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Protocols for Regulation and Study of Diphosphoinositol Polyphosphates

S. T. Safrany

Division of Cell Signalling, University of Dundee, Dundee, Scotland, United Kingdom

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ABSTRACT

The roles of diphosphoinositol polyphosphates (DIPs) in mammalian cell biology have been difficult to determine because of the lack of tools known to regulate their levels. I have determined a series of protocols that regulate these DIPs, and these can be used to further our understanding of these molecules. Sorbitol and sucrose significantly raised levels of bis-diphosphoinositol tetrakisphosphate ($[PP]_2$-$InsP_4$) but slightly lowered levels of diphosphoinositol pentakisphosphate (PP-$InsP_5$) in DDT_1 MF-2 cells. These effects correlate with the ability of hyperosmotic stress to interfere with protein trafficking described previously and suggest that $[PP]_2$-$InsP_4$ specifically impedes protein trafficking. The effects on $[PP]_2$-$InsP_4$ were not regulated by extracellular signal-regulated kinase or phospholipase D, as exemplified by the lack of effect of U0126 and butan-1-ol. I have also found that genistein potently and rapidly lowers levels of $[PP]_2$-$InsP_4$, whereas a similar inhibitor, herbimycin, was without effect. Thapsigargin, a sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor previously shown to selectively lower PP-$InsP_5$ after short-term treatment, also selectively raises PP-$InsP_5$ after a longer treatment. The calmodulin inhibitors *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and chlorpromazine significantly lowered all higher inositol phosphates, as well as DIPs, whereas the calmodulin-dependent kinase inhibitors methyl 9-(*S*)-12-(*R*)-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-2,3,9,10,11,12-hexahydro-10-(*R*)-hydroxy-9-methyl-1-oxo-10-carboxylate (K-252a) and 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93) were without effect. W-7 and chlorpromazine also lowered levels of phosphatidylinositol 4,5-bisphosphate and ATP but greatly increased levels of phosphatidylinositol 4-phosphate. Trypan blue exclusion deemed that these doses were not cytotoxic. These results identify an increasing number of reagents that regulate DIP levels. Using these tools, and those described previously, we can further understand the roles of the DIPs in cell biology.

There is increasing evidence of the important roles played by inositol phosphates in cell biology. Although the importance of inositol 1,4,5-trisphosphate as a regulator of intracellular free calcium has been understood for more than 20 years, the functions of many of the other inositol phosphates remains less well defined. It is not unreasonable to believe that some of the more than 60 inositol phosphates found in eukaryotes are intermediates. Nevertheless, an increasing number of inositol phosphates are being identified that have varied and diverse functions. In addition to calcium signaling, they have been implicated in nuclear mRNA export (York et al., 1999), chromatin remodelling (Shen et al., 2003; Steger et al., 2003), DNA repair (Hanakahi et al., 2000; Byrum et al., 2004), membrane trafficking (Ye et al., 1995; Saiardi et al., 2002), and control of cell proliferation (Orchiston et al., 2004).

The least studied members of all inositol phosphates are the diphosphoinositol polyphosphates (DIPs). These are phosphorylation products of the most abundant inositol phos-

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ABBREVIATIONS: DIP, diphosphoinositol polyphosphate; $InsP_5$, inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol hexakisphosphate; PP-$InsP_5$, diphosphoinositol pentakisphosphate; $[PP]_2$-$InsP_4$, bisdiphosphoinositol tetrakisphosphate; cNMP, cyclic nucleotide monophosphate; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; ZD7288, 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)pyridinium chloride; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; HIP, higher inositol phosphates; KN-93, 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; mpV(pic), monoperoxo(picolinato)oxovanadate(V); K-252a, methyl 9-(*S*)-12-(*R*)-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-2,3,9,10,11,12-hexahydro-10-(*R*)-hydroxy-9-methyl-1-oxo-10-carboxylate; HA1077, (5-isoquinolinesulfonyl)homopiperazine; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PKA, protein kinase A.

phates found in cells, inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) and inositol hexakisphosphate ($InsP_6$) (Safrany et al., 1999). Until recently, $InsP_6$ was believed to be the endpoint in the inositol phosphate kinase cascade. Three human $InsP_6$ kinases has been identified, producing diphosphoinositol pentakisphosphate (PP-$InsP_5$) (Saiardi et al., 1999; Schell et al., 1999), and a *Saccharomyces cerevisiae* homolog (KCS1) has also been characterized (Saiardi et al., 2000). $InsP_6$ kinases can also phosphorylate $InsP_5$, producing diphosphoinositol tetrakisphosphate. PP-$InsP_5$ can be further phosphorylated by a kinase that has yet to be cloned (Huang et al., 1998). The structure of the product, bisdiphosphoinositol tetrakisphosphate ($[PP]_2$-$InsP_4$), remains to be determined in mammalian systems. Despite their low levels, these DIPs are the most rapidly turned over inositol phosphates in unstimulated cells.

