Protocols for regulation and study of diphosphoinositol polyphosphates.*

S.T. Safrany
Division of Cell Signalling
University of Dundee
MSI/WTB Complex
Dow Street
Dundee
DD1 5EH
Running Title Page

Running title: A diphosphoinositol polyphosphate toolkit.

Corresponding author:
S.T. Safrany
Division of Cell Signalling
University of Dundee
MSI/WTB Complex
Dow Street
Dundee
DD1 5EH
Tel (01382) 348018
Fax (01382) 345893
E-mail s.t.safrany@dundee.ac.uk

Number of text pages: 23
Number of tables: 0
Number of figures: 6
Number of references: 40
Number of words in
  Abstract: 250
  Introduction: 587
  Discussion: 1237
Abbreviations

cNMP, cyclic nucleotide monophosphate; DIP, diphosphoinositol polyphosphate; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylene-diamine-tetra-acetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIP, higher inositol phosphates; HPLC, high performance liquid chromatography; InsP₃, inositol 1,3,4,5,6-pentakisphosphate; InsP₆, inositol hexakisphosphate; 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; KN-93, 2-[N-(2-Hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; mpV(pic), monoperoxo(picolinato)oxovanadate(V); PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; PP-InsP₄, diphosphoinositol tetrakisphosphate; PP-InsP₅, diphosphoinositol pentakisphosphate; [PP]₂-InsP₄, bis-diphosphoinositol tetrakisphosphate; SERCA, sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase; W-7, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide; ZD7288, 4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino)pyridinium chloride.
Abstract

The roles of diphosphoinositol polyphosphates (DIPs) in mammalian cell biology have been difficult to determine, due to the lack of tools known to regulate their levels. I have determined a series of protocols which 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$_4$), whilst slightly lowering 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 previously described 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$_1$, 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 SERCA pump inhibitor previously shown to selectively lower PP-InsP$_5$ following acute treatment also selectively raises PP-InsP$_5$ following a longer-term treatment. The calmodulin inhibitors W-7 and chlorpromazine significantly lowered all higher inositol phosphates, as well as DIPs, whereas the calmodulin-dependent kinase inhibitors, K-252a and KN-93, were without effect. W-7 and chlorpromazine also lowered levels of phosphatidylinositol 4,5-bisphosphate and adenosine 5’-triphosphate 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 previously described, we can further understand the roles of the DIPs in cell biology.
Introduction

There is increasing evidence of the important roles played by inositol phosphates in cell biology. While the importance of inositol 1,4,5-trisphosphate as a regulator of intracellular free calcium has been understood for over 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 60+ inositol phosphates found in eukaryotes are intermediates. Nevertheless, there are an increasing number of inositol phosphates being identified having varied and diverse functions. In addition to calcium signalling, they have been implicated in nuclear mRNA export (York et al., 1999), chromatin remodelling (Shen et al., 2003; Steger et al., 2003), DNA repair (Byrum et al., 2004; Hanakahi et al., 2000), membrane trafficking (Saiardi et al., 2002; Ye et al., 1995) 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 phosphates 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 end point 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), a *Saccharomyces cerevisiae* homologue (KCS1) has also been characterised (Saiardi et al., 2000). InsP$_6$ kinases can also phosphorylate InsP$_5$, producing diphosphoinositol tetrakisphosphate (PP-InsP$_4$). PP-InsP$_5$ can be further phosphorylated by a kinase that is 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 previously been shown that PP-InsP$_5$ can be regulated by the SERCA 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 (Safrany et al., 1998; Shears et al., 1995). As such it was not a surprise to observe that incubating cells with low levels (<mM) fluoride increased levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$. Higher levels caused a paradoxical decrease in [PP]$_2$-InsP$_4$ levels which 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-InsP₃ and [PP]₂-InsP₄ 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 paper, I present, for the first time, new experimental protocols which 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 DDT₁ MF-2 with sorbitol or sucrose, a treatment that is known to inhibit protein trafficking, also raises [PP]₂-InsP₄ levels. These results identify a possible mechanism through which hyperosmotic stress acts, and that [PP]₂-InsP₄ 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 determine further the roles played by these inositol phosphates in physiology.
Materials and Methods

