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Mechanical force-activated phospholipase D is mediated by $G\alpha_{12/13}$-Rho and calmodulin-dependent kinase in renal epithelial cells

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Ziembicki, Jenny, Rajnish Tandon, Jeffrey R. Schelling, John R. Sedor, R. Tyler Miller, and Chunfa Huang. Mechanical force-activated phospholipase D is mediated by $G\alpha_{12/13}$-Rho and calmodulin-dependent kinase in renal epithelial cells. *Am J Physiol Renal Physiol* 289: F826–F834, 2005. First published May 24, 2005; doi:10.1152/ajprenal.00412.2004.—The renal glomerulus, the site of plasma ultrafiltration, is exposed to mechanical force in vivo arising from capillary blood pressure and fluid flow. Studies of cultured podocytes demonstrate that they respond to stretch by altering the structure of the actin cytoskeleton, but the mechanisms by which physical force triggers this architectural change and the signaling pathways that lead to generation of second messengers are not defined. In the present study, we found that in renal epithelial cells [podocytes and Madin-Darby canine kidney (MDCK) cells], application of mechanical force to the cell surface through fibronectin-coated ferric beads and exposure of the cells to magnetic force lead to Rho translocation and actin cytoskeleton reorganization. This application of force recruited Rho and filamentous actin (F-actin) to bead loci and subsequently stimulated phospholipase D (PLD), a downstream effector of Rho. Using MDCK cells that stably express regulators of G protein-signaling (RGS) proteins [RGS4 attenuates $G\alpha_i$ and $G\alpha_q$, and the p115RhoGEF-RGS domain (p115-RGS) attenuates $G\alpha_{12/13}$] to define the signaling pathway, we found that mechanical force induced $G\alpha_{12/13}$-Rho activation and increased F-actin to stimulate PLD activity. The activation can be partially prevented by the C_3 exoenzyme. Pretreatment of the cells with chemical inhibitors of several kinases showed that calmodulin-dependent kinase is also involved in stretch-induced PLD activation by a separate pathway. Taken together, our data demonstrate that in cultured podocytes and MDCK cells, mechanical force leads to actin cytoskeleton reorganization and PLD activation. The signaling pathways for PLD activation involve $G\alpha_{12/13}$/Rho/F-actin and calmodulin-dependent kinase.

podocytes; signaling

THE GLOMERULAR CAPILLARY WALL consists of three layers: glomerular endothelial cells, the glomerular basement membrane, and glomerular epithelial cells (podocytes). The capillary wall is exposed to mechanical force in vivo arising from capillary blood pressure and fluid flow (46). Many renal diseases including diabetic nephropathy and focal segmental glomerulosclerosis are characterized by reduced nephron mass. With loss of nephron mass, intraglomerular pressure increases and resident glomerular cells are exposed to increased mechanical force (29, 30, 44, 45, 50). At the cellular level, mechanical forces trigger a variety of architectural and biochemical changes. For instance, the responses of cells to mechanical force lead to the accumulation of filamentous actin at focal adhesions, increased rigidity of the cell membrane, the activation of a large number of enzymes, and the regulation of gene expression (10, 24, 44, 49, 50). Although it is well known that mechanical force has many effects on the structure and function of cells, little is known about how physical signals are converted into the intracellular signals that lead to cell responses.

Podocytes are terminally differentiated, highly specialized cells consisting of the cell body, major processes, and foot processes and cover the outer aspect of the glomerular capillary as the final glomerular filtration barrier (46). Through an actin-based contractile apparatus, podocytes counterbalance the pressure within the underlying capillary to prevent outward ballooning of the vessel and to preserve the normal architecture of the cells (44, 50). Kriz et al. (31) first demonstrated that podocytes play a role in the counteraction of capillary wall tension. Later, Endlich et al. (8) showed that in response to biaxial cyclic mechanical stress, differentiated mouse podocytes reorganize their actin cytoskeleton to form radial stress fibers and an actin-rich center. The F-actin reorganization in response to mechanical force depends on Ca^{2+} influx and Rho kinase (8). These studies indicate that glomerular podocytes are mechanosensitive and that members of the Rho family play an important role in the reorganization of the actin cytoskeleton. Knockout mice that lack the Rho-GDP dissociation inhibitor develop glomerulosclerosis, which indicates the important role of Rho in maintaining kidney structure and function (53). However, the signal transduction cascade(s) that allows podocytes to respond to force and that leads to Rho activation, actin cytoskeleton alteration, and beyond remains largely unknown.

The physiological responses to mechanical force include simultaneous activation of a large number of enzymes, such as extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal protein kinases, tyrosine kinases, and protein kinase C (49). Mechanical force has been documented to stimulate PLA_2 (2), PLC (3), and phosphatidylcholine biosynthesis (34) in membrane phospholipid metabolism. Phospholipase D (PLD), a recognized downstream effector of the small GTPase Rho (37), is an important enzyme in cell signaling that hydrolyzes phosphatidylcholine to produce choline and phosphatidic acid. Phosphatidic acid subsequently can be converted to diacylglycerol by phosphatidate phosphatase and lysophosphatidic acid by PLA_2. PLD has been implicated in many critical cellular processes including secretion, vesicular trafficking, mitosis, actin cytoskeletal reorganization, and production of reactive

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oxygen species (11, 35, 39). Earlier evidence has also shown that PLD directly associates with the actin cytoskeleton (7, 23, 25) and that actin can bidirectionally modulate PLD activity in intact cell and cell-free assays: globular actin (G-actin) inhibits PLD activity (32, 35), whereas filamentous actin (F-actin) stimulates PLD activity (32, 33). Stretch-induced Rho activation and actin cytoskeleton reorganization may be involved in PLD activation and generation of signaling molecules. The role of PLD in the physiological condition of renal epithelial cells and the way this enzyme responds to mechanical forces in such cells are still unknown.