It has been shown previously that PP-$InsP_5$ can be regulated by the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor thapsigargin (Glennon and Shears, 1993). The phosphatases involved in hydrolysis of $[PP]_2$-$InsP_4$ and PP-$InsP_5$, termed diphosphoinositol polyphosphate phosphohydrolases, are sensitive to fluoride (Shears et al., 1995; Safrany et al., 1998). As such, it was not a surprise to observe that incubating cells with low levels (below millimolar) of fluoride increased levels of PP-$InsP_5$ and $[PP]_2$-$InsP_4$. Higher levels caused a paradoxical *decrease* in $[PP]_2$-$InsP_4$ levels that was found to be mediated by cyclic nucleotide monophosphates (cNMPs) via an unidentified mechanism (Safrany and Shears, 1998).

The function of these DIPs remains a target for research. It is clear that they play a role in protein trafficking (Saiardi et al., 2000). In vitro experiments show that PP-$InsP_5$ and $[PP]_2$-$InsP_4$ can inhibit formation of clathrin triskelia (Ye et al., 1995). Yeast strains in which the KCS1 gene has been removed show clear trafficking defects (Saiardi et al., 2002). The major interest is determining which of the DIPs plays a role in vivo.

In this article, I present, for the first time, new experimental protocols that alter levels of DIPs. Together with previously published data, these treatments will help determine the roles of the DIPs. To this end, I have determined that hyperosmotic stress, caused by treating the Syrian hamster vas deferens smooth muscle cell line DDT_1 MF-2 with sorbitol or sucrose, a treatment that is known to inhibit protein trafficking, also raises $[PP]_2$-$InsP_4$ levels. These results identify a possible mechanism through which hyperosmotic stress acts and reveals that $[PP]_2$-$InsP_4$ is a regulator of protein trafficking in vivo. These data clearly show that these inositol phosphates are rapidly turned over and that their pools can be selectively regulated. The protocols developed can be used to further determine the roles played by these inositol phosphates in physiology.

Materials and Methods

DDT_1 MF-2 Syrian hamster vas deferens smooth muscle cells [originally provided by Dr. D. Gill, University of Maryland School of Medicine (Baltimore, MD), then by Dr. S.B. Shears, National Institute of Environmental Health Sciences, National Institutes of Health (Research Triangle Park, NC)] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 'high glucose' (i.e., 25 mM), supplemented with 2 mM glutamine and 5% fetal calf serum at 37°C in 5% CO_2/95% humidified air. Cells were harvested and plated at a density of ~200,000 cells/well (16-mm diameter, 24-well multiplates) in the DMEM-based culture medium described above, supplemented with 50 μCi/ml [^{3}H]inositol. On the fourth day, cell monolayers were washed (2 × 250 μl) and then incubated (250 μl) in [^{3}H]inositol-free, HEPES-buffered Krebs-like media (115 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 11 mM glucose, 1.36 mM $CaCl_2$, 25 mM HEPES, pH 7.4 with NaOH). Cells were maintained at 37°C for 3 h before the beginning of any experiments. Otherwise, overnight treatments were performed in DMEM containing [^{3}H]inositol. All experimental protocols were time-matched with control incubations. Experiments were quenched by rapid aspiration of the Krebs-like (or DMEM) media, followed by addition of 250 μl of ice-cold 0.6 M perchloric acid and neutralized by the addition of 70 μl of 1 M K_2CO_3 containing 5 mM Na_2EDTA. After being kept at 4°C for 30 min, the perchlorate precipitate was removed by centrifugation (10,000*g*, 2 min). The supernatants were finally diluted with 3 volumes of 1 mM Na_2EDTA. Samples were stored at −20°C before being loaded onto a 4.6 × 125-mm Partisphere 5 μm SAX HPLC column. Inositol phosphates were eluted at 1 ml/min by the following gradient generated by mixing buffer A (1 mM Na_2EDTA) and buffer B [buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.85 with H_3PO_4; total $[P_i]$ = 2.6 M] as follows: 0 to 5 min, 0% B; 5 to 10 min, 0 to 50% B; 10 to 60 min, 50 to 100% B; 60 to 70 min, 100% B. Fractions were collected at 1-min intervals, mixed with 4.2 volumes of Flo-Scint IV scintillant, and radioactivity was determined using liquid scintillation spectrometry. EC_{50} values were derived using Prism (GraphPad, San Diego, CA). Inositol lipids were extracted from the perchloric acid pellet, prepared, and analyzed as described previously (Batty and Downes, 1994). Peaks were ascribed by coelution of standards in parallel runs.

