Hypertonic Stress Increases Phosphatidylinositol 4,5-Bisphosphate Levels by Activating PIP5KIβ

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Hyperosmotic stress increases phosphoinositide levels, reorganizes the actin cytoskeleton, and induces multiple acute and adaptive physiological responses. Here we showed that phosphatidylinositol 4,5-bisphosphate (PIP2) level increased rapidly in HeLa cells during hypertonic treatment. Depletion of the human type I phosphatidylinositol 4-phosphate 5-kinase β isoform (PIP5KIβ) by RNA interference impaired both the PIP2 and actin cytoskeletal responses. PIP5KIβ was recruited to membranes and was activated by hypertonic stress through Ser/Thr dephosphorylation. Calycin A, a protein phosphatase 1 inhibitor, blocked the hypertonicity-induced PIP5KIβ dephosphorylation/activation as well as PIP2 increase in cells. Urea, which raises osmolarity without inducing cell shrinkage, did not promote dephosphorylation nor increase PIP2 levels. Disruption or stabilization of the actin cytoskeleton, or inhibition of the Rho kinase, did not block the PIP2 increase nor PIP5KIβ dephosphorylation. Therefore, PIP5KIβ is dephosphorylated in a volume-dependent manner by a calycin A-sensitive protein phosphatase, which is activated upstream of actin remodeling and independently of Rho kinase activation. Our results establish a cause-and-effect relation between PIP5KIβ dephosphorylation, lipid kinase activation, and PIP2 increase in cells. This PIP2 increase can orchestrate multiple downstream responses, including the reorganization of the actin cytoskeleton.

All cells experience fluctuations in osmolarity. Unicellular organisms and plants continuously confront osmotic challenges in their environment. In higher animals, the kidney and the gastrointestinal system are routinely exposed to severe osmotic fluctuation, while the majority of cells in other organs are confronted with transient osmolarity variations due to changes in the transmembrane transport of solutes or shifts in the balance between low molecular weight precursors and their macromolecular products. Recently, there has been a renewed interest in understanding the mechanism of hypertonic response in the clinical arena (1), due to the discovery that treatments using hypertonic resuscitation in experimental models of trauma, hemorrhagic shock, sepsis, and burn injury are more beneficial than conventional isotonic resuscitation (2, 3). While the fundamental mechanism for such protection is not completely understood, the actin cytoskeleton, which is reorganized during hypertonic stress, has been implicated (3, 4).

Actin remodeling as well as many of the other hyperosmotic responses are evolutionary conserved. These include large shifts in phosphoinositide metabolism, activation of the mitogen-activated protein and tyrosine kinase pathways, volume regulation and the reprogramming of gene transcription (3, 5).

Phosphatidylinositol 4-phosphate (PIP4P)4 and phosphatidylinositol 4,5-bisphosphate (PIP2) levels increase dramatically in mammalian cardiac muscle and tissue culture cells that were exposed to hypertonic sucrose or NaCl (6). Other phosphoinositides, including phosphatidylinositol 3,5-bisphosphate (7, 8), phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate are increased in some other types of cells as well (9).

Hyperosmotic stress acutely induces cell shrinkage, which is subsequently corrected by volume regulation. The response cascade can be classified into four mechanistic components: volume sensing, signal generation, signal transduction, and effector activation (10). Given that PIP2 is a multifunctional regulator and adaptor, the PIP2 increase may coordinate many of the responses to hypertonic stress. Nevertheless, the role of PIP2 in the response hierarchy, and the mechanism for PIP2 increase, have yet to be elucidated.

PIP2 levels are maintained by a dynamic balance between PIP2 synthesis and degradation. PIP2 is synthesized primarily by the type I PIP5Ks, which phosphorylate PIP4P on the D5 position of the inositol ring. PIP2 is degraded primarily by phosphoinositide phosphatases and by PI-phospholipase Cs. Three major PIP5K isoforms, called α, β, and γ, have been identified (11). Additionally, the γ isoform is differentially spliced to generate several variants (12, 13).

