Signaling by Higher Inositol Polyphosphates

SYNTHESIS OF BISDIPHOSPHOINOSITOL TETRAKISPHOSPHATE ("InsP₅") IS SELECTIVELY ACTIVATED BY HYPEROSMOTIC STRESS*†

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Xavier Pesesse, Kuicheon Choi, Tong Zhang, and Stephen B. Shears‡

From the Inositide Signaling Group, NIEHS, National Institutes of Health, Department of Health and Social Services, Research Triangle Park, North Carolina 27709

Evidence has accumulated that inositol polyphosphates (diphosphoinositol pentakisphosphate (PP-InsP₅) and bisdiphosphoinositol tetrakisphosphate ([PP]₂-InsP₄)) are intracellular signals that regulate many cellular processes including endocytosis, vesicle trafficking, apoptosis, and DNA repair. Yet, in contrast to the situation with all other second messengers, no one studying multicellular organisms has previously described a stimulus that acutely and specifically elevates cellular levels of PP-InsP₅ or [PP]₂-InsP₄. We now show up to 25-fold elevations in [PP]₂-InsP₄ levels in animal cells. Importantly, this does not involve classical agonists. Instead, we show that this [PP]₂-InsP₄ response is a novel consequence of the activation of ERK1/2 and p38MAPK/β kinases by hyperosmotic stress. JNK did not participate in regulating [PP]₂-InsP₄ levels. Identification of [PP]₂-InsP₄ as a sensor of hyperosmotic stress opens up a new area of research for studies into the cellular activities of higher inositol phosphates.

†Pivotal to the signaling functions of PP-InsP₅ and [PP]₂-InsP₄ are their highly negative electrostatic potential and the considerable free energy of their hydrolysis (for review, see Ref. 1). The former attribute facilitates their functionally significant binding to PH (2) and other protein domains (3, 4). Additionally, the high energy phosphate groups may phosphorylate proteins (5). These effects are believed to underlie the roles of inositol pyrophosphates in regulating clathrin assembly by adaptor proteins (3), apoptosis (6), vesicle trafficking (7), chemotaxis (2), and DNA repair (5). It has also been pointed out (e.g. Ref. 2) that many of the important cellular functions currently attributed to InsP₅ may in vivo be more effectively performed by PP-InsP₅ and [PP]₂-InsP₄. The Dicystelidosts have uniquely exploited the physico-chemical properties of the inositol diphosphates by synthesizing near-millimolar levels of these molecules (8). However, animal cells have 300-fold lower levels of PP-InsP₅ and [PP]₂-InsP₄ (1). It has therefore generally been anticipated that, in animal cells, there should be substantial, stimulus-dependent increases in cellular levels of PP-InsP₅ and [PP]₂-InsP₄ to elevate levels of these polyphosphates to a functionally significant threshold. However, no such phenomenon has been described previously. Instead, only quite small stimulus-dependent changes in PP-InsP₅ and [PP]₂-InsP₄ turnover have been reported, and in each case the result was a decrease rather than an increase in cellular levels of the pyrophosphates. For example, depletion of endoplasmic reticulum Ca²⁺ stores by thapsigargin transiently decreases PP-InsP₅ levels by 50% (9). Activation of β-adrenergic receptors decrease levels of [PP]₂-InsP₄ by up to 40% (10).

This absence of a substantial, regulatory context for PP-InsP₅ and [PP]₂-InsP₄ has been addressed in the current study, in which we selected a biological paradigm of fundamental and widespread significance, namely, defense against osmotic stress. The maintenance of osmotic balance across plasma membranes is an ongoing cellular burden in the face of controlled changes in both cell size and intracellular hydration as well as exchange of metabolites and ions across plasma membranes (11). There are also clinically relevant osmotic challenges that can impose strain upon the cytoskeleton, perturb chromatin structure, damage DNA, and inhibit DNA repair (12, 13). One way to examine how cells sense and adapt to hyperosmotic stress is to add sorbitol to the medium (11). In this report, we show that the addition of 0.2 m sorbitol can elevate [PP]₂-InsP₄ levels up to 25-fold, and we also study the molecular mechanisms that are involved.

EXPERIMENTAL PROCEDURES

Cellular levels of inositol phosphates were determined as previously described (10). InsP₅ and PP-InsP₅ kinase activities were assayed as described (15). Several colleagues kindly supplied the following cDNA constructs: DN-p38α and DN-p38β in pCDNA3 (Dr. J. Han, Scripps Research Institute, La Jolla, CA); PIPKH in pCMV2 (Dr. J. Chang, Beth Israel Deaconess Medical Center, Boston, MA); DN-ERK1 and DN-ERK2 in pCMV5 (Dr. C. Der, University of North Carolina).