Using radiolabeling of cells and mechanical stretch induced by exposure of iron bead-coated cells to magnetic force, we employed conditionally immortalized glomerular podocytes and Madin-Darby canine kidney (MDCK) cells as models to study the effect of mechanical force on the dynamics of the actin cytoskeleton and signaling pathways involved in PLD activation. In the present study, we demonstrate that mechanical force induces PLD activation via signaling pathways that require $G\alpha_{12/13}$/Rho/F-actin and calmodulin-dependent kinase activation in both differentiated podocytes and MDCK cells.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless specified otherwise. Cell culture reagents were purchased from Mediatech (Herndon, VA). [^{3}H]choline chloride (86 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Ceramic permanent magnets (Duramax) were purchased from Dura Magnetics (Sylvania, OH). The monoclonal anti-α-actin, the polyclonal anti-c-Myc (A-14), and the polyclonal anti-RhoA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-hemagglutinin (HA) antibody was described previously (15). The polyclonal antibodies against total ERK and phospho-ERK were obtained from New England Biolabs (Beverly, MA). Texas red-phalloidin was purchased from Molecular Probes (Eugene, OR). PD-98059, Ro-318220, AG-1478, and C_3 exoenzyme were purchased from Biomol Research (Plymouth Meeting, PA). KN92 and KN93 were obtained from Calbiochem (La Jolla, CA).

Cell culture. Conditionally immortalized mouse podocytes were obtained from Dr. Leslie Bruggeman (MetroHealth Medical Center, Cleveland, OH) and cultured as previously described (41). Briefly, podocytes were propagated at 33°C in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 U/ml mouse recombinant γ-interferon to ensure expression of the SV40 T antigen. To induce differentiation, podocytes were seeded on type I collagen-coated plates and the cultures were maintained at 37°C without γ-interferon for 10–14 days. MDCK cells that stably express regulators of G protein signaling (RGS) proteins, either the HA-tagged p115-RGS or the Myc-tagged RGS4, were established by retroviral infection as previously described (20). MDCK cells expressing vector alone or RGS proteins were cultured in DMEM containing 10% FBS, 5 U/ml of penicillin, and 5 μg/ml of streptomycin at 37°C. Overexpression of the desired proteins was documented by immunoblotting. The experiments were performed with several different clones, and similar results were obtained with all clones.

Radiolabeling, treatment, and measurement of PLD activity. The differentiated podocytes and MDCK cells expressing vector alone or RGS proteins were grown to 85–90% confluence in six-well plates and then prelabeled with 1 μCi/ml [^{3}H]choline chloride in 1 ml of 1% FBS-RPMI or -DMEM overnight. Cells were equilibrated with serum-free RPMI or DMEM for 1 h, then loaded with fibronectin-coated ferric oxide beads using a method similar to that previously described (14). Briefly, ferric oxide microparticles were coated with 10 μg/ml of fibronectin in 1× PBS and equilibrated overnight. The beads were then washed three times with 1× PBS and sonicated before their application to the cells. The coated ferric oxide beads were attached to the cells, incubated at 37°C for 10 min, and then washed three times with RPMI for differentiated podocytes and DMEM for MDCK cells. The cells were incubated in the same medium for 1 h, and magnetic force was applied to the cells using a ceramic permanent magnet (25 × 101 × 152 mm) at a height of 3.5 cm for differentiated podocyte and 2.5 cm for MDCK cells for 0–60 min. The manufacturer's value for the magnetization is 3.1×10^5 A/m. In the experiments utilizing chemical inhibitors, the equilibrated media contained the inhibitors or vehicle at the concentrations indicated. In the experiments with C_3 exoenzyme treatment, 100 ng/ml C_3 exoenzyme was added to the medium during the radiolabeling period. The radiolabeled metabolitic product in the media was collected, centrifuged at 12,000 rpm for 5 min, and measured by liquid scintillation spectrometry. The [^{3}H]choline metabolites in the media were evaporated, resuspended in 50% ethanol, and resolved using thin-layer chromatography (TLC) to identify [^{3}H]choline. [^{3}H]choline was identified by comigration with a commercial standard in a solvent system containing methanol-0.9% NaCl-ammonium hydroxide (50:50:5, vol/vol) (21, 54). The standard was visualized with iodine vapor, and the area corresponding to choline was scraped into scintillation vials and quantitated with liquid scintillation spectrometry.

Immunoblotting. The MDCK cells that stably express vector, HA-p115Rho-GEF-RGS, or Myc-RGS4 were harvested in SDS-PAGE loading buffer and boiled for 5 min. The samples were subjected to 11% SDS-PAGE and processed for immunoblotting with the appropriate antibodies. To determine actin and Rho translocation to at the magnetic bead loci, the bead/membrane complexes from differentiated podocytes and MDCK cells were isolated at 4°C using a side-pull magnetic-isolation apparatus (Dynal-MPC, Dynal, Lake Placid, NY) (47). The bead-free supernatant was collected, and the bead/membrane complex was washed once with 1× PBS and resuspended in SDS-PAGE loading buffer without bromophenol blue. The protein concentration of the samples was determined using bicinchoninic acid with BSA as a standard. Equal amounts of protein were subjected to 11% SDS-PAGE, processed for immunoblotting using anti-RhoA, anti-α-actin, or anti-total ERK antibody, and visualized with enhanced chemiluminescence. For quantitative measurement, the Rho-specific band on the immunoblot films from three individual experiments was scanned and the optical density was measured using Scion Image software. In experiments to determine the effect of different inhibitors on phospho-ERK activation, MDCK cells were cultured to 75–90% confluence in 12-well plates. Cells were incubated in media containing 1% FBS-DMEM for 18–20 h, then equilibrated for 1 h in the presence or absence of the inhibitors at the concentrations indicated. After removal of media, cells were washed with 1× PBS and then loaded with fibronectin-coated ferric oxide beads as described above. Magnetic force was applied for 30 min. The MDCK cells were harvested in SDS-PAGE loading buffer and boiled for 5 min. The protein concentration of the samples was determined. Equal amounts of protein were subjected to 11% SDS-PAGE and processed for immunoblotting with the phospho-ERK antibody.

F-actin staining. MDCK cells were plated in 100-mm dishes containing glass coverslips and grown to 60–85% confluence. After serum deprivation for 18–20 h, the cells were washed with 1× PBS, loaded with fibronectin-coated ferric oxide beads at 37°C for 10 min, and washed 3× with DMEM to remove unbound beads. Magnetic force was applied with a ceramic magnet for 0–60 min. The coverslips were washed with ice-cold 1× PBS, fixed in acetone for 10 min, and permeabilized with 0.1% saponin for 10 min. The cells were stained with Texas red X-phalloidin. The samples were visualized using a Zeiss fluorescence microscope (model LSM-5, Pascal), and imaged using the Axiovert 200 program (Zeiss).