PIP5KIs are predominantly cytosolic proteins that are recruited to plasma and organelle membranes through associ-
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Despite the large cadre of potential regulatory mechanisms, nothing is known about the regulation of the PIP5KIs by hypertonic stress. In this paper, we have identified the hypertonic stress regulated PIP5KI and examined the relation between its activation by Ser/Thr dephosphorylation to the hypertonicity-induced increase in PIP$_2$ production and actin remodeling.

**EXPERIMENTAL PROCEDURES**

**PIPKI Overexpression**—We use the isoform designation for human PIP5KIs, which is different from that for mouse (30). Hemagglutinin (HA)-tagged PIP5KI$\alpha$-b, -γ87, and -γ90 were cloned from cDNA provided by other laboratories. In some experiments, recombinant adenovirus vectors expressing β-galactosidase, HA-tagged human PIP5KIβ or myc-tagged human PIP5KIα were used. These were generated using the AdEasyTM adenoviral vector system (Stratagene) (23).

**Hypertonic Treatments**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 250 mM sucrose or 100 mM NaCl was added to the isotonic growth medium during hypertonic stimulation.

**TLC**—Cells were labeled for 4 h in phosphate-free Dulbecco’s modified Eagle’s medium and 40 μCi/ml $[^{32}$P]PO$_4$ and were then exposed to sucrose or NaCl. Samples were processed for TLC as described previously (23).

**High Performance Liquid Chromatography (HPLC)**—Lipids were extracted, deacylated, and analyzed on anion exchange HPLC columns. Negatively charged glycerol head groups were separated from the different types of phosphoinositides better than PI4P (Table 1). Therefore, hypertonic stress induced by these two stimuli preferentially increases PIP$_2$ level.

**RNA Interference**—The small interfering RNA (siRNA) sequences targeting the three human PIP5KI isoforms individually were as described previously (33, 34). Firefly luciferase siRNA (nucleotides 695–715) was used as a negative control.

**Immunoprecipitation**—HeLa cells overexpressing the epitope-tagged PIP5KI isoforms were lysed in buffer containing 25 mM HCl, pH 7.5, 0.15 M NaCl, 5 mM MgCl$_2$, 1 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 0.5% Nonidet P-40, and protease inhibitors. The overexpressed PIP5KIs were immunoprecipitated with monoclonal anti-epitope tag antibody bound to protein G-Sepharose. Anti-HA and anti-myc are from Convence.

**In Vitro PIP5KI Kinase Assay**—Lipid kinase activity was measured by phosphorylation of PI4P using $[^{32}$P]ATP as a phosphate donor. Sepharose G beads containing immunoprecipitated epitope-tagged PIP5KI were suspended in a solution containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 10 mM EDTA, 0.1 mM EGTA, 0.4% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 70 μM PI4P (Biomol Inc.) and 35 μM phosphatidylinerine (Avanti). Kinase assay was initiated by adding $[^{32}$P]ATP (1 μCi/50 μl reaction with a final concentration of 0.2 mM ATP, PerkinElmer Life Sciences), and reaction proceeded at room temperature for 15 min. The reactions were stopped by adding CHCl$_3$:MeOH:HCl, and lipids were extracted as described above and separated by TLC. Radioactivity associated with the PIP band that comigrates with a PI4P standard was quantitated by PhosphorImager analysis, and the amount of PIP5KI protein in the equivalent immunoprecipitate was determined by Western blotting. Kinase activity was normalized to the amount of immunoprecipitated protein and expressed as percent of the activity without sucrose stimulation. Under the conditions of our experiments, the kinase activity was linear from 5 to 30 min.

**Fluorescence Microscopy**—Immunofluorescence labeling was as described previously (34, 35). Anti-PI5P was from BioAssay Systems.

**Membrane Fractionation**—Cell homogenates were centrifuged at 100,000 × g to obtain the cytosol and membrane fractions (33). Organelles and organelle membranes were separated according to their density by a multistep centrifugation procedure (36). Samples obtained by centrifugation sequentially at 19,000 × g, 41,000 × g (low speed), and 180,000 × g (high speed) were analyzed. Plasma membrane enriched sample was obtained by floatation on a sucrose cushion. The pellet fractions were adjusted to 100 μl, and equal volumes of membrane fractions were analyzed by Western blotting with anti-HA antibody.