RESULTS AND DISCUSSION

Cells are constantly adapting to the osmotic challenges brought on by normal cellular activities, such as the breakdown of macromolecules, controlled changes in both cell size and intracellular hydration, as well as exchange of metabolites and ions across plasma membranes (11). We investigated whether inositol diphosphates might be regulated by hyperosmotic stress, which we introduced by the addition of 0.2 m sorbitol. It is notable that many earlier studies into hyperosmotic stress have frequently employed much higher doses of sorbitol (0.5–0.7 m). Cells adapt successfully to 0.2 m sorbitol, since it failed...
even after 24 h to affect viability of either HEK cells (101 ± 2%) or the DDT1 MF-2 vas deferens smooth muscle line (96 ± 1%) compared with non-sorbitol controls. A novel, immediate and selective response of DDT1 MF-2 cells and HEK cells to 0.2 M sorbitol is a dramatic elevation of [PP]2-InsP4 levels, in the range of 10–25-fold (Fig. 1). It is worth emphasizing the novelty of this observation; there is no previous report of any extracellular stimulus that can specifically and acutely increase levels of an inositol diphosphate.

Both PP-InsP5 and [PP]2-InsP4 are hydrolyzed by DIPPs (14, 16). Any DIPPII inhibition following osmotic stress should elevate both of the diphosphoinositol polyphosphates. On the contrary, a 26 ± 4% (n = 8) drop in PP-InsP5 accompanies the sorbitol-dependent increase in [PP]2-InsP4 levels (Fig. 1A). These effects are consistent with hyperosmotic stress elevating [PP]2-InsP4 levels by activating the PP-InsP5 kinase. However, it is possible that sorbitol stress inhibits an [PP]2-InsP4-specific phosphatase or [PP]2-InsP4-specific phosphorylation of proteins (5). Time course data indicated that [PP]2-InsP4 levels were elevated within 5–10 min of the initiation of hyperosmotic stress (Fig. 1B). As little as 0.05 M sorbitol promoted a severalfold [PP]2-InsP4 response (Fig. 1C). Within minutes of terminating the hyperosmotic challenge, [PP]2-InsP4 levels declined exponentially, although they did not return to pre-activated levels within the 2-h experimental time frame (Fig. 1D); instead, the cell’s memory of their stress history persisted.

To assess the mechanisms underlying the sorbitol-dependent increase in [PP]2-InsP4 levels, we considered how this enzyme might interface with other signaling entities. Hyperosmotic stress is known to activate Ser/Thr phosphoprotein phosphatases (17) and Tyr phosphoprotein phosphatases (18), but inhibitors of either pathway (20 nM okadaic acid or 10 μM phenylarsine oxide, respectively) did not modify the [PP]2-InsP4 response to sorbitol (data not shown). The JNK MAP kinase pathway can be activated by 0.5 M sorbitol (19). In agreement with this earlier study, we also found that 0.5 M sorbitol activated JNK (Fig. 2A). However, JNK was not activated by 0.2 M sorbitol (Fig. 2A), yet this dose of sorbitol was sufficient to maximally elevate [PP]2-InsP4 levels (Fig. 1C). Thus, we conclude that JNK activation is not necessary for [PP]2-InsP4 levels to be increased by hyperosmotic stress.

The ERK and p38 MAP kinase pathways are activated by osmotic stress (Fig. 2, B and C, and Refs. 20 and 21). Pretreatment of cells with the MEK inhibitors U0126 (10 μM) or PD098059 (50 μM) inhibited ERK1/2 phosphorylation (Fig. 2, B and C, and Ref. 22). This was accompanied by a 45–50% reduction of the sorbitol-dependent increase in [PP]2-InsP4 levels (Fig. 3A). The similarity of the results obtained with two structurally unrelated MEK inhibitors makes it unlikely that reversal of the sorbitol effect results from unsuspected nonspecific actions of U0126 or PD098059. Pretreatment of cells with SB203580 inhibited phosphorylation of p38α/β (Fig. 2C and Ref. 23) and also elicited a 23% attenuation of the sorbitol-dependent increase in [PP]2-InsP4 levels (Fig. 3A). The relatively mild effect of SB203580 when it was added alone may be in part explained by its ability to non-specifically activate ERK1/2