Cell-free PLD activity assay. To determine PLD activity in the bead/membrane complexes using a cell-free assay, MDCK cells were cultured in 100-mm dishes and grown to 85% confluence. The cultures

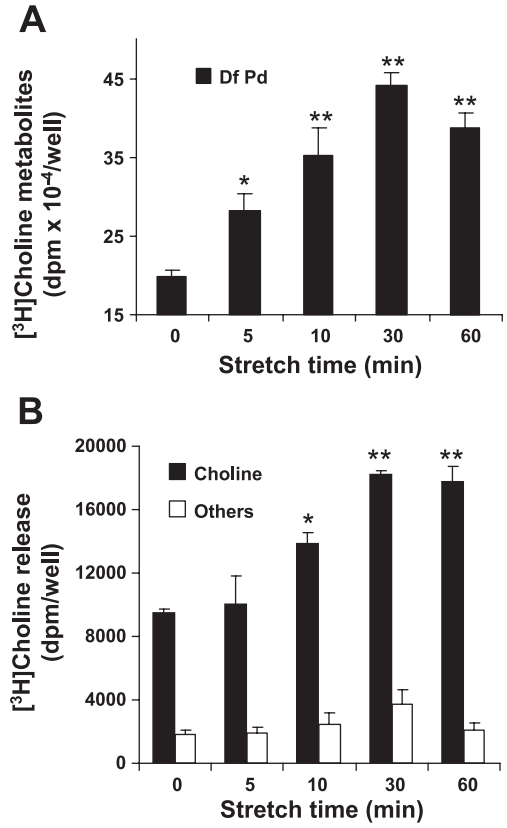

Fig. 1. Time course of stretch-induced [^{3}H]choline release in differentiated podocytes (Df Pd). The differentiated podocytes were prelabeled with ~1 μCi/ml [^{3}H]choline chloride of 1% FBS-RPMI overnight. Fibronectin-coated ferric oxide beads were loaded onto cells, the cultures were incubated in RPMI for 1 h, and were then exposed to magnetic force for 0–60 min. The media containing [^{3}H]choline metabolites were collected and quantitated using liquid scintillation counting (*A*). The media were evaporated, and [^{3}H]choline as an index of PLD activity was resolved by thin-layer chromatography and identified by comigration with a commercial standard. The standard was visualized with iodine vapor, and the area corresponding to choline (choline) and all other areas (others) were scraped into scintillation vials and quantitated with liquid scintillation spectrometry (*B*). The data represent the average of 3 experiments performed with triplicate samples. dpm, Disintegrations/min. The values with mechanical force were statistically different from that without force. $*P < 0.05$. $**P < 0.01$.

were starved for 18–20 h in 1% FBS-DMEM, loaded with fibronectin-coated ferric oxide beads as described above, and then exposed to magnetic force for 0–60 min. Cells were harvested with 0.75 ml of HME buffer containing 10 mM HEPES, pH 7.5, 2 mM $MgCl_2$ and 1 mM EDTA with protease inhibitors, and homogenized with 40 strokes of a Dounce homogenizer. The bead/membrane complexes were isolated as described earlier, and the samples were resuspended in 100 μl of buffer containing 20 mM HEPES, 154 mM NaCl, 0.05% Triton X-100, and 1.25 mM EDTA, pH 8.0. The amount of cellular protein was determined. Purified [^{3}H]choline-phosphatidylcholine was prepared as described earlier (21). Samples containing 50 μg of cellular protein and 200,000 dpm [^{3}H]choline-phosphatidylcholine in 100 μl final volume were incubated at 37°C for 40 min. The reaction was terminated with 0.55 ml methanol/1% HCl and extracted by adding 0.55 ml chloroform and 0.5 ml water. The aqueous phase containing [^{3}H]choline was quantitated with liquid scintillation spectrometry.

Data analysis. The data were analyzed for significance using one-way repeated measures of ANOVA followed by Tukey's test for comparisons between the experimental groups shown in the figures. The data represent means of three or more experiments performed with duplicate or triplicate samples.

RESULTS

Mechanical force activates PLD in renal epithelial cells. PLD hydrolyzes glycerophospholipids such as phosphatidylcholine to produce phosphatidic acid and choline. To evaluate stretch-induced PLD activity in renal epithelial cells, we prelabeled differentiated podocytes with ~1 μCi/ml [^{3}H]choline