**Isolation of the Triton X-100-insoluble Actin Cytoskeleton**—Cells were lysed in Triton X-100 and centrifuged sequentially at low speed (15,900 × g) to collect cross-linked actin stress fibers and networks (low speed pellet) and at high speed (336,000 × g) to sediment long filaments that were not cross-linked (high speed pellet).

**RESULTS**

**Hypertonic Stress Increased the PIP$_2$ Level in Cells**—When HeLa cells were exposed to 250 mM sucrose for 10 min, the $[^{32}$P]PI$_4$P level increased to a greater extent than $[^{32}$P]PI4P (Fig. 1A and Table 1). Likewise, addition of 100 mM NaCl to normal culture medium also increased PIP$_2$ levels to a much higher extent than PI4P (Table 1). Therefore, hypertonic stress induced by these two stimuli preferentially increases PIP$_2$ level.

The increase in phosphoinositide levels was evident within 2 min of sucrose stimulation and reached a maximum by 10 min (Fig. 1A). While there was a subsequent slow decline, PIP$_2$ was still above prestimulation levels at 20 min. The rapid increase in $[^{32}$P]PI$_4$P and $[^{32}$P]PI4P suggests that the change in phosphoinositide turnover/biosynthesis is an early response to hypertonic stimulation.

HPLC, which measures the amount of each lipid and can separate the different types of phosphoinositides better than the TLC method (31), confirmed that there was a 2-fold increase in PIP$_2$ (Fig. 1B and Table 1). Unexpectedly, there was almost no change in PI4P mass, despite a modest increase in its labeling by $[^{32}$P (Fig. 1A). No new peak corresponding to either PI3P, PI(3,4)P$_2$, or PI(3,4,5)P$_3$ was detected (Fig. 1B). The response profile of the HeLa cell establishes that PIP$_2$ is increased selectively. This increase is most likely due to the direct activation of PIP5KIs and/or inactivation of PIP$_2$ phos-
phosphatases and is unlikely to be due to increased availability of the PI4P substrate.

The sites of PIP2 increase were identified by immunofluorescence microscopy. As shown previously (34), the PIP2 antibody stained small punctae that lined the plasma membrane (PM pool) and a perinuclear region (internal organelle pool) (Fig. 1C). Hypertonic treatment increased anti-PIP2 staining intensity, especially in large punctae that line the plasma membrane. Some of these punctae were at the tips of retraction fibers that were formed when the cell shrunk (see Fig. 2B).

**TABLE 1**

| Treatment     | Lipid mass | |
|---------------|------------|--|---|---|
|               | % of isotonic control | % of isotonic control | |
| 250 mM sucrose| 105 ± 2 (n = 4) | 93 ± 17 (n = 4) | 219 ± 33 (n = 4) | |
| 100 mM NaCl    | ND         | ND            | ND            | |

a From HPLC analyses.

b From PhosphorImager analyses of TLC data.

PI5KIs Depletion Blunted the Hypertonicity-induced PIP2 and Actin Responses—siRNA oligonucleotides were used to identify the PI5KI isoform that is primarily responsible for the hypertonic stress-induced PIP2 increase. As reported previously, PI5KIβ depletion by RNAi (33, 34) decreased the basal [32P]PIP2 level to the greatest extent compared with depletion of the other PI5KI isoforms (Fig. 2A). PI5KIβ completely blocked the hypertonicity-induced PIP2 increase, while depletion of the other PI5KIs had much less effect. These results showed that PI5KIβ accounts for most of the hypertonicity-induced PIP2 increase. We therefore focused on its behavior for the remainder of this paper.

HeLa cells normally have long actin stress fibers and cortical actin filaments. After hypertonic stimulation, the stress fibers became thicker, and retraction fibers were formed at the cell periphery as the cell shrunk (Fig. 2B). An increase in stress fibers and polymerized actin was confirmed biochemically by isolating the Triton X-100-insoluble cytoskeleton (Fig. 2C). The amount of actin in the Triton-X-100-insoluble low speed pellet (23), which contains cross-linked actin filaments such as stress fibers, and the Triton-insoluble high speed pellet, which contains long actin filaments that are not cross-linked sufficiently to be sedimented by centrifugation are increased, while actin in the high speed supernatant (representing actin monomers and small oligomers) decreased.