ATP levels were determined using a luciferase-based assay kit (Merck Biosciences, Nottingham, UK), following the manufacturer's recommended protocol. Samples were either prepared as described above (omitting the addition of [^{3}H]inositol) or, for the determination of extracellular ATP, snap-frozen and assayed directly. Trypan blue exclusion was determined by incubating cells for 20 to 30 min in the presence of trypan blue (Sigma-Aldrich, Gillingham, UK) and determining a ratio of trypan blue-excluding (alive) cells to blue (dead) cells.

Materials. [*myo*-^{3}H]inositol (10–25 Ci/mmol; 10 mCi/ml; in sterile water), was provided by Amersham Biosciences UK Ltd. (Chalfont, Bucks). Tissue culture reagents were purchased from Invitrogen Ltd., (Paisley, UK). Thapsigargin was purchased from Alexis Corporation UK (Nottingham, UK), and ZD7288 was purchased from Tocris Cookson Ltd. (Bristol, UK). All other reagents used in this study were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK) or Merck Biosciences Ltd. (Nottingham, UK).

Results

HPLC was used to resolve the ^{3}H-labeled inositol phosphates in DDT_1 MF-2 cells, all of which have been observed previously in other mammalian cells (see Safrany and Shears, 1998). Although $[PP]_2$-[^{3}H]$InsP_4$ was present at low levels, they were of sufficient magnitude to exceed those of all the $InsP_4$ isomers combined. No more polar ^{3}H-labeled material was detected in these or any further experiments described in this study, even when $[P_i]$ in the HPLC eluate was increased to 2.6 M. It is noted that levels of $[PP]_2$-$InsP_4$ accumulated during incubation of the cells in Krebs-like media, reaching a plateau after 2 to 3 h. The reasons for this have yet to be determined.

In an attempt to understand the roles played by DIPs, I have tested a number of reagents that regulate key cellular functions. The finding, some 5 years ago, that cNMPs lowered $[PP]_2$-$InsP_4$ still awaits explanation. It is still unclear

why cyclic AMP and cyclic GMP lower $[PP]_2$-$InsP_4$, and what the consequences of this regulation are.

There is some evidence that DIPs are involved in protein trafficking. Much of this information has been obtained using in vitro binding assays, and some preliminary information is now available on protein trafficking in yeast lacking the IP_6 kinase, KCS1. No studies have yet been published on whether DIPs are involved in protein trafficking in mammalian cells. A protocol in which hyperosmotic stress, afforded by treating cells with sorbitol or sucrose, is known to block such trafficking was used to determine whether such treatment altered levels of DIPs. Treatment of DDT_1 MF-2 cells with either reagent caused a rapid and dose-dependent decrease in PP-$InsP_5$, accompanied by an increase in $[PP]_2$-$InsP_4$ levels. The effects of sucrose were short-lived, but sorbitol gave a robust and prolonged increase in $[PP]_2$-$InsP_4$ (Fig. 1). This is consistent with in vitro binding assays showing that $[PP]_2$-$InsP_4$ was able to inhibit clathrin triskelion formation and block protein trafficking. The ability of sorbitol to raise $[PP]_2$-$InsP_4$ was not blocked by U0126 (10 μM), or by butan-1-ol (2%), excluding a role for extracellular signal-regulated kinase and phospholipase D.

I have also identified that genistein, a broad-range protein tyrosine kinase inhibitor (Akiyama et al., 1987), is a potent regulator of $[PP]_2$-$InsP_4$ levels. Treatment of DDT_1 MF-2 cells with 100 μM genistein caused a rapid reduction in the levels of $[PP]_2$-$InsP_4$ without affecting the levels of other HIPs. The decline in $[PP]_2$-$InsP_4$ levels was both time- (Fig. 2) and dose- (Fig. 3) dependent. Genistein lowered $[PP]_2$-$InsP_4$ levels in a dose-dependent manner, with an EC_{50} of 28 μM. This is in close agreement with the published IC_{50} for $pp60^{v\text{-}src}$ of 26 μM (Akiyama et al., 1987). These effects were not mimicked by a similar protein kinase inhibitor, herbimycin A (10 μM, data not shown), therefore excluding the role of a $pp60^{v\text{-}src}$ -like kinase in regulating PP-$InsP_5$ phosphorylation. Protein tyrosine phosphatase inhibitors dephostatin (100 μM) and mpV(pic) (100 μM), like the broad inhibitor okadaic acid (1 μM), were without effect. mpV(pic) did, however, greatly raise levels of $InsP_4$, by more than 15-fold over control levels (data not shown). This is the first observation of mpV(pic) raising inositol phosphate levels, presumably by activating phospholipase C. The mechanism by which genistein lowered $[PP]_2$-$InsP_4$ is unclear. The roles of MEK, LCK, p70S6 kinase, phosphoinositide 3-kinase, and smooth muscle myosin light chain kinase were excluded by the lack of effect of SB203580 (10 μM), PD98059 (1 mM), rapamycin (100 nM), and wortmannin (100 nM) (data not shown).