DDT<sub>1</sub> 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, NIEHS, NIH, 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% foetal calf serum at 37°C in 5% CO<sub>2</sub>/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 [³H]inositol. On the 4th day, cell monolayers were washed (2×250 µl) and then incubated (250 µl) in [³H]inositol-free HEPES-buffered Krebs-like media (115 mM NaCl, 5 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 11 mM glucose, 1.36 mM CaCl<sub>2</sub>, 25 mM HEPES, pH 7.4 with NaOH). Cells were maintained at 37°C for 3 h prior to the beginning of any experiments. Alternatively, overnight treatments were performed in DMEM containing [³H]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<sub>2</sub>CO<sub>3</sub> containing 5 mM Na<sub>2</sub>EDTA. 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<sub>2</sub>EDTA. Samples were stored at -20°C prior to 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<sub>2</sub>EDTA) and buffer B [buffer A plus 1.3 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, pH 3.85 with H<sub>3</sub>PO<sub>4</sub>; total [Pi] = 2.6 M] as follows: 0-5 min, 0% B; 5-10 min, 0-50% B; 10-60 min, 50-100% B; 60-70 min, 100% B. Fractions were collected at 1-min intervals, mixed with 4.2 vols of Flo-Scint IV scintillant, and radioactivity was determined using liquid scintillation spectrometry. EC<sub>50</sub> values were derived using Prism (GraphPad, San Diego, CA). Inositol lipids were extracted from the perchloric acid pellet, prepared and analysed as previously described (Batty and Downes, 1994). Peaks were ascribed by co-elution 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 [3H]inositol), or, for the determination of extracellular ATP, snap frozen and assayed directly.

Trypan blue exclusion was determined by incubating cells for 20-30 minutes 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

[3H]myo-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]-labelled 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 2-3 hours. 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$_4$ (Figure 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$\mu$M), 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$\mu$M 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 (Figure 2) and dose (Figure 3) dependent. Genistein lowered [PP]$_2$-InsP$_4$ levels in a dose-dependent manner, with an
EC$_{50}$ of 28µM. This is in close agreement with the published IC$_{50}$ for pp60$^{v-src}$ of 26µM (Akiyama et al., 1987). These effects were not mimicked by a similar protein kinase inhibitor, herbimycin A (10µM, data not shown), therefore excluding the role of a pp60$^{v-src}$-like kinase in regulating PP-InsP$_5$ phosphorylation. Dephostatin (100µM) and mpV(pic) (100µM), protein tyrosine phosphatase inhibitors, like the broad inhibitor, okadaic acid (1µM) were without effect. mpV(pic) did, however greatly raise levels of InsP$_4$, by over 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 (10µM), PD98059 (1mM), rapamycin (100nM) and wortmanin (100nM) (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 (>10µM), however, caused a marked and rapid decline of HIPs and DIPs, InsP$_3$ to [PP]$_2$-InsP$_4$, although the ratios between each of these remained constant, identifying that there was a specific block at the level of InsP$_3$ production (Figure 4). Similar data were obtained with chlorpromazine (Byrum et al., 2004), whereas KN-93 (500µM) and K-252a (50µM), calmodulin kinase inhibitors were without effect. As W-7 has been proposed as being cytotoxic (Jan et al., 2000), its effects were more systematically assessed. Cells treated with W-7 or chlorpromazine (up to 1mM) for 1 hour excluded trypan blue (>97% exclusion), even following a 24 hour recovery period in DMEM. At this highest dose, a morphological change occurred. Cells lost their usual stellate shapes are became rounded. This is consistent with a significant decrease in cellular phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) levels, which is accompanied by an increase in phosphatidylinositol 4-phosphate (PtdIns(4)P) (Figure 5a). Adenosine 5’-triphosphate (ATP) levels were also determined. These, like inositol phosphate levels, were seriously compromised at higher doses of both W-7 and chlorpromazine (Figure 5b). Measurement of extracellular ATP and inositol phosphate levels indicated that much of that lost was recoverable from outside the cells. This indicates that, despite being impermeant to trypan blue, the cell membranes 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, MAP kinase-activated protein kinase -1b, and p70 ribosomal protein S6 kinase, as HA1077 (3mM) and H-89 (1µM), which are known not to affect [PP]_2-InsP_3 levels (Safrany and Shears, 1998) have subsequently found to inhibit these kinases in addition to PKA and PKG (Bain et al., 2003; Davies et al., 2000).