**FIGURE 1.** Effects of hypertonic stress on PI2. HeLa cells were exposed to 250 mM sucrose for 10 min, and their phosphoinositides were analyzed. A, TLC. HeLa cells were labeled with [32P] for 4 h and exposed to sucrose. Left panel, triplicate samples from a single experiment are shown. Data are representative of four independent experiments. [32P]PIP2 and [32P]PI4P levels increased to 180 ± 10% (n = 8) and 121 ± 10% (n = 8) of control level, respectively. Right panel, time course of [32P] incorporation into PIP2 and PI4P. The amount of [32P], expressed in arbitrary intensity units, is shown as mean ± S.E. from three independent experiments, each done in triplicate. B, HPLC quantitation. Phospholipids were deacylated, and negatively charged glycerol head groups were eluted and detected on-line by suppressed conductivity (μS, microsiemens units). Glycerol-PIP2 levels increased to 219 ± 33% of control value (n = 4). Results are from a single experiment and are representative of three independent experiments. C, PIP2 distribution as detected by anti-PIP2 with and without a 10-min sucrose treatment.
Therefore, hypertonicity promotes actin polymerization and cross-linking into stress fibers and/or networks.

**FIGURE 2.** PIP5KI RNAi inhibits the hypertonic PIP, and actin responses. A, HeLa cells were transfected with siRNA oligonucleotides targeting the PIP5Kια, -β, and -γ isoforms or an irrelevant sequence (control). Cells were stimulated with sucrose, and 32P incorporation into PIP2 was quantitated after TLC. Radioactivity of siRNA-treated samples was expressed as a percentage of that from cells transfected with the control siRNA sample. Data are mean ± S.E. of five independent experiments. B, phalloidin actin staining. Control and PIP5KIβ RNAi cells with and without sucrose treatment were fixed, permeabilized, and stained with FITC-phalloidin. C, partitioning of actin in Triton-soluble and -insoluble fractions. Two samples from each condition are shown, and the mean percentages of actin in each were calculated. The low speed pellets of control and sucrose-treated samples contained 23 and 33% of the total actin, respectively. The high speed pellets of control and sucrose-treated samples were 12 and 19% of total, respectively. D, HeLa cells overexpressing HA-PIP5KIβ were incubated with NaCl and stained with anti-HA.

**Therefore,** hypertonicity promotes actin polymerization and cross-linking into stress fibers and/or networks.

**PIP5KIβ** depletion dramatically changed the cell shape and decreased the amount of stress fibers (Fig. 2B). Although hypertonic stress shrunk the PIP5KIβ RNAi-treated cells, it did not promote stress fiber formation. Since PIP5KIβ depletion blocked the actin polymerization/reorganization response, we conclude that PIP5KIβ has a major role in orchestrating the hypertonic stress fiber response.

**PIP5KIβ Was Recruited to Membranes by Hypertonic Stress—** Immunofluorescence was used to examine the effect of hypertonic stress on the subcellular distribution of PIP5KIβ. PIP5KIβ was cytosolic and also associated with plasma membrane and endomembranes (Fig. 2D). Hypertonic stress increased the amount of PIP5KIβ at the cell periphery and the retraction fibers also contained PIP5KIβ.

The increase in PIP5KIβ membrane association was confirmed by subcellular fractionation using two different methods. Centrifugation at 100,000 x g showed that under isotonic conditions, ~45% of the kinase was recovered in the pellet (membranes) (Fig. 3A). HA-PIP5KIβ migrated as a doublet in both the supernatant (cytosol) and pellet, which, as will be shown later, was due to a difference in the extent of phosphorylation. Hypertonic stimulation collapsed the doublet into a single band and increased the recovery of PIP5KIβ (70% of total) in the pellet fraction.