That $InsP_3$ 3-kinases are regulated by calmodulin-dependent kinase (Dewaste et al., 2000), suggested that the gateway to all the HIPs could be regulated by calmodulin kinase inhibitors. Low levels of W-7, a calmodulin inhibitor, were without effect. Higher levels (>10 μM), however, caused a marked and rapid decline of HIPs and DIPs, $InsP_5$ to $[PP]_2$-$InsP_4$, although the ratios between each of these remained constant, identifying the presence of a specific block at the level of $InsP_5$ production (Fig. 4). Similar data were obtained

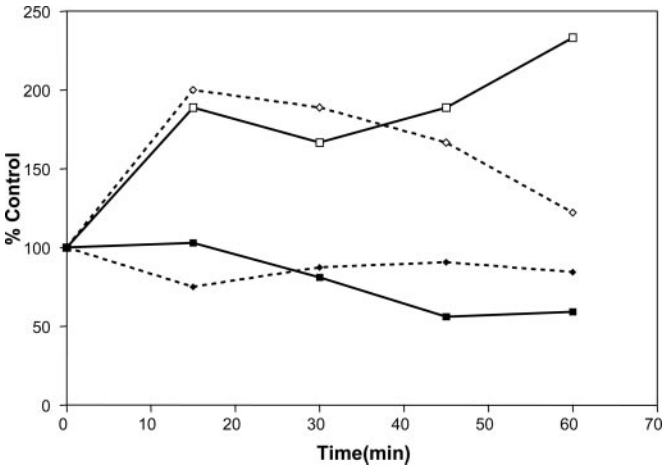

Fig. 1. Time-dependent effects of sucrose (squares) and sorbitol (diamonds) on cellular levels of $[PP]_2$-$InsP_4$ (open symbols) and PP-$InsP_5$ (filled symbols). $[^3H]$Inositol-labeled cells were incubated for the indicated times with 0.5 M sucrose or sorbitol. Cells were quenched, extracted, and analyzed by HPLC as described under *Materials and Methods*. The levels of $[PP]_2$-$InsP_4$ and PP-$InsP_5$ are shown as a percentage of vehicle-treated controls. Data are representative of three to five experiments.

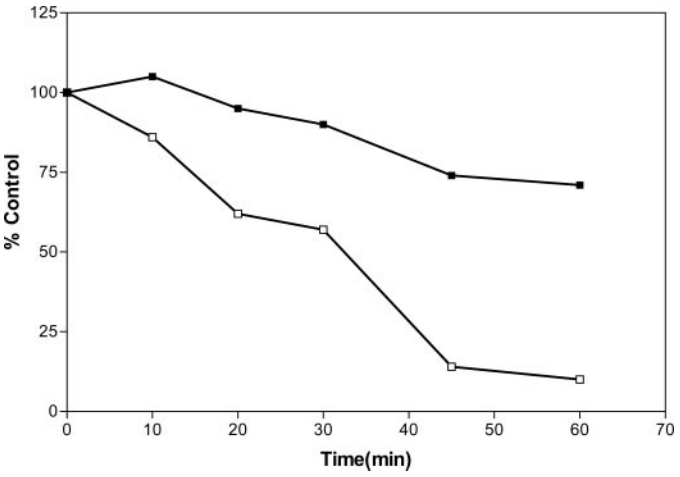

Fig. 2. Time-dependent effects of genistein on cellular levels of $[PP]_2$-$InsP_4$ (□) and [PP]-$InsP_5$ (■). $[^3H]$Inositol-labeled cells were incubated for the indicated times with 100 μM genistein. Cells were quenched, extracted, and analyzed by HPLC as described under *Materials and Methods*. The levels of $[PP]_2$-$InsP_4$ and PP-$InsP_5$ are shown as a percentage of vehicle-treated controls. Data are representative of three experiments.

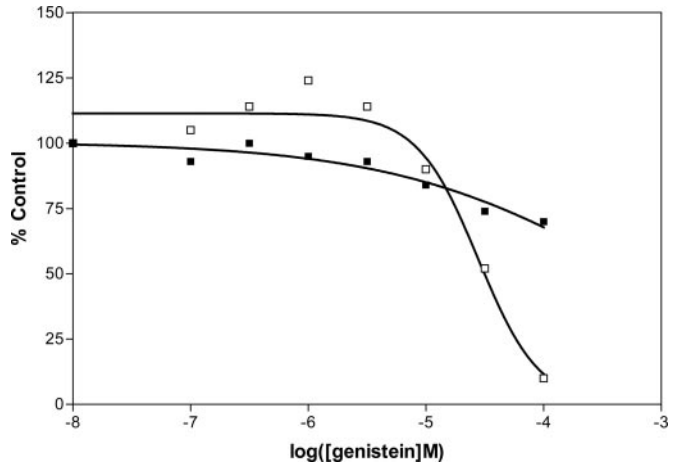

Fig. 3. Dose-dependent effects of genistein upon cellular levels of $[PP]_2$-$InsP_4$ and PP-$InsP_5$. $[^3H]$Inositol-labeled cells were incubated for 60 min with the indicated concentrations of genistein. Cells were quenched, extracted, and analyzed by HPLC as described under *Materials and Methods*. Data are presented as percentages of vehicle-treated controls. Data are representative of three experiments.