Potassium depletion of cells has also been shown to inhibit protein trafficking, and so potassium regulation of DIPs has also been investigated. Amiloride (1mM), ouabain (100µM), Cs⁺ (10mM), Ba⁺ (1mM) and tetraethylammonium (20mM) were all found to have no effect. Equally oxidative stress, afforded by treatment of cells with hydrogen peroxide (100µM), was without effect. Arachidonic acid (30µM) also did not alter levels of DIPs. No effect on levels was observed treating cells with histamine (acting on H1 receptors (Cowlen et al., 1990)), lipopolysaccharide (10µM), ZD7288 (1mM), cycloheximide (100µg/ml), pertussis toxin (50ng/ml) or dexamethazone (1nM). Also tested without effect were PP2 (10µM), and its control PP3 (10µM) and platelet-derived growth factor (50ng/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 PKG, 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_3 levels, and raises [PP]_2-InsP_4 levels, whereas treatment of cells with high (≥10mM) levels of fluoride causes an increase in PP-InsP_3 and decrease in [PP]_2-InsP_4 levels. Lower levels of fluoride (<1mM) cause a selective increase in PP-InsP_3 levels.

The SERCA pump inhibitors thapsigargin and cyclopiazonic acid show a selective effect on PP-InsP_3, lowering this inositol phosphate (Glennon and Shears, 1993), whereas W-7, a calmodulin inhibitor lowers all HIPs and DIPs from InsP_3 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_3 levels to rebound. Levels of PP-InsP_3 were doubled by overnight treatment with thapsigargin (10µM) (Figure 6). Interferons α, β and γ were unable to affect levels of inositol phosphates, despite interferon β having been shown to upregulate InsP_6 kinase (Morrison et al., 2001).
These effects were not limited to DDT1 MF-2 cells. Treatment of human embryonic kidney (HEK293) and human cervix epitheloid 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 labelling cells with high amounts of $[^{3}H]$myo–inositol, it is difficult to determine their levels. Levels of these DIPs do appear to increase if cultured cells are maintained in a Krebs-like buffer for 2-3 hours before analysis (compare figures 4 and 6). Despite this, they do appear to undergo a rapid turnover, and are acutely 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 that implicate cNMPs as acting 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, as 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 was tested. ZD7288 had no reproducible effect alone, nor did it alter the ability of cNMPs to lower $[PP]_2$-InsP$_4$.

The potent effects of genistein in lowering $[PP]_2$-InsP$_4$ levels, and subsequently PP-InsP$_3$ levels 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 reanalysing, 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 sulphhydryl groups, genistein competes with ATP at the active site (Simonson and Herman, 1993). These data suggest that the active site of PP-InsP$_3$ 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, as \([PP]_{2}\)-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 from this and earlier (Jones et al., 1999) studies, it is apparent that levels of 3-phosphorylated inositides is 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 \(S.\) \textit{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 paper by Sajan et al. (Sajan et al., 2002) showed that sorbitol activated atypical PKC 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 whereby InsP\(_3\) 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). PtdIns(4,5)P\(_2\) 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\(_5\) 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 InsP₃ levels can be selectively lowered by overexpressing PTEN M-CBR3. Such treatment of U87-MG cells decreased their proliferative rate (Orchiston et al., 2004). While no manipulation that selectively regulates InsP₆ 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 InsP₃ by 60%, and InsP₆ levels 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 papers. A combination of these two approaches in parallel may yield insight into the roles of InsP₆ 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 InsP₃ and cytosolic multiple inositol polyphosphate phosphatase lowers InsP₅ and InsP₆ levels. Acute treatment with thapsigargin selectively raises PP-InsP₅ levels, whereas chronic treatment selectively lowers PP-InsP₃ levels. Low levels of fluoride raise PP-InsP₅ and [PP]₂-InsP₄; higher levels raise PP-InsP₅ and lower [PP]₂-InsP₄. Genistein, along with cNMPs, lower [PP]₂-InsP₄ levels. Sucrose and sorbitol lower PP-InsP₃ and raise [PP]₂-InsP₄. In summary, there are a number of treatments that 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.
Acknowledgments