Multistep fractionation (36) provided additional information about the partitioning of PIP5KIβ among different organelle fractions (Fig. 3B). Under isotonic conditions, slightly less than half of the total PIP5KIβ was membrane associated, and of this, half was recovered in the PM enriched fraction (Fig. 3B). Sucrose stimulation decreased the percentage of PIP5KIβ in the cytosolic fraction by 60% and almost doubled the percentage recovered in the PM and high speed pellet fraction. Therefore, the immunofluorescence and biochemical data both show that there is an increase PIP5KIβ membrane association.

**Hypertonic Stress Dephosphorylated PIP5KIβ but Not the Other PIP5KIs—** Park et al. (29) showed that PIP5KI isoforms are constitutively phosphorylated and that they can be activated by Ser/Thr dephosphorylation. Hypertonic stress col-
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Unlike PIP5KIβ, neither PIP5K1α nor PIP5K1γ (both L and S variants) was dephosphorylated by hypertonic stimulation (Fig. 4B). PIP5KIβ is phosphorylated by protein kinase A on its Ser-241 residue (29). However, we found that the HA-PIP5KIβS241A mutant was dephosphorylated normally during hypertonic stimulation (data not shown). Therefore, Ser-241 is not a major site of hypertonicity-induced dephosphorylation.

Effects of PIP5KIβ Dephosphorylation—The effect of hypertonic stress on the lipid kinase activity of PIP5KIβ was examined using an in vitro kinase assay. HA-PIP5KIβ that was immunoprecipitated from hypertonicity-stressed cells had three times higher lipid kinase activity than those from the unstimulated control, while the activities of PIP5K1α, γL, and γS were not changed significantly (Fig. 4C). Therefore, hypertonic stress selectively activates PIP5KIβ.

We next examined the effect of inhibiting Ser/Thr protein phosphatases on the hypertonic response. We tested the following Ser/Thr phosphatase inhibitors: caly A inhibits PP1 and PP2A; okadaic acid inhibits PP2A at 1–10 nM concentrations (IC50 0.51 nM) and PP1 at higher concentrations (IC50 42 nM). Cyclosporine A inhibits PP1B but not PP1A.

Caly A increased the intensity of the 32P label in the upper band of the PIP5KIβ doublet under isotonic conditions and blocked the sucrose-induced dephosphorylation (Fig. 5A). Okadaic acid had no effect at 10 nM (data not shown) but did increase HA-PIP5KIβ basal phosphorylation and blocked dephosphorylation at 100 nM. The differential effects of caly A and okadaic acid on PIP5KIβ dephosphorylation suggest that the PP1 phosphatases promote PIP5KIβ dephosphorylation during hypertonic stress. Cyclosporine A did not block dephosphorylation (data not shown), ruling out a PP2B involvement.

Caly A was used to evaluate the relationship between the hypertonicity-induced PIP5KIβ dephosphorylation and lipid kinase activation. We found that caly A decreased basal PIP5KIβ activity by 63% and blocked PIP5KIβ activation by sucrose (Fig. 5B). We also used caly A to determine whether PIP5KIβ dephosphorylation is a primary trigger for the hypertonic PIP2 response. Caly A dampened the PIP2 response (Fig. 5C), and this effect was specific for PIP2, because PI4P increased normally. It is curious though that caly A had minimal effect on the PIP2 level of the cell under isotonic condition (Fig. 5C), even though it inhibited PIP5KIβ in vitro (Fig. 5B). It is possible that PIP2 did not decrease in the calyculin A-treated cells because of compensatory changes that restore the ambient isotonic PIP2 level. However, these compensations are not able to raise PIP2 to a sufficiently high level to compensate for the lack of PIP5KIβ activation during hypertonic stress. Taken together, the series of experiments establish that there is a cause and effect relationship between hypertonicity-induced PIP5KIβ dephosphorylation, lipid kinase activation, and PIP2 increase in cells.

Effects of PIP5KIβ Dephosphorylation on Its Steady State Membrane Association—Since hypertonicity induces PIP5KIβ dephosphorylation and also promotes its recruitment to membranes (Figs. 2–4), we examined the possibility that the more dephosphorylated PIP5KIβ is preferentially membrane associated. However, the ratio of the two bands in the PIP5KIβ doublet in the 100,000 × g supernatant and pellet fractions (Fig. 3A)
were similar. Therefore, the more phosphorylated and less phosphorylated PIP5K1β associate with membranes to a similar extent under the steady state isotonic conditions used here. We conclude that the increase in membrane association during hypertonic stress cannot be simply attributed to dephosphorylation.