with chlorpromazine (Byrum et al., 2004), whereas KN-93 (500 μM) and K-252a (50 μM) calmodulin kinase inhibitors had no effect. Because it has been proposed that W-7 is cytotoxic (Jan et al., 2000), its effects were more systematically assessed. Cells treated with W-7 or chlorpromazine (up to 1 mM) for 1 h excluded trypan blue (>97% exclusion), even after a 24-h recovery period in DMEM. At this highest dose, a morphological change occurred. Cells lost their usual stellate shapes and became rounded. This is consistent with a significant decrease in cellular phosphatidylinositol 4,5-bisphosphate levels, which is accompanied by an increase in phosphatidylinositol 4-phosphate (Fig. 5a). ATP levels were also determined. These, like inositol phosphate levels, were seriously compromised at higher doses of both W-7 and chlorpromazine (Fig. 5b). Measurement of extracellular ATP and inositol phosphate levels indicated that much of that lost was recoverable from outside the cells. This indicates that the cell membranes, despite their impermeability to trypan blue, allowed the passage of ATP and inositol phosphates.

One is also able to exclude any roles played by Rho-associated, coiled-coil-forming protein kinase II, protein kinase C-related protein kinase 2, mitogen- and stress-activated protein kinase 1, mitogen-activated protein kinase-activated protein kinase-1b, and p70 ribosomal protein S6 kinase, because HA1077 (3 mM) and H-89 (1 μM), which are known not to affect $[PP]_2$-$InsP_4$ levels (Safrany and Shears, 1998), have been subsequently found to inhibit these kinases in addition to PKA and cyclic GMP-dependent protein kinase (Davies et al., 2000; Bain et al., 2003).

Potassium depletion of cells has also been shown to inhibit protein trafficking, and so potassium regulation of DIPs has also been investigated. Amiloride (1 mM), ouabain (100 μM), Cs^+ (10 mM), Ba^+ (1 mM), and tetraethylammonium (20 mM) were all found to have no effect. Oxidative stress afforded by treatment of cells with hydrogen peroxide (100 μM) also had no effect. Arachidonic acid (30 μM) did not alter levels of DIPs. No effect on levels was observed after treating cells with histamine [acting on H1 receptors (Cowlen et al., 1990)], lipopolysaccharide (10 μM), ZD7288 (1 mM), cycloheximide (100 μg/ml), pertussis toxin (50 ng/ml), or dexamethasone (1 nM). Also tested without effect were 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (10 μM), and its control 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (10 μM), and platelet-derived growth factor (50 ng/ml).

Using these data, along with those previously published, we can now address the roles of DIPs and HIPs in a cellular context. cNMPs can lower $[PP]_2$-$InsP_4$ levels selectively, these effect are not mediated by PKA or cyclic GMP-dependent protein kinase, nor are they mediated by cyclic nucleotide-gated cation channels. The mechanism and consequences of this regulation await to be determined. Hyperosmotic stress, which inhibits protein trafficking, significantly lowers PP-$InsP_5$ levels, and raises $[PP]_2$-$InsP_4$ levels, whereas treatment of cells with high (≥10 mM) levels of fluoride causes an increase in PP-$InsP_5$ and decrease in $[PP]_2$-$InsP_4$ levels. Lower levels of fluoride (<1 mM) cause a selective increase in PP-$InsP_5$ levels.

The sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase pump inhibitors thapsigargin and cyclopiazonic acid show a selective effect on PP-$InsP_5$, lowering this inositol phosphate