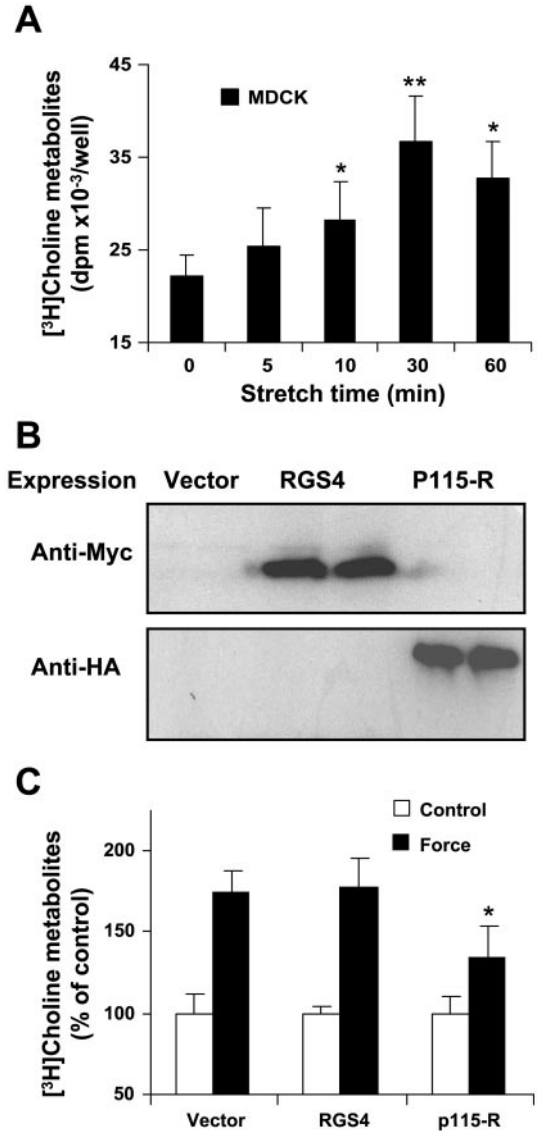

Fig. 2. Effect of RGS4 and p115-RGS on stretch-induced [^{3}H]choline metabolite release in Madin-Darby canine kidney (MDCK) cells. *A*: MDCK cells were prelabeled with ~1 μCi/ml [^{3}H]choline chloride, loaded with fibronectin-coated ferric oxide beads, and exposed to magnetic force for 0–60 min. The [^{3}H]choline metabolites were measured. The data represent the average of 3 experiments performed with triplicate samples. The values with mechanical force were statistically different from that without force. $*P < 0.05$. $**P < 0.01$. *B*: MDCK cells that stably overexpress RGS4, p115-RGS (P115-R), or vector alone were lysed with loading buffer. Duplicate samples were processed for immunoblotting using the anti-Myc (A-14) or anti-HA antibody, respectively. *C*: cells were prelabeled with ~1 μCi/ml [^{3}H]choline chloride overnight and then coated with iron beads and exposed to magnetic force for 30 min. The release of [^{3}H]choline metabolites was measured. The results represent the average of 4 experiments performed with duplicate or triplicate samples in 2 different clones. The values with mechanical force in MDCK cells were statistically different from that in p115-RGS-expressing cells. $*P < 0.05$.

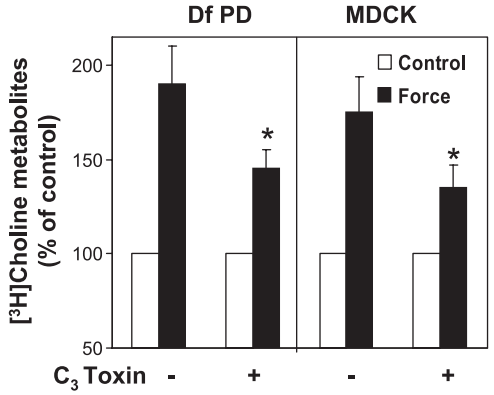

Fig. 3. Effect of C_3 exoenzyme on stretch-stimulated PLD activity in differentiated podocytes and MDCK cells. The differentiated podocytes and MDCK cells were prelabeled with 1 μCi/ml [^{3}H]choline overnight in the presence or absence of 100 ng/ml of C_3 exoenzyme. The cells were coated with ferric oxide beads and then exposed to magnetic force for 30 min. The PLD products in the media were collected and measured using liquid scintillation spectrometry. The data represent means of 3 experiments performed with duplicate samples. The values with C_3 exoenzyme were statistically different from that without C_3 exoenzyme. *$P < 0.05$.

chloride in 1 ml of 1% FBS-RPMI for 18–20 h. The cells were then loaded with fibronectin-coated ferric oxide beads. After removal of unattached beads, the cultures were incubated in fresh RPMI for 1 h and exposed to magnetic force for 0–60 min. Figure 1 shows a time-dependent increase in [^{3}H]choline metabolite release from differentiated podocytes (Fig. 1*A*). Using TLC to analyze [^{3}H]choline metabolites, we found that the major product, [^{3}H]choline, as an index of PLD activity, showed the same time-dependent pattern (Fig. 1*B*). This result demonstrates PLD activation by stretch in renal epithelial cells.

Effect of RGS proteins on stretch-induced PLD activation. Cells that respond to mechanical force can simultaneously activate multiple second messenger systems (49). To determine whether members of the heterotrimeric G protein family are involved in mechanical force-induced PLD activation, we employed MDCK cells that stably express vector alone or RGS proteins (RGS4 or p115-RGS). MDCK cells were used because both MDCK cells and podocytes are renal epithelial cells, their signaling pathways may be similar, and podocytes are very difficult to transfect so that is difficult to use them for expression of interfering proteins. Figure 2*A* shows a similar time course of stretch-induced PLD activation in MDCK cells. RGS4 is a GTPase-activating protein for $G\alpha_i$ and $G\alpha_q$ (19), whereas p115RhoGEF functions as a GTPase-activating protein for $G\alpha_{12/13}$ and a guanine nucleotide exchange factor for the small G protein Rho (17, 27). The p115RhoGEF construct we used, p115-RGS (amino acids 1–252), contains the RGS domain and attenuates $G\alpha_{12/13}$ signaling (27, 56). RGS protein expression was documented by immunoblotting using an anti-Myc antibody for RGS4-Myc or anti-HA antibody for HA-p115-RGS (Fig. 2*B*). To assess the effect of RGS proteins on stretch-induced PLD activation, the stable MDCK cells were prelabeled with 1 μCi/ml [^{3}H]choline chloride overnight, loaded with ferric oxide beads, and then exposed to magnetic force for 30 min. Cells that stably overexpress p115-RGS showed significantly diminished stretch-stimulated PLD activity (~50% inhibition), whereas RGS4-expressing cells were not affected (Fig. 2*C*). These results demonstrate that mechanical force stimulates PLD activity via a $G\alpha_{12/13}$-dependent signaling pathway.

Effect of C_3 exoenzyme on stretch-stimulated PLD activity. $G\alpha_{12/13}$ is an upstream mediator of agonist-induced Rho activation (52). In addition, Rho has been linked to the activation of PLD in vitro by addition of recombinant RhoA and in intact cells by hormone stimulation (11, 36, 37, 39). To determine whether Rho is involved in stretch-induced PLD activation in renal epithelial cells, we treated differentiated podocytes and MDCK cells with C_3, the exoenzyme of *Clostridium botulinum*