STS is a Royal Society University Research Fellow. The author thanks the reviewers for improving this manuscript.
References


Gaidarov I, Chen Q, Falck JR, Reddy KK and Keen JH (1996) A functional phosphatidylinositol 3,4,5-
trisphosphate/phosphoinositide binding domain in the clathrin adaptor AP-2 alpha subunit.

Glennon MC and Shears SB (1993) Turnover of inositol pentakisphosphates, inositol hexakisphosphate and

Hanakahi LA, Bartlet-Jones M, Chappell C, Pappin D and West SC (2000) Binding of inositol phosphate to

Hansen SH, Sandvig K and Vandeurs B (1993) Clathrin and Ha2 Adapters - Effects of Potassium-

Regulation of AP-3 function by inositides. Identification of phosphatidylinositol 3,4,5-

Holmsen H and Rygh T (1990) Chlorpromazine makes the platelet plasma membrane permeable for low-

of diphosphoinositol pentakisphosphate kinase, which synthesizes the inositol pyrophosphate

Jan CR, Lu CH, Chen YC, Cheng JS, Tseng LL and Jun-Wen W (2000) Ca(2+) mobilization induced by


Morrison BH, Bauer JA, Kalvakolanu DV and Lindner DJ (2001) Inositol hexakisphosphate kinase 2
mediates growth suppressive and apoptotic effects of interferon-beta in ovarian carcinoma cells. J

Niebuhr K, Giuriato S, Pedron T, Philpott DJ, Gaits F, Sable J, Sheetz MP, Parsot C, Sansonetti PJ and
Payrastre B (2002) Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD


transport through proline-rich tyrosine kinase-2, the extracellular signal-regulated kinase pathway and phospholipase D. *Biochem J* **362**:665-74.


Footnotes

*This work was funded by The Royal Society and Tenovus-Scotland.
Legends for figures

Figure 1. Time-dependent effects of sucrose (squares) and sorbitol (diamonds) on cellular levels of \([\text{PP}]_2\text{-InsP}_4\) (open symbols) and PP-InsP\(_5\) (filled symbols). \(^{[3]}\text{H}\)Inositol-labelled cells were incubated for the indicated times with 0.5M sucrose or sorbitol. Cells were quenched, extracted and analyzed by HPLC as described in Materials and Methods. The levels of \([\text{PP}]_2\text{-InsP}_4\) and PP-InsP\(_5\) are shown as a percentage of vehicle-treated controls. Data are representative of 3-5 experiments.

Figure 2. Time-dependent effects of genistein on cellular levels of \([\text{PP}]_2\text{-InsP}_4\) (open squares) and [PP]-InsP\(_5\) (filled squares). \(^{[3]}\text{H}\)Inositol-labelled cells were incubated for the indicated times with 100\(\mu\)M genistein. Cells were quenched, extracted and analyzed by HPLC as described in Materials and Methods. The levels of \([\text{PP}]_2\text{-InsP}_4\) and PP-InsP\(_5\) are shown as a percentage of vehicle-treated controls. Data are representative of 3 experiments.

Figure 3. Dose-dependent effects of genistein upon cellular levels of \([\text{PP}]_2\text{-InsP}_4\) and PP-InsP\(_5\). \(^{[3]}\text{H}\)Inositol-labelled cells were incubated for 60 min with the indicated concentrations of genistein. Cells were quenched, extracted and analyzed by HPLC as described in Materials and Methods. Data are presented as percentages of vehicle-treated controls. Data are representative of 3 experiments.

Fig. 4. HPLC analysis