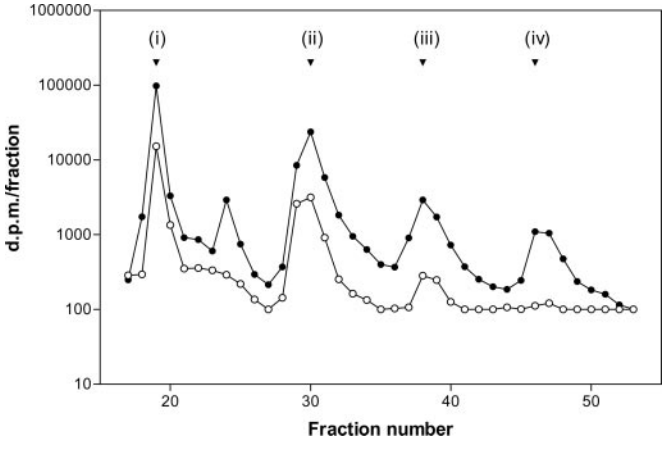

Fig. 4. HPLC analysis of inositol polyphosphates in DDT_1 MF-2 cells. [^{3}H]Inositol-labeled cells were vehicle-treated (3% ethanol, ●) or treated with W-7 (1 mM, 60 min) (○), quenched, extracted, and analyzed by HPLC as described under *Materials and Methods*. The identity of each peak was ascertained by reference to previous data using this HPLC system (Glennon and Shears, 1993; Shears et al., 1995) and by using standards of [^{3}H]$InsP_6$, PP-[^{3}H]$InsP_5$ and $[PP]_2$-[^{3}H]$InsP_4$. The ordinate is presented on a $\log_{10}$ scale to allow visualization of larger and smaller peaks in the same figure. The ^{3}H dpm in each peak for control (●) and W-7-treated (○) cells were as follows: peak i, $InsP_5$ = 103,400/16,210; peak ii, $InsP_6$ = 40,750/6684; peak iii, PP-$InsP_5$ = 5877/366; peak iv, $[PP]_2$-$InsP_4$ = 2256/41. The peak eluting at 24 min (3778/0) was an uncharacterized inositol pentakisphosphate.

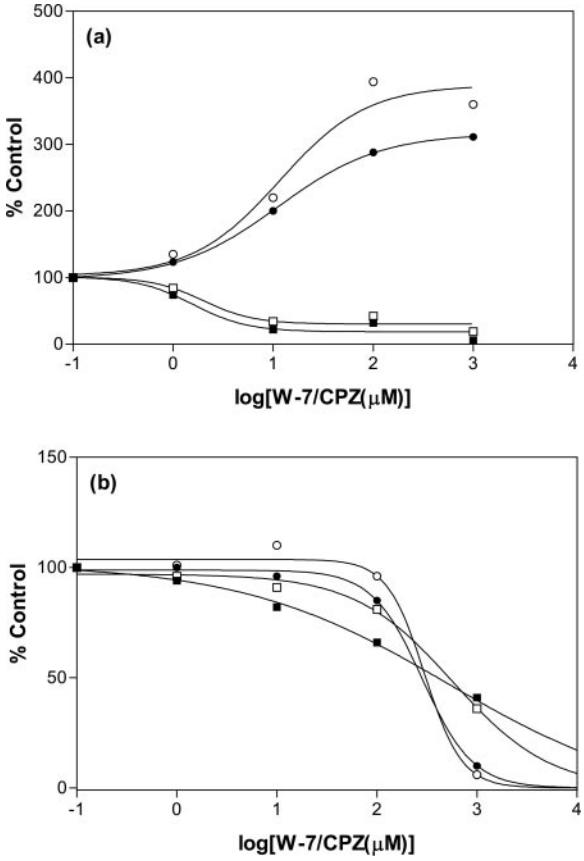

Fig. 5. Dose-dependent effects of W-7 (filled symbols) and chlorpromazine (open symbols) upon cellular levels of phosphatidylinositol 4-phosphate (circles) and $PtdIns(4,5)P_2$ (squares) (a) and ATP (circles) and combined HIPs and DIPs (squares) (b). Cells were incubated for 60 min with the indicated concentrations of drug. Cells were quenched, processed, and analyzed as described under *Materials and Methods*. Data are presented as percentages of vehicle-treated controls. Data are representative of two to four experiments

(Glennon and Shears, 1993), whereas W-7, a calmodulin inhibitor, lowers all HIPs and DIPs from $InsP_5$ to $[PP]_2$-$InsP_4$, although this reagent had greater effects on inositol lipids and ATP. In contrast, overnight treatment of cells with thapsigargin allow for PP-$InsP_5$ levels to rebound. Levels of PP-$InsP_5$ were doubled by overnight treatment with thapsigargin (10 μM) (Fig. 6). Interferons α, β, and γ were unable to affect levels of inositol phosphates, despite interferon β having been shown to up-regulate $InsP_6$ kinase (Morrison et al., 2001).

These effects were not limited to DDT_1 MF-2 cells. Treatment of human embryonic kidney 293 and human cervix epithelioid carcinoma HeLa cells afforded similar results. This suggests that these treatments can be used in a wide variety of cell types to allow the study of DIPs.

Discussion

The DIPs, discovered some 10 years ago, have remained the Cinderellas of the inositol phosphate field. Their low levels and lability make them difficult to study. Even when labeling cells with high amounts of [*myo*-^{3}H]inositol, it is difficult to determine their levels. Levels of these DIPs do seem to increase if cultured cells are maintained in a Krebs-like buffer for 2 to 3 h before analysis (compare Figs. 4 and 6). Despite this, they do seem to undergo a rapid turnover and are quickly regulated by physiological and pharmacological stimuli. I describe here a number of ways in which the levels of the DIPs are regulated. As with the previously published observation that cNMPs regulate $[PP]_2$-$InsP_4$, these current observations identify new pathways by which well established treatments may cause their effects.