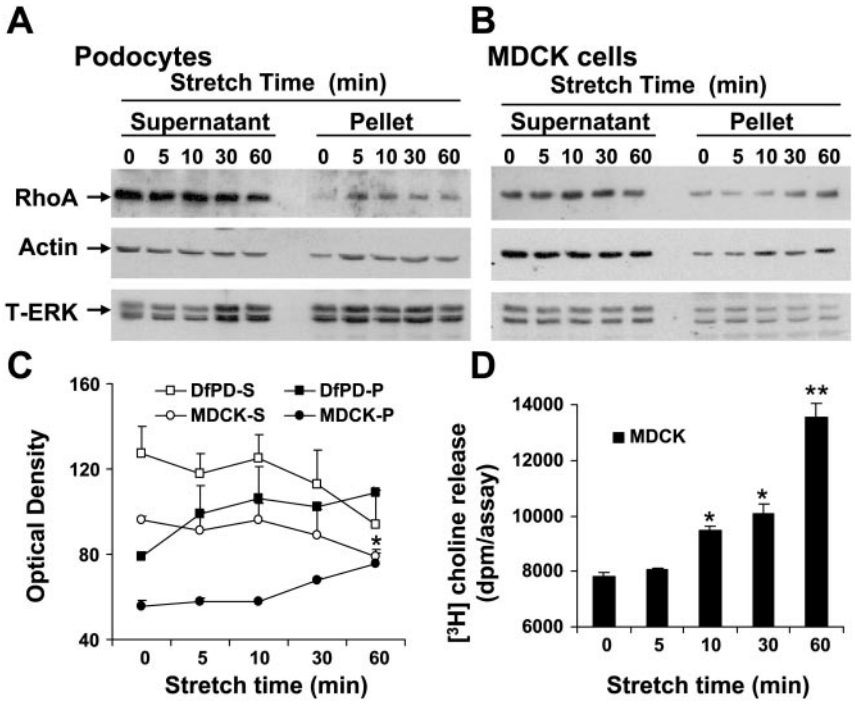

Fig. 4. Stretch-induced increases in RhoA and actin content at bead/membrane locus in differentiated podocytes and MDCK cells. The differentiated podocytes (*A*) and MDCK cells (*B*) were cultured in 100-mm dishes to 85% confluence. The cells were coated with iron beads and exposed to magnetic force for 0–60 min. Cells were harvested in 0.75 ml of HME buffer (10 mM HEPES, pH 7.5, 2 mM $MgCl_2$ and 1 mM EDTA with protease inhibitors) and then homogenized. The bead/membrane complex was separated using a Dynal-MPC. Equal amounts of cellular proteins were subjected to 11% SDS-PAGE, and the samples (bead-free supernatant and bead/membrane complex) were processed for immunoblotting using an anti-RhoA, an anti-α-actin, or an anti-total ERK (T-ERK) antibody. A representative blot from 3 experiments is shown. *C*: Rho-specific bands on the immunoblot films taken from 3 individual experiments were scanned, and the optical density was measured using Scion Image software. DfPD-S, differentiated podocyte supernatant; DfPD-P, differentiated podocyte pellet; MDCK-S, MDCK cell supernatant; and MDCK-P, MDCK cell pellet. *D*: PLD activity was analyzed in a cell-free assay system using [^{3}H]choline-phosphatidylcholine as the substrate and the bead/membrane complex as the enzyme source at 37°C for 40 min. The reaction was terminated, and the hydrolytic products were quantitated using liquid scintillation counting. The data represent means of 4 separate experiments performed with duplicate samples. The values with mechanical force were statistically different from that without force. *$P < 0.05$. **$P < 0.01$.

that ADP ribosylates asparagine-41 of RhoA and inhibits its function by preventing interaction with downstream effectors (1, 52). As shown in Fig. 3, C_3 exoenzyme inhibited ~50% of [^{3}H]choline metabolite release in both differentiated podocytes and MDCK cells. The blockade of C_3 exoenzyme indicates that in renal epithelial cells, stretch-induced PLD activation is partially $G\alpha_{12/13}$-Rho dependent.

Stretch activates Rho and increases F-actin in bead loci. The activation of Rho requires its translocation from cytosol to membrane (13, 20). Rho activation leads to actin polymerization and the formation of stress fibers in many cells (52). Mechanical force induces Rho activation and actin assembly and changes its distribution in a manner that can rapidly reinforce Rho and cytoskeletal linkages at force application sites (10, 24, 49). Actin has been shown in both intact cells and cell-free systems to modulate PLD activity in an isoform-specific manner: G-actin inhibits and F-actin stimulates PLD activity (33, 35). The next series of experiments was conducted to determine whether mechanical force induces Rho activation, actin polymerization, and stress fiber formation and whether F-actin stimulates PLD activity. Differentiated podocytes and MDCK cells were cultured in 100-mm dishes, loaded with ferric oxide beads, and then exposed to magnetic force for 0–60 min. Equal amounts of protein from the bead/membrane complexes and bead-free supernatant were processed for immunoblotting using antibodies against α-actin and RhoA. Total ERK1/2 was used as a protein loading control. In differentiated podocytes and MDCK cells, the contents of both RhoA and actin increased at the magnetic bead loci and decreased in the bead-free supernatant (Fig. 4, *A* and *B*). Figure 4*C* shows the measurement of RhoA translocation in differentiated podocytes and MDCK cells. To determine the relationship of the change in the actin cytoskeleton to PLD activation, we also analyzed PLD activity in a cell-free system. In the differentiated podocytes, the bead/membrane complexes contain very little protein (~10 μg/100-mm dish), making it difficult to determine PLD activity using the cell-free assay. To avoid this limitation, we again chose MDCK cells. Equal amounts of protein from the bead/membrane complex were processed for the cell-free PLD activity assay using [^{3}H]choline-phosphatidylcholine as a substrate. Figure 4*D* depicts a time-dependent increase in PLD activity, measured as [^{3}H]choline production. The results show parallel increases in stretch-induced actin content and PLD activity in the cell-free system and indicate that PLD activation by mechanical force is associated with $G\alpha_{12/13}$-Rho activation and actin accumulation in renal epithelial cells. To demonstrate that mechanical force induces actin polymerization and stress fiber formation, MDCK cells were plated in 100-mm dishes with two coverslips, loaded with ferric oxide beads, and stretched for 0–60 min. The coverslips were stained with Texas red X-phalloidin for selectively labeling F-actin. As shown in Fig. 5, mechanical force increased Texas red-X-phalloidin staining and stress fiber formation at the magnetic bead loci at both 5 (Fig. 5*B*) and 10 min (Fig. 5*C*).

