not detectably disrupt the plasma membrane. Using confocal microscopy we were able to monitor the changes of fluorescence intensity in the dorsal focal plane during force application (Fig. 1B). These data were used to estimate quantitatively the relative extension of the cell during force application (Fig. 1C). During the initial phase of force application the proportion of total cell fluorescence in the dorsal focal plane increased gradually and reached a peak at about 15 to 20 minutes (passive force-induced extension phase). The calcein fluorescence slowly returned toward baseline levels over the subsequent period in spite of ongoing force application (Fig. 1D), suggesting that the contraction of
the extended dorsal third of the cell was an active process (contractive phase).

Whole cell actin
As cell stretching apparently invoked an actin-dependent shape change we used western blots to measure total cell actin. There was no apparent increase in total actin in force-treated samples. In fixed cells stained with TRITC-phalloidin, no significant difference in F-actin between controls and force samples (30 minutes) was observed by confocal microscopy of whole cell fluorescence (C = 10.0±1.2 fluorescence units (FU);  F = 12.1±1.5 FU;  P>0.2). To determine if there was an overall shift in the actin pools in response to force, the ratio of the filamentous (F-actin) form of actin to the monomeric (G-actin) form of actin (F/G ratio) was determined. There was no significant difference in the F/G ratio at 30 minutes of force application.
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cation compared to pre-force levels (C = 0.91±0.07; F = 0.79±0.04; P>0.4).

Bead-associated actin

As previous work with stretch-induced actin assembly has implicated the subcortical region as a prominent site for accumulation of actin filaments (Pender and McCulloch, 1991) we assessed the amount of actin in the attachment sites of collagen-bead complexes. Identification of cell membrane proteins bound to collagen-coated magnetic beads showed that the bead attachment sites were enriched with vinculin, talin, and β-actin. The actin accumulation at the bead/membrane complex increases during constant force exposure while vinculin and talin levels remain constant (results are representative of three separate experiments). Non-force samples exhibited no change in actin accumulation over a 30 minute time period (No force lane at 30 minutes). (B) Difference in α2-integrin vinculin and β-actin association between BSA-coated and collagen-coated beads isolated from cell membranes. Note the increased association of α2-integrin and β-actin with the collagen beads compared to BSA beads.

2 integrin were associated with the BSA beads (Fig. 2B) compared to the collagen-beads. Further, as shown by measurements of the % area of cells covered with beads before and after force (Fig. 3), when cells were equally loaded with BSA or collagen-coated beads and then subjected to force application BSA beads did not bind as strongly to cells as collagen-coated beads. Confocal microscopy of cells incubated with collagen-coated beads demonstrated localization of vinculin (Fig. 4A) and talin (Fig. 4B) to the regions of the cell membrane associated with bead contact whereas membrane-attached BSA beads did not show significant recruitment of talin or vinculin to attachment sites (Fig. 4C,D).

We next determined if force applied to the magnetic beads regulated actin filament formation in focal adhesions. Isolation of proteins from collagen-coated beads demonstrated a slight increase within 5 minutes of force application with an ~1.5-fold increase in actin associating with beads within 30 minutes after force application (Fig. 2A) while the levels of talin and vinculin remained unchanged. We noted that the increase in actin was not due to force-induced recruitment of focal adhesions or simply variations in the numbers of beads since equal numbers of membrane-bound beads were observed in pre-force and post-force samples. Further, vinculin and talin levels did not change during the duration of force application. Confocal microscopy also showed that in force-treated cells actin filaments accumulated at membrane sites associated with beads (Fig. 5).

We reasoned that stromal cells might exhibit alterations in the stiffness of membrane structures as a cytoprotective response to force application. To determine if force application altered membrane rigidity, atomic force microscopy was performed on cells which had been incubated with beads (controls) or on cells incubated with beads and then subjected to 30 minutes of force (force). For controls there was no observed increase in rigidity (i.e. no change in Δh) associated with the membrane in close proximity to the bead compared to distant sites (close: E=1.29±0.08 MN/m²; distant (beyond 200 nm from bead): 1.48±0.08 MN/m²; P=0.11). However force samples demonstrated a greater than 6-fold increase in rigidity
adjacent to the bead compared to membrane rigidity at greater distances from the bead (close: 4.90±0.94 MN/m²; distant: 0.74±0.07 MN/m²; P=0.0006; Fig. 6A,B).