The Relationship between the Hypertonicity-induced PIP₂ Response, Volume Change, and Actin Remodeling—Hypertonic NaCl or sucrose induces cell shrinkage and reorganization of the actin cytoskeleton. In contrast, 200 mM urea, which is cell permeant and therefore increases osmolarity without inducing cell shrinkage, did not increase PIP₂ nor PIP5K1β dephosphorylation (Fig. 6A). Therefore, the PIP₂ and dephosphorylation responses are both dependent on volume changes.

Cell shrinkage deforms the actin cytoskeleton and imposes mechanical tension on the integrins (37). Since the actin cytoskeleton can potentially act as a volume sensor and PIP5KIs are activated by integrin signaling (4, 15, 38, 39), we investigated the possibility that PIP5K1β dephosphorylation depends on signals transmitted by the actin cytoskeleton. Latrunculin A, which depolymerizes actin filaments, and jasplakinolide, which stabilizes actin filaments, were used to interfere with the cytoskeletal response. We found that neither blocked the PIP₂ increase nor PIP5K1β dephosphorylation (Fig. 6B). Therefore,
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FIGURE 6. Effects of urea, actin poisons, and Rho kinase inhibition. A, effect of urea on the PIP2 response. HeLa cells were exposed to either 100 mM NaCl or 200 mM urea for 10 min. Top panel, [32P]PIP2 expressed as a percent of control. Data shown are mean ± S.E. of two independent experiments. Bottom panel, Western blot of HA-PIP5K1β. B, effects of actin depolymerization or stabilization. Cells were incubated with 100 nM latrunculin B (lat B) or 500 nM jasplakinolide for 30 min prior to exposure to 100 mM NaCl. Top panel, [32P]PIP2, mean ± S.E. of three independent experiments. Bottom panel, Western blotting of HAPIP5K1β. C, effect of Rho kinase inhibition. Cells were incubated with 10 μM Y23187 for 30 min prior to exposure to NaCl. HAPIP5K1β was detected by Western blotting.

PIP5K1β dephosphorylation and PIP2 increase do not directly depend on cytoskeletal remodeling.

Under isotonic conditions, the small GTPase Rho and its downstream effector Rho kinase stimulate stress fiber formation and promote PIP5K1 targeting and activation (15, 22, 40). Since Rho is activated by hypertonic stress (41, 42), we examined the possibility that Rho kinase activation promotes PIP5K1β dephosphorylation. This is not the case because the Rho kinase inhibitor, Y27843, did not block PIP5K1β dephosphorylation (Fig. 6C) even though it inhibited actin stress fiber formation (data not shown).

DISCUSSION

In this paper, we report that hypertonic stress activates and targets PIP5K1β to promote high levels of site-specific PIP2 generation. The increase in PIP2 levels is a direct result of PIP5K1β activation by Ser/Thr dephosphorylation.

The involvement of PIP5K1β was established by RNA interference. The lack of response in cells depleted of PIP5K1β was not due to nonspecific cell death, because PIP5K1β RNAi has surprisingly little effect on another signaling pathway (34). Furthermore, although the other PIP5Ks are not major players in the hypertonic response, they have important roles that have been identified by RNAi depletion (34) and by gene knock-out

(43). Our results support the growing realization that the PIP5Ks are functionally specialized, and by extension, the PIP2 pools of the cell may be functionally and perhaps even physically segregated.

The phosphorylation status of PIP5K1β is likely to be maintained by a balance between protein kinases and phosphatases. Theoretically, the hypertonicity-induced PIP5K1β dephosphorylation could be achieved either by inhibiting a PIP5K1β protein kinase or activating its phosphatase. However, although hypertonicity activates many kinases (5, 44), there are relatively few examples of hypertonicity-inactivated kinases. Therefore, our current working hypothesis is that PIP5K1β is dephosphorylated primarily by activation of a caly A-sensitive PPI. The involvement of PPI1 is supported by the ability of caly A to inhibit PIP5K1β dephosphorylation as well as activation, and also to block PIP2 increase in cells. Our conclusion is further supported by the finding that PPI1 dephosphorylates and activates PIP5K1β in vitro (29).