Effect of chemical inhibitors on stretch-induced PLD activation. The signaling pathway involving $G\alpha_{12/13}$, Rho, and F-actin does not completely account for stretch-induced PLD activation. Mechanical force-induced signals include the activation of multiple pathways, i.e., ERK, tyrosine kinases, protein kinase C, and PLC, that results in the elevation of intracellular Ca^{2+} (49). Some of these proteins have been impli-

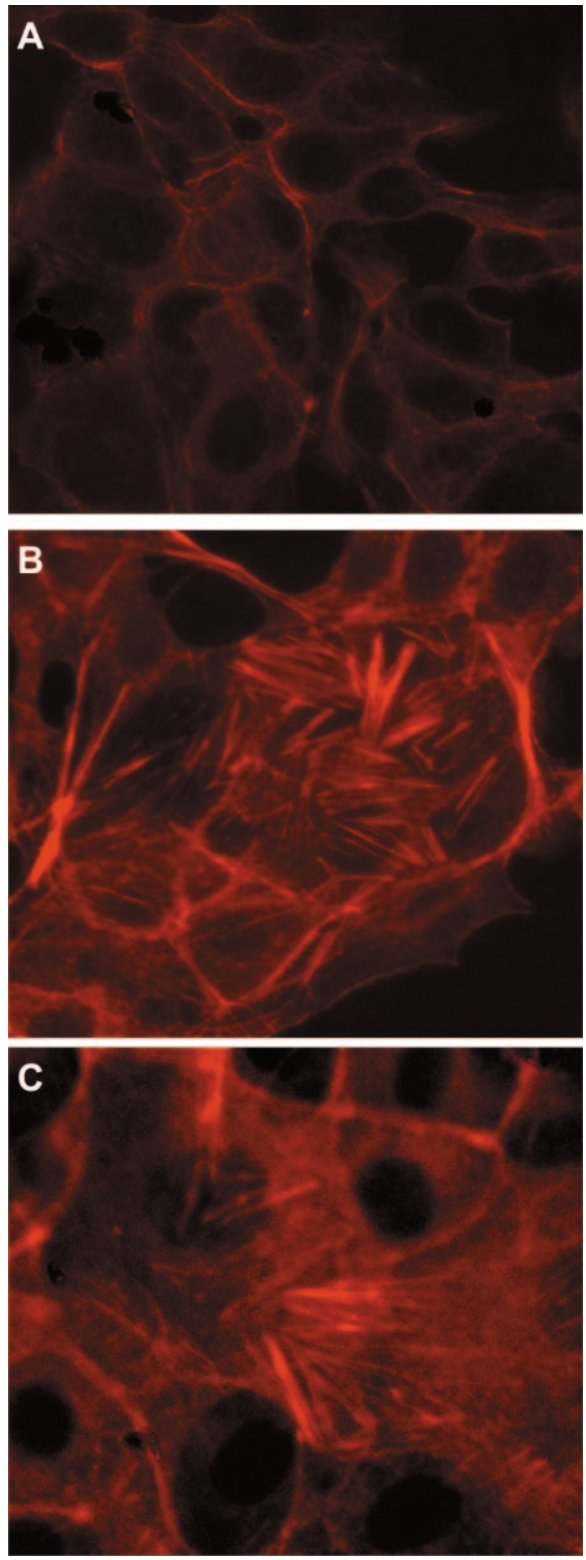

Fig. 5. Effect of stretch on the formation of actin stress fibers in MDCK cells. MDCK cells were plated on coverslips and grown to 40–50% confluence. After equilibration in serum-free DMEM for 18 h, cells were coated with ferric oxide beads and exposed to magnetic force for 0–10 min. The coverslips were fixed and stained with Texas red-phalloidin. *A*: no force. *B*: force for 5 min. *C*: force for 10 min.

cated in the regulation of PLD activity (11, 36, 39). To determine whether other signaling pathway(s) may be involved in stretch-induced PLD activation, differentiated podocytes and MDCK cells were pretreated with 30 μM PD-98059 (an MEK inhibitor that blocks the phosphorylation of p42/p44 ERK), 10 μM Ro-318220 (a calmodulin-dependent kinase inhibitor), and 1 μM AG-1478 (a tyrosine kinase inhibitor) for 1 h followed by 30 min of force application. PLD activity was measured as the formation of [^{3}H]choline metabolites. The data show that only Ro-318220 partially inhibits stretch-induced PLD activity in differentiated podocytes (Fig. 6*A*) and MDCK cells (Fig. 6*C*). To confirm that the inhibitors are effective for their respective kinases, we analyzed the cells from the same experiments for phospho-ERK activity. Figure 6*B* shows the Ponceau S staining for protein loading and immunoblotting with the anti-phospho-ERK antibody to document inhibitor effect. Both PD-98059 and AG-1478 inhibited activation of p42/44 ERK, whereas Ro-318220 had no effect. These data demonstrate that calmodulin-dependent kinase participates in stretch-induced PLD activation but that ERK and tyrosine kinases inhibited by AG-1478 do not.

To confirm the effect of calmodulin-dependent kinase on stretch-induced PLD activation, we employed another calmodulin-dependent kinase inhibitor (KN93) to verify the signaling pathway in both cell lines. KN92 is a negative control compound for KN93. Differentiated podocytes and MDCK cells were prelabeled with [^{3}H]choline overnight, loaded with ferric oxide beads, pretreated with 15 μM KN92 or KN93 for 1 h, and then exposed to magnetic force for 30 min. Like Ro-318220, KN93 also partially inhibited stretch-induced PLD activation in both differentiated podocytes and MDCK cells, whereas the negative control (KN92) had no effect (Fig. 7*A*). Our results suggest that two different signaling pathways are involved in stretch-induced PLD activation in renal epithelial cells: one involving $G\alpha_{12/13}$, Rho, and F-actin and the other involving calmodulin-dependent kinase. We next sought to determine whether $G\alpha_{12/13}$-Rho and calmodulin-dependent kinase operate in the same pathway, or in independent pathways, to activate PLD in response to mechanical force. Figure 7*B* shows that either C_3 exoenzyme or KN93 can partially inhibit stretch-induced PLD activation, whereas the combination results in a significantly greater reduction in PLD activity, suggesting that the pathways are parallel.

DISCUSSION

Stretch-induced architectural and biochemical changes in cardiomyocytes and other cells have been widely investigated (10, 24, 49). The phenomenon has been extended to renal epithelial cells such as glomerular podocytes, which are also exposed to mechanical force in vivo that arises from blood pressure and fluid flow. One recent study showed that biaxial cyclic stress induced a unique reorganization of the actin cytoskeleton in podocytes via a Rho-dependent pathway that resulted in the formation of radial stress fibers and an actin-rich center (8). However, the signaling cascades regulating actin cytoskeleton reorganization in response to stretch have yet to be fully defined. To study stretch-induced signaling pathways, we chose PLD, a recognized downstream effector Rho, the small GTPase (11, 36) and an important enzyme in cell signaling that hydrolyzes phosphatidylcholine to produce choline and phosphatidic acid. Using a whole cell model in which cells are prelabeled with a radioactive lipid precursor, coated with ferric oxide beads, exposed to magnetic force, and deter-