Tyrosine phosphorylation
Blots of proteins isolated from force-treated samples demonstrated an increase in tyrosine-phosphorylation of several protein species over a five minute period with a noticeable decrease at ten minutes (Fig. 7A). Prominent phosphorylated species were located between 35 and 70 kDa. As a prominent band at ~68 kDa was strongly tyrosine phosphorylated by 5 minutes, we investigated whether this might represent paxillin. Blots of paxillin immunoprecipitated from cell lysates and then probed for phosphotyrosine showed that paxillin was one protein which was tyrosine phosphorylated rapidly in response to force (Fig. 7B).

Fig. 4. Confocal microscopy of cells incubated with collagen-coated (A,B) or BSA beads (C,D) and immunostained for either vinculin (A,D) or talin (B,C). Note co-localization of vinculin and talin to regions of cell membrane associated with collagen bead contact. Left panel: corresponding phase contrast image to the fluorescent micrograph showing bead distribution (corresponding controls incubated only with secondary antibody were negative). Cells were optically sectioned and the image was obtained at the level of the bead-membrane interface. (C,D) Micrographs of membrane-attached BSA-coated beads stained for talin and vinculin.

Fig. 5. Confocal microscopy of TRITC-phalloidin stained cells. (A) Force-dependent cortical actin rearrangement with actin accumulation at membrane sites associated with beads and some loss of stress fibers. (B) No detectable F-actin accumulation at bead/membrane sites in ‘no force’ samples. Bar, 25 μm.
Role of extracellular calcium and tyrosine phosphorylation

To assess if the increase of $[\text{Ca}^{2+}]_i$ or tyrosine kinase activity that followed physical force application was involved in the observed actin reorganization, cells were pretreated with BAPTA/AM, or genistein prior to exposing the cells to 30 minutes of force. BAPTA/AM is a cell permeable EGTA analogue which binds to free Ca$^{2+}$ in the cytoplasm and reduces $[\text{Ca}^{2+}]_i$ whereas genistein blocks tyrosine-specific protein kinases (Wijetunge et al., 1992) and inhibits ATP binding to the kinase domain of several tyrosine kinases (Akiyama et al., 1987). The force-induced increase in actin associated with the beads observed in control cells was inhibited by treatment of samples with either genistein or BAPTA/AM. Tyrosine phosphorylation and calcium are required for force-induced actin rearrangement ($n=4$). Proteins were isolated by the magnetic bead procedure. Actin was quantified by densitometry of western blots using scanner and NIH Image software.

Calcium ion influx

As intentional depolymerization of cortical actin filaments increases calcium flux through SACs in response to mechan-
These cells demonstrated progressively lower increases of 
$[Ca^{2+}]_i$ in response to the applied force (Fig. 9B,C). Conversely, cells exposed to the same force level for 1 second every 10 minutes demonstrated reproducible cytoplasmic calcium responses on a consistent basis (Fig. 9D). These results were not due to cell death as basal $[Ca^{2+}]_i$ was unchanged after force application, confirming membrane integrity and maintenance of calcium homeostatic mechanisms. In addition, monitoring of the fura2 isosbestic point showed no dye leakage in cells exposed to a constant force of 2 N/m² for 30 minutes (Fig. 9 inset), confirming that there was no detectable disruption of the plasma membrane.

**DISCUSSION**

Membrane tension induces cytoplasmic calcium transients which are partially regulated by the actin cytoskeleton (Glogauer et al., 1995). Since force alters both the organization (Gottlieb, 1990; Pender and McCulloch, 1991) and the physical properties (Wang et al., 1993) of the cytoskeleton, we wanted to determine which types of force-induced signals mediate actin assembly and whether force-induced actin assembly in turn regulates force-induced calcium signalling. The major findings in this study are that the actin assembly induced by mechanical perturbation is dependent on calcium and tyrosine phosphorylation and that actin in turn regulates calcium signalling. This coordination of cytoskeleton and signalling processes suggests a reflexive cytoprotective system to maintain homeostasis despite potentially damaging physical forces. These conclusions are dependent in part on the validity of the model system.

**Validity of model**

Our collagen-bead, magnetic force model applies a 2 N/m² pulling force perpendicular to the cell membrane of substrate-attached fibroblasts. These forces induce tension in the membrane/cytoskeletal system and extension of the dorsal surface. Previous studies using other physical force models have used similar force magnitudes (4 N/m², Wang et al., 1993; 15 N/m², Girard et al., 1993) to study force-mediated structural and biochemical changes.