In attempting to understand the mechanism by which cNMPs, I have tested a number of treatments implying that cNMPs act through novel pathways. cNMPs cause activation of the glucocorticoid receptor; these effects may be partly PKA-independent (Eickelberg et al., 1999). The ability of dexamethasone to alter $[PP]_2$-$InsP_4$ was tested. The lack of effects suggests that GR activation by cNMPs is not upstream of the effects on DIPs. A report that cNMPs can also regulate Na^+ channels (Niisato et al., 1999) was also investigated. Amiloride, a Na^+ transport blocker was found to be without effect, as was ouabain, a Na^+/K^+ ATPase inhibitor, suggesting, again, that cNMP-mediated Na^+ transport is not upstream from DIPs. The ability of genistein to mimic cNMPs on lowering $[PP]_2$-$InsP_4$ also suggests that the effects on $[PP]_2$-$InsP_4$ mediated by both cNMPs and genistein are not related to Na^+ channels, because genistein blocks cAMP-mediated Na^+ channel flux (Niisato et al., 1999). Because cNMPs are known to activate Ih currents (Bosmith et al., 1993), the effects of ZD7288, an effective and selective blocker of Ih channels, were tested. ZD7288 had no reproducible effect alone, and it did not alter the ability of cNMPs to lower $[PP]_2$-$InsP_4$.

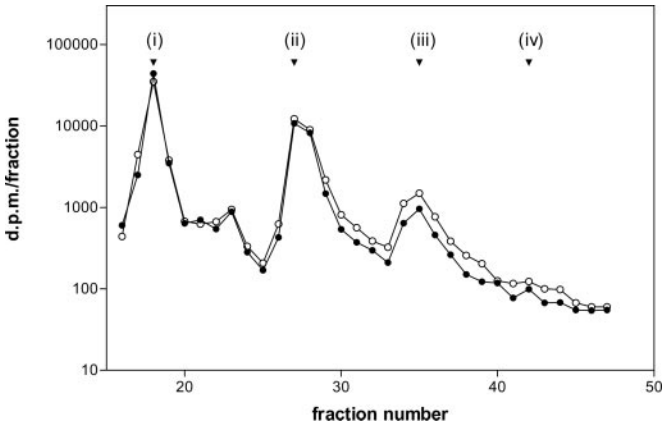

Fig. 6. HPLC analysis of inositol polyphosphates in DDT_1 MF-2 cells. [^{3}H]Inositol-labeled cells were untreated (●) or treated overnight with thapsigargin (10 μM) (○), quenched, extracted, and analyzed by HPLC as described under *Materials and Methods*. The ordinate is presented on a $\log_{10}$ scale, to allow visualization of larger and smaller peaks in the same figure. The ^{3}H dpm in each peak for control and thapsigargin-treated cells were as follows: peak i, $InsP_5$ = 51,400/43,885; peak ii, $InsP_6$ = 21,070/24,420; peak iii, PP-$InsP_5$ = 2116/3872; peak iv, $[PP]_2$-$InsP_4$ = 93/99. The peak eluting at 23 min (474/450) was an uncharacterized inositol pentakisphosphate.

The potent effects of genistein in lowering $[PP]_2$-$InsP_4$ levels and subsequently PP-$InsP_5$ levels, and raises further concerns regarding the specificity of this drug as a protein kinase inhibitor. One could suggest that the recorded effects of genistein now need reanalysis to determine whether some of its effects can be attributed to its regulation of $[PP]_2$-$InsP_4$. The mechanism of action of genistein and herbimycin differ greatly. Herbimycin acts via binding to protein sulfhydryl groups, and genistein competes with ATP at the active site (Simonson and Herman, 1993). These data suggest that the active site of PP-$InsP_5$ kinase resembles a protein tyrosine kinase ATP-binding pocket.