Fig. 1. Hyperosmotic challenge resets the dynamic poise of the diphosphoinositol polyphosphate metabolic cycle. A, cellular levels of [3H]InsP5, PP-[3H]InsP5, and [PP]2-[3H]InsP4, and below a schematic describing their interconversion by InsP6 kinases (IP6K), PP-InsP5 kinase (IP7K), and phosphatases (DIPPs). [3H]InsP5 levels in control cells (DDT1 MF-2 = 188,985 dpm, HEK = 110954 dpm) were assumed to be 40 μM so that other inositol phosphate levels, for presentation purposes, could be estimated from relative [3H]peak sizes. DDT1 MF-2 cells are shown: control (open circles) InsP5 = 37 μM, PP-InsP5 = 1.6 μM, PP-InsP4 = 10 μM, [PP]2-InsP4 = 0.1 μM; sorbitol (filled circles) InsP5 = 39 μM, PP-InsP5 = 0.91 μM, [PP]2-InsP4 = 7.6 μM, PP-InsP5 = 2.5 μM. HEK cells (data not shown): control InsP5 = 84 μM, PP-InsP5 = 2.3 μM, PP-InsP4 = 2.3 μM, [PP]2-InsP4 = 0.06 μM; sorbitol InsP5 = 89 μM, PP-InsP4 = 0.9 μM, InsP6 = 34 μM, PP-InsP5 = 2.2 μM, [PP]2-InsP4 = 0.57 μM. Also shown: [PP]2-InsP4 levels in DDT1 MF-2 cells treated with 0.2 M sorbitol for various times (n = 4, B) or with various concentrations of sorbitol for 30 min (representative experiment, C). D, after 30-min treatment with 0.2 M sorbitol the medium was replaced with control culture medium. The broken line denotes original [PP]2-InsP4 levels in control cells (n = 4).
We eliminated the possibility that the MAP kinase inhibitors might non-specifically block synthesis of the diphosphoinositol polyphosphates: native preparations of PP-InsP$_5$ kinase showed 103 ± 2% activity when incubated with 10 μM SB203580, 105 ± 3% with 10 μM U0126, and 103 ± 2% with 50 μM PD098059 (n = 3), all relative to vehicle controls. The recombinant InsP$_5$ kinase showed 97 ± 6% activity when incubated with 10 μM SB203580, 105 ± 2% with 10 μM U0126 and 96 ± 5% with 50 μM PD098059 (n = 3–4), all relative to vehicle controls. Although MAP kinase inhibitors did not affect PP-InsP$_5$ synthesis in vitro, the level of PP-InsP$_5$ in cells treated with either PD098059 or U01276 was 25% lower than control cells (Fig. 3B). These data suggest that the ERK activity that is ongoing even in resting cells (Fig. 2B) may weakly enhance InsP$_5$ kinase activity. Consistent with this explanation, the sorbitol-dependent decrease in PP-InsP$_5$ levels was not reversed by the MEK inhibitors (Fig. 3B). The absence of any significant effect of the MAP kinase inhibitors upon PP-InsP$_5$ levels in sorbitol-treated cells (Fig. 3B) is of additional interest; this observation indicates that the attenuation of sorbitol-mediated increases in PP-InsP$_5$ levels by the MAP kinase inhibitors is not a secondary consequence of a reduction in substrate supply for the PP-InsP$_5$ kinase.

To substantiate the inhibitor experiments, we next transiently transfected cells with dominant negative (DN) MAP kinase cDNA constructs. It is first important to note that environmental stresses provide “lateral” as well as “top-down” input into MAP kinase responses (25). The DN-ERK constructs block the MAP kinase pathway at a point that is downstream of the site of action of the MEK inhibitors, so these two empirical approaches could yield quantitatively different degrees of attenuation of the sorbitol response if there were any lateral input between MEK and ERK. When co-expressed, DN-ERK1 (K71R) and DN-ERK2 (K52R) (26) reduced the effect of sorbitol upon [PP]$_2$-InsP$_4$ levels by 30% (Fig. 4). Neither DN construct had any significant effect when added alone (data not shown). Also when co-expressed, the TGY-to-AGF DN-p38α and DN-p38β proteins (27, 28) reduced the effect of sorbitol upon [PP]$_2$-InsP$_4$ levels by 30% (Fig. 4). Again, neither DN construct had any significant effect when added alone (data not shown). Transient transfection of DDT1 MF-2 cells with all four DN constructs reduced by 50% the effect of hyperosmotic stress upon [PP]$_2$-InsP$_4$ levels (Fig. 4). This is approximately the maximum effect that could be anticipated, given the limited transfection efficiency (50%; determined by GFP co-transfection). We found that the activity of the native PP-InsP$_5$ kinase was unaffected by recombinant, phosphorylated ERK1/2 (data not shown). It therefore seems likely that another signaling protein that lies downstream of ERK mediates its activation of cellular [PP]$_2$-InsP$_4$ accumulation. Nevertheless, [PP]$_2$-InsP$_4$ signaling offers a new repertoire for MAP kinase actions upon cellular physiology. Its unusual nature is also of interest; MAP kinases do not typically recruit diffusible second messengers.

There is a growing appreciation that the inositol diphos-
output from the MAP kinase pathway when it is utilized by so many extracellular and intracellular stimuli? Much additional work may be required to solve this problem, but in any case, our data are still significant in their demonstration that it is stress-dependent and not agonist-initiated increases in [PPI]2-InsP levels that provides a regulatory context for the actions of inositol pyrophosphates. It is intriguing that hyperosmotic shock can damage DNA and affect vesicle trafficking processes (10). Hence these are both processes that can be regulated by inositol pyrophosphates (see Introduction). In any case, our demonstration of a stimulus that can specifically elevate one of the higher inositol pyrophosphates, in a specific and acute manner, is an unprecedented observation that opens up a new area of research for inositol signaling.

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Fig. 5. Effect of EGF upon ERK phosphorylation, and cellular levels of PP-InsP2 and [PPI]2-InsP2. [3H]Insitol-labeled DDT1 MF-2 cells were treated for 30 min with either vehicle (C) or 100 ng/ml EGF (E) and levels of PP-InsP2 and [PPI]2-InsP2 were determined as described under “Experimental Procedures” (n = 3). Representative phospho-ERK Western analyses are also presented.