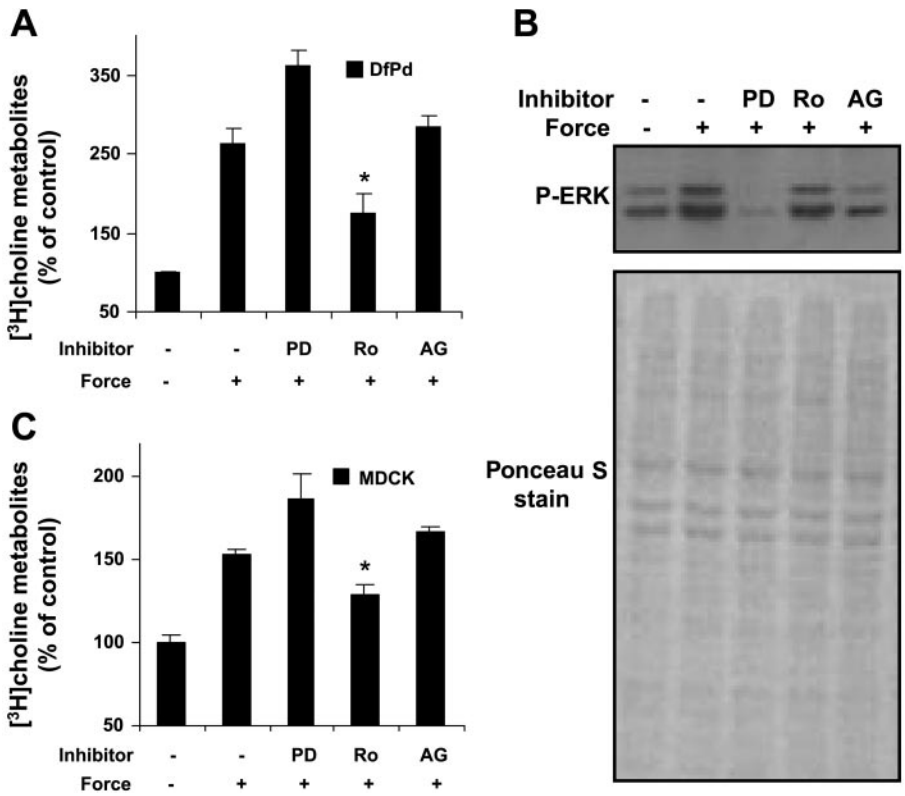

Fig. 6. Effect of kinase inhibitor on stretch-induced PLD activation in differentiated podocytes and MDCK cells. The differentiated podocytes (*A*) and MDCK cells (*C*) were cultured on 6-well plates and prelabeled with 1 μCi/ml [^{3}H]choline chloride for 18 h. Cells were coated with iron beads, pretreated with 30 μM PD-98059 (PD), 10 μM Ro-318220 (Ro), or 1 μM AG-1478 (AG) for 1 h, and exposed to magnetic force for 30 min. The release of [^{3}H]choline metabolites was measured. The data represent means of 3 experiments performed with triplicate samples. *B*: MDCK cells were coated with iron beads, pretreated with 30 μM PD-98059, 10 μM Ro-318220, or 1 μM AG-1478 for 1 h, and exposed to magnetic force for 30 min. The samples were harvested with HME buffer and homogenized. The bead/membrane complex was separated using a Dynal-MPC as described. Equal amounts of cellular proteins were subjected to 11% SDS-PAGE and processed for immunoblotting using an anti-phospho-ERK antibody. The blot shown is representative of 3 similar blots. *Top*: phospho-ERK blot. *Bottom*: Ponceau S stain of the cellulose membrane. Values for Ro-318220 were statistically different from that without Ro-318220 (*$P < 0.05$).

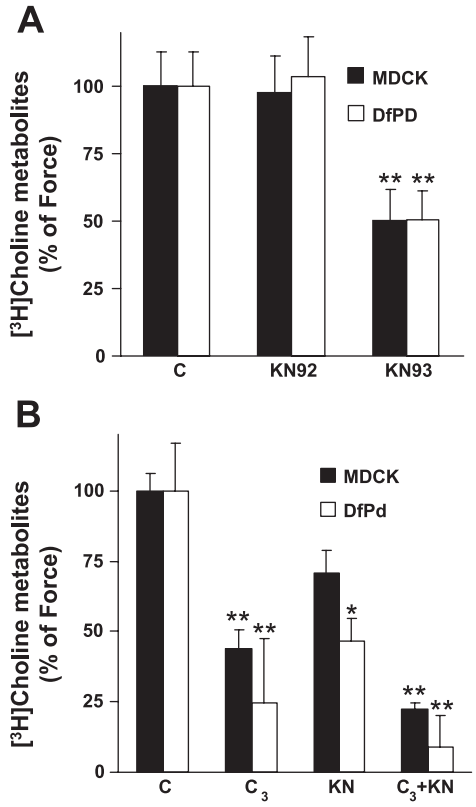

Fig. 7. Effect of C_3 exoenzyme and KN93 on stretch-induced PLD activation in differentiated podocytes and MDCK cells. MDCK cells and differentiated podocytes were prelabeled with 1 μCi/ml [^{3}H]choline chloride in the presence or absence of 100 ng/ml of C_3 exoenzyme (C_3) for 18 h. The cells were coated with iron beads, equilibrated with or without either 15 μM KN92 or KN93 for 1 h (*A*), equilibrated with or without 15 μM KN93 (KN) for 1 h and pretreated with C_3 exoenzyme and KN93 (C_3/KN; *B*), and exposed to magnetic force for 30 min. The radiolabeled products in the media were collected and measured using liquid scintillation spectrometry. PLD activity was measured as the formation of [^{3}H]choline metabolites. The data represent means of 3 experiments performed with triplicate samples. Values with inhibitors were statistically different from that without inhibitors (*$P < 0.05$, **$P < 0.01$).

mined [^{3}H]choline release is used as a index of PLD activity, we have demonstrated that mechanical force stimulates two signaling pathways to activate PLD through *1*) activating $G\alpha_{12/13}$ and Rho and increasing F-actin content and *2*) stimulating calmodulin-dependent kinase by presumably increasing intracellular Ca^{2+}. To our knowledge, this is the first example of PLD activation in response to mechanical force and of stretch-induced signaling pathways that activate PLD in renal epithelial cells.