**Actin**

As applied force did not alter total cell actin or the relative size of F and G actin pools, we considered that the force-induced actin changes were restricted to actin pools in close proximity to the membrane (Pender and McCulloch, 1991) and that whole cell methods were not sensitive enough to detect these localized changes. Although demonstration of actin reorganization in response to physical force in fibroblasts is not novel, the precise nature of the ‘change in F-actin content’ (Bibby and McCulloch, 1994; Pender and McCulloch, 1991) has not been resolved. To determine actin changes at the force transfer points subjacent to the membrane, we used the cell surface-bead isolation technique developed by Plopper and Ingber (1994) to isolate the integrin-associated cytoskeleton. This
method enabled examination of localized actin changes at bead/integrin sites in close proximity to the membrane. Our results demonstrate that in response to force there was significant recruitment of F-actin to the bead/membrane adhesion sites at 30 minutes after force application. The addition of actin to the membrane-receptor complex occurred without any change in vinculin, talin or α-2 integrin levels. A possible site for the increased actin assembly could be direct binding to the α-2 subunit of the integrin receptor, as previous work demonstrated strong and specific binding of actin to the cytoplasmic domain of the α-2 integrin subunit (Kieffer et al., 1995). Notably, the spatial location of actin assembly in response to a force applied perpendicular to the membrane in fibroblasts is quite different from the actin stress fiber changes which occur in endothelial cells after hemodynamically applied stress (Gottlieb, 1990) and is similar to hypoosmotic swelling-induced actin assembly in the subcortical layer (Hallows et al., 1996). Collectively these studies demonstrate the importance of characterizing force-mediated actin changes in terms of spatial redistribution and not just F-actin content. They also demonstrate the variability of actin responses to forces in different directions relative to the cell membrane.

**Regulation of actin assembly**

We considered that the specific mechanotransduction signals involved in actin assembly may involve cytoplasmic calcium and tyrosine phosphorylation. The force-induced actin recruitment to bead/membrane sites was dependent on both calcium influx and tyrosine-phosphorylation, both of which have been implicated in actin reorganization. For example intracellular calcium ion transients induced by growth factors and antigens are associated with dramatic structural changes of the cytoskeleton (Forscher, 1989). The observed calcium-dependent nature of actin accumulation in focal adhesions may be due in part to the important role of calcium in regulating the activity of many actin-binding proteins, molecules which regulate not only actin polymerization but also the global organization of the actin cytoskeleton (Forscher, 1989; Janmey, 1994).

We found that force-induced actin assembly required tyrosine phosphorylation. Ligands such as lysophosphatidic acid can induce increased tyrosine phosphorylation and subsequently alter actin polymerization and organization (Chrzanoski-Wodnicka and Burridge, 1994). Apparently, tyrosine phosphorylation of actin binding proteins may be important in regulation of actin assembly. For example in fibroblasts (Burridge et al., 1992) and macrophages (Greenberg et al., 1994), tyrosine phosphorylation of paxillin has been implicated in actin assembly. As tyrosine phosphorylation of paxillin is enhanced in sites where F-actin interacts with the plasma membrane (Greenberg et al., 1994), this process may integrate extracellular signalling events with dynamic changes in the cytoskeleton. Thus our observation of early tyrosine phosphorylation of paxillin may be part of a general mechanism to stabilize the extracellular matrix adhesion complex during the early stages of force application and actin reorganization. We speculate that paxillin phosphorylation stabilizes membrane-actin complexes when the plasma membrane is under tensile stress. Support for this contention comes from findings that genistein inhibits the accumulation of F-actin at phagocytic cups (Greenberg et al., 1993) and, as shown here, genistein blocks the force-induced actin accumulation. It is possible that paxillin may signal for the recruitment of actin filaments at stress sites to help strengthen adhesive contacts.