The ability of sorbitol to raise $[PP]_2$-$InsP_4$ levels suggests that the effects of hyperosmotic stress on protein trafficking (Oka et al., 1989) may be mediated by these effects, in that $[PP]_2$-$InsP_4$ has been previously shown to inhibit clathrin triskelion formation (Ye et al., 1995). Much discussion has taken place as to whether inositol phosphates or inositol lipids are the key ligands for proteins involved in the inhibition of clathrin triskelia (see Gaidarov et al., 1996; Hao et al., 1997), yet based on this and earlier (Jones et al., 1999) studies, it is apparent that levels of 3-phosphorylated inositides are decreased and only levels of $[PP]_2$-$InsP_4$ are increased. In contrast, K^+ depletion and cytosolic acidification, other treatments known to inhibit protein trafficking (Hansen et al., 1993), did not mimic sorbitol in its ability to raise $[PP]_2$-$InsP_4$. This suggests that these reagents have their effects by different mechanisms; it will be interesting to determine how these other treatments alter trafficking. It is noted that in *Saccharomyces cerevisiae*, a reduction in levels of DIPs leads to inhibited trafficking, and it is proposed that inositol phosphate-binding proteins could be regulated in a fashion analogous to heterotrimeric G-proteins (Saiardi et al., 2002). A recent article by Sajan et al. (2002) showed that sorbitol activated atypical protein kinase C and GLUT4 glucose transporter translocation and that these events were mediated by extracellular signal-regulated kinase and phospholipase D. That U0126 and butan-1-ol failed to inhibit sorbitol-mediated increases in $[PP]_2$-$InsP_4$ excludes these components from being upstream of $[PP]_2$-$InsP_4$. It is possible that $[PP]_2$-$InsP_4$ is upstream from these cellular components.

We have recently used W-7 and chlorpromazine to study the effects of depleting HIPs on Ku70 function (Byrum et al., 2004). Under conditions in which $InsP_5$ to $[PP]_2$-$InsP_4$ are reduced, Ku70 mobility is perturbed. These data suggest that some of these inositol phosphates represent physiological

regulators of DNA-dependent protein kinase, whereby Ku70 requires inositol phosphates to relocate to areas of DNA damage (Byrum et al., 2004). W-7 and chlorpromazine also affected levels of inositol lipids and depleted ATP. These observations mirror those made previously in human platelets (Strunecka et al., 1987; Tharmapathy et al., 2000). Phosphatidylinositol 4,5-bisphosphate is required for cytoskeletal integrity (Niebuhr et al., 2002), its depletion leading to a change in cellular morphology. Significant loss of ATP and inositol phosphates to the media would suggest that these cells are permeable to these small molecules (Holmsen and Rygh, 1990) and could explain how exogenous $InsP_6$ could effect Ku70 mobilization in W-7-treated cells (Byrum et al., 2004). The effects observed suggest that the cationic amphiphilic nature of these compounds allows shielding of the negative charge of ATP and inositol phosphates (among other components), thus allowing them to pass through the membrane down their concentration gradients. The effects on adenine nucleotides could be mimicked by the cationic detergent cetyl-trimethylammonium bromide, whereas in contrast, the anionic detergent dioctyl sulfosuccinate was without effect (Tharmapathy et al., 2000). The complex nature of the effects of W-7 and chlorpromazine may bring into question their usefulness in further studies of HIP and DIP function. It is clear that they have a number of other effects above that of calmodulin antagonists, although the ability of extracellular inositol phosphates to rescue a response can act as a good control.

I have also recently shown that $InsP_5$ levels can be selectively lowered by overexpressing PTEN M-CBR3. Such treatment of U87-MG cells decreased their proliferative rate (Orchiston et al., 2004). Although no manipulation that selectively regulates $InsP_6$ levels has yet been described, the overexpression of cytosolic multiple inositol polyphosphate phosphatase, achieved by removal of the C-terminal ER recycling signal (SDEL), has been shown previously to lower levels of $InsP_5$ by 60% and $InsP_6$ by 40%. This treatment, too, was found to cause a decrease in the rate of cell proliferation (Chi et al., 2000). It would be expected that such decreases in the levels of these DIP precursors would also lead to a decrease in the DIPs themselves, although this was not addressed in the above articles. A combination of these two approaches in parallel may yield insight into the roles of $InsP_6$ in mammalian cells.

These results, complemented by earlier studies, identify means by which DIPs can be regulated. The calmodulin inhibitors W-7 and chlorpromazine lower all HIPs and DIPs, PTEN M-CBR3 selectively lowers $InsP_5$, and cytosolic multiple inositol polyphosphate phosphatase lowers $InsP_5$ and $InsP_6$ levels. Short-term treatment with thapsigargin selectively raises PP-$InsP_5$ levels, whereas long-term treatment selectively lowers PP-$InsP_5$ levels. Low levels of fluoride raise PP-$InsP_5$ and $[PP]_2$-$InsP_4$; higher levels raise PP-$InsP_5$ and lower $[PP]_2$ to $InsP_4$. Genistein, along with cNMPs, lower $[PP]_2$-$InsP_4$ levels. Sucrose and sorbitol lower PP-$InsP_5$ and raise $[PP]_2$-$InsP_4$. In summary, a number of treatments can alter levels of DIPs, either *en masse* or selectively. These tools can now be used to regulate the DIPs and determine their roles and functions in intact cells.

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Address correspondence to: S. T. Safrany, Division of Cell Signaling, University of Dundee, MSI/WTB Complex, Dow Street, Dundee, DD1 5EH. E-mail: s.t.safrany@dundee.ac.uk