In glomerular hyperfiltration, podocytes may experience mechanical force in a number of ways. They may experience lateral stretch as a capillary dilates, they may experience force perpendicular to the plane of the basement membrane (vertical traction) as they balloon in response to increased filtrate flow, or they may experience shear force from increased filtrate flow. We chose to study vertical traction because it could be applied for a discrete time period, a feature that we believed would be valuable in studying signaling cascades. Our experimental system differs in this respect from that used by other investigators who have used cyclic biaxial stretch. However, stretch in both systems has similar effects on the cells, causing traction on the cytoskeleton and membrane, increased Ca^{2+} entry, altered cytoskeletal structure, and, as we have shown here, activation of Rho (42, 43). Although fibronectin through ferric beads can be bound to its receptor and plays an important role in cellular signal transduction through integrins, we measure that PLD activity in the cells under the same bead loading and washing is only in response to stretch.

PLD can catalyze the hydrolysis of phospholipids to generate phosphatidic acid and related head groups and a transphosphatidylation reaction in which the phosphatidyl group of phospholipids is transferred to appropriate nucleophiles. Our method for stretching cells involves loading fibronectin-coated ferric oxide beads on cell surfaces and exposing the cells to magnetic force. Although PLD activity can be measured more specifically as transphosphatidylation in the presence of alcohol, the heavy ferric oxide beads make it difficult to extract total lipids. Nevertheless, we and others have found that measuring [^{3}H]choline release or transphosphatidylation as a mark of PLD activity provides similar results in hormone-stimulated PLD activation in MDCK and other cell types (18, 26). Consequently, [^{3}H]choline release also indicates that mechanical force induces the hydrolysis of phosphatidylcholine.

The formation of actin stress fibers in response to mechanical force has previously been demonstrated in numerous cell types, including cardiac myocytes, fibroblasts, and endothelial cells (10, 22, 24, 49, 57). Concerning glomerular cells, actin cytoskeletal reorganization in response to stretch has also been demonstrated in mesangial cells (16) and podocytes (8). Notably, there is no information on how mechanical force regulates dynamic rearrangement of the actin cytoskeleton. Using the bead-magnet technique, we demonstrate that mechanical force leads to a significant increase in RhoA and actin translocation in the bead/membrane complexes in both differentiated podocytes and MDCK cells. Measuring stretch-induced PLD activation in RGS protein-expressing MDCK cells, we demonstrated that mechanical force through some mechanosensor stimulates $G\alpha_{12/13}$ and RhoA, a downstream target of $G\alpha_{12/13}$ (17). Rho activation leads fibroblasts to form actin stress fibers (52) and is also capable of binding to and activating PLD (6, 37). The involvement of Rho in this signaling pathway is demonstrated by RhoA translocation and inhibition of stretch-induced PLD activity in differentiated podocytes and MDCK cells pretreated with C_3 exoenzyme, which specifically ADP ribosylates Rho (not Rac or Cdc42). The coordination of actin and PLD is of critical importance in cellular functions, including cell proliferation, migration, vesicle trafficking, and secretion (11, 24, 36, 49). At the cellular level, mechanical forces induce actin cytoskeleton reorganization, which leads to activation of multiple signaling pathways. Kusner et al. (32, 33) recently showed that F-actin stimulated PLD activity in both intact cells and cell-free assay systems. Our data show that the parallel increase in PLD activity in the cell-free assay and increase in actin content in the bead/membrane complex implicates the role of the actin cytoskeleton in PLD activation. To determine whether the increase in actin in the bead/membrane complexes involves F-actin, stretched MDCK cells were stained with Texas red-phalloidin, which can selectively label F-actin. The results indicate that F-actin is recruited to stretch sites and that it may polymerize at the magnetic bead loci in MDCK cells during force application (Fig. 5). Similar experiments were not performed in differentiated podocytes due to the large amount of actin fibers under basal conditions. Taken

together, our data demonstrate that mechanical force stimulates $G\alpha_{12/13}$ and Rho activation, that active Rho regulates cell shape changes through effects on the actin cytoskeleton, and that increasing F-actin stimulates PLD activation to hydrolyze phospholipids and generate second messenger molecules.

Mechanical effects can also activate many other signaling pathways including ion channels, ERK, and tyrosine kinases (10, 24, 49). As a result of stretch-stimulated ion channels, cells can rapidly increase intracellular Ca^{2+} (3). Calmodulin is a ubiquitous and highly conserved Ca^{2+} sensor. Increases in intracellular Ca^{2+} can activate calmodulin-dependent kinase, which associates with carbachol-induced PLD activation in Chinese hamster ovary cells (22, 40). In human airway epithelial cells, genistein (a tyrosine kinase inhibitor) abolishes both agonist-induced PLD activation and actin stress fiber formation (48). Vanadate, an inhibitor of tyrosine phosphatases, has also been shown to stimulate PLD activity in other cell types (5). In addition, ERK, which can activate PLD_2 in PC12 cells (55), was shown to be activated by cyclic stretch in mesangial cells (28). Both human PLD_1 and PLD_2 can be regulated by calcium and protein kinase C in Sf9 cells (51). Pretreating differentiated podocytes and MDCK cells with several specific kinase inhibitors, we found that only the calmodulin-dependent kinase inhibitors Ro-318220 and KN93 significantly reduced stretch-induced PLD activity (Figs. 6 and 7). Our data indicate that calmodulin-dependent kinase is also involved in stretch-activated PLD. This signaling pathway is very complex and will be further studied.

Glomerular podocytes experience mechanical force as a consequence of the hydrostatic pressure in glomerular capillaries. In renal diseases with reduced nephron mass, the pressure in capillaries, particularly the regions closest to the afferent arteriole, is increased, the capillaries dilate, the podocytes stretch, become injured, ultimately may die, and the glomerulus undergoes a sclerotic process. The mechanism by which physical signals are converted into biochemical signals that ultimately govern cell responses is only beginning to be defined. Two lines of evidence indicate that Rho is very important in this process. Biaxial stretch induces actin cytoskeletal rearrangement via the small G protein Rho, and Rho-GDP dissociation inhibitor knockout mice develop glomerulosclerosis (8, 53). The current studies demonstrate that mechanical force stimulates Rho to activate PLD and to produce stretch-induced second messengers: PA, diacylglycerol, and lysophosphatidic acid. Rho activation and the formation of these second messengers may contribute to glomerular injury by a variety of mechanisms, including stimulation of superoxide production, alterations in cytoskeletal structure, and ultimately by alterations in gene expression (24, 38, 53).

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