**Actin regulation of mechanotransduction**

The majority of data on the role of actin in force transduction (Glogauer et al., 1995), SAC activity (Sachs, 1989) and regulatory volume changes (Hallows et al., 1996) has been obtained by decreasing whole cell F-actin content with cytochalasins prior to force application. However, cytochalasin treatment may not disrupt all portions of the actin cytoskeleton (Hallows et al., 1996). The effect on cell function of increasing F-actin in the cortex has not been determined previously. As our force application model increases F-actin subjacent to the membrane, we have applied this method to study actin regulation of signalling. We hypothesized that changes in the physical properties of membrane-associated structures provide a mechanism to control SAC activity during long term force exposure (Glogauer et al., 1995). Consequently we determined if actin accumulation at force transfer sites subjacent to the plasma membrane altered membrane rigidity. Atomic force microscopy demonstrated that membrane rigidity in the vicinity of bead sites increased substantially with force application and that these were the same sites of force-induced actin assembly. These findings are consistent with acoustic microscopy data demonstrating that increased membrane rigidity corresponds spatially with increased densities of subcortical actin fibers (Luers et al., 1991). Although Wang et al. (1993) have demonstrated a stiffening of the cytoskeleton in response to directly applied torque through focal adhesions, their result is more indicative of a direct connection between the bead adhesion complex and the actin cytoskeleton as opposed to the time-dependent stretch-induced actin assembly we have demonstrated. Furthermore the resulting changes in membrane rigidity we observed were due to localized cytoskeletal changes whereas the stiffening observed by Wang et al. (1993) is due to the entire cytoskeletal network (tensility model). Notably, in the force samples, there was a slightly lower measured rigidity at greater distances (>123 nm) from the beads compared to control samples. We interpret this difference as a redistribution of localized G and F actin pools in the underlying actin cortex. However, since the rigidity near the bead is more than eight times larger than at greater distances (>123 nm), the slight reduction at greater distances compared to control cells may have no real functional significance.

Our rationale for examining membrane rigidity after force exposure originates from the idea that deformation of the membrane and associated cytoskeleton may activate SAC activity (Morris, 1990) and that the applied membrane tension generated by our model induces a calcium influx through SACs (Glogauer et al., 1995). Thus subsequent changes in membrane rigidity may play a role in modulating SAC activity during prolonged force exposure. However, the relationship between membrane tension and mechanotransduction is poorly understood. It has been proposed (Sheetz and Dai, 1996) that cells communicate with the extracellular environment by regulating membrane tension. To test this notion we have demonstrated that repeated force exposure decreased the influx of calcium into the cytoplasmic compartment. First, the reduction of stretch-induced calcium transients occurs 7-8 minutes after the
initial stretch and is potentiated over 30 minutes. Second, the increased rigidity of the cell membrane and the resultant effect on the activity of the stretch-activated cation permeable channels is mediated by both the global subcortical actin meshwork as well as the subpopulation of filaments that insert into focal adhesions. Indeed stretch-induced subcortical actin assembly is detectable within 30 seconds after stretch (Pender and McCulloch, 1991). In the present study we were able to detect a small increase in actin binding to beads in the focal adhesion complexes within 5 minutes after initiation of stretch and that the actin increased up to 30 minutes (Fig. 2a). Thus the proposed time line in which stretch-induced actin assembly and the subsequent inhibition of stretch-activated cation channels seems to be consistent overall. This phenomenon is likely mediated by a combination of the whole subcortical actin filament pool as well as those filaments that insert into focal adhesions.

**Model**

We have noted a reflexive, interactive relationship between actin assembly and calcium increase in our experiments. It has been suggested (Glogauer et al., 1995; Sachs, 1989) that microfilaments are involved in regulating the activity of mechanosensitive channels. Depolymerization of actin filaments with cytochalasin D increased the calcium influx through stretch-activated channels, possibly by altering the physical characteristics of the membrane and consequently the number of channels activated by a given stress (Glogauer et al., 1995). As our present results show that cytoplasmic calcium responses to repeated force applications were progressively diminished over time, it is possible that the increases in F-actin and membrane rigidity as a result of sustained force application may be part of a general mechanism for absorbing forces so that stretch-activated ion channels are not constantly subjected to threshold membrane tension. The increased F-actin content of cells such as periodontal ligament fibroblasts which are chronically exposed to high levels of physical force (Pender and McCulloch, 1991) may be part of a phenotypic compensatory mechanism to alter membrane properties. Thus not only does the increased actin provide structural stability to withstand increased physical stress, it also serves to shield cellular elements involved in mechanotransduction from the full effect of the physical stress, namely the SACs (Fig. 10). The reciprocity between actin assembly and regulation of calcium ions demonstrates the potential signalling modulating role of actin in mechanotransduction. The importance of this reflexive relationship is suggested by the observation that prolonged elevation of cytoplasmic calcium ion levels induces apoptosis (McConkey et al., 1991; Clapham, 1995).

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REFERENCES


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