Our results show for the first time that PIP5K1β depletion decreases the amount of actin filaments under isotonic conditions and blocks hypertonicity-induced actin polymerization/reorganization. These results place PIP5K1β activation and PIP2 increase upstream of actin remodeling. This placement is further supported by additional lines of evidence. Urea, which increases osmolarity without causing cell shrinkage, does not promote PIP5K1β dephosphorylation nor increase PIP2. Since urea also does not promote actin assembly (38), these events are inter-related. Our results with actin poisons and Rho kinase inhibitor clearly establish that PIP5K1β dephosphorylation is not dependent on mechanical transduction by the actin cytoskeleton.

Rho is activated by hypertonic stress, and Rho recruits PIP5K1β to the plasma membrane under isotonic conditions (14, 15). However, we find that PIP5K1β is dephosphorylated normally in the presence of the Rho kinase inhibitor, and dephosphorylated PIP5K1β is not preferentially recruited to membranes. We propose that the current result can be explained by hypothesizing that PIP5K1β is subject to regulation at multiple levels. Exposure to hypertonicity immediately triggers PIP5K1β dephosphorylation by a volume sensing, Rho kinase-independent protein phosphatase. In addition, Rho kinase is also activated either independently or downstream of PIP5K1β dephosphorylation. The latter possibility is suggested by the finding that PIP2 activates a Rho GEF in yeast (45). Rho and Rho kinase
can then stimulate PIP5Kβ further by direct binding and targeting. Thus, PIP5Kβ activation by dephosphorylation is an apical signal, which can be further modulated or propagated by other downstream regulators and crosstalk at multiple levels. The actin cytoskeleton may be remodeled initially by the PIP2 generated through PIP5Kβ activation and then further downstream by Rho activation.

It is hypothesized that a stronger cortical actin network tempers the inflammatory cascade during traumatic injury (4, 46) by blocking leukocyte exocytosis (4, 46, 47). In addition, the reorganized cytoskeleton reinforces endothelial cell:cell and cell:matrix adhesion, to minimize the monolayer disruption that exacerbates the injury response (48). The type of actin remodeling appears to be cell-specific. For example, hypertonicity induces cortical actin assembly in CHO cells by activating Rho and Rho kinase (47). We find that hypertonicity increases actin assembly as well as actin filament cross-linking into stress fiber in HeLa cells, suggesting that PIP2 activates regulatory proteins that favor actin nucleation and polymerization or severing (49). These results are consistent with our previous finding that overexpression of PIP5Kβ induces NWASP:Arp2/3 dependent actin polymerization (50) and actin stress fiber formation in CV1 cells (23).

Our study suggests that the increase in PIP2 levels may explain how hypertonic resuscitation protects against the inappropriate inflammatory responses in burn and trauma patients. This model is supported by the finding that knock-out mice that do not express the mouse equivalent of human PIP5Kβ exhibit enhanced passive cutaneous and systemic anaphylaxis (32). Like the PIP5Kβ RNAi HeLa cells, the mast cells of these null animals have decreased actin filaments, and they are hyperactive in degranulation and cytokine production. We predict that these mice will be more susceptible to burn induced complications, and hypertonic resuscitation might be less effective in protecting against these injuries because of the lack of a PIP2 response.

In conclusion, PIP5Kβ is the major source of the PIP2 generated during hypertonic stress, and this PIP2 is necessary for the hypertonicity-induced reorganization of the actin cytoskeleton. This study provides mechanistic understanding of how hypertonicity induces PIP5Kβ activation, increases PIP2, and reorganizes the actin cytoskeleton. PIP5Kβ is dephosphorylated in response to volume changes upstream of cytoskeletal reorganization, and it generates an apical signal that has a central role in regulating the actin cytoskeleton and most likely a host of other hypertonic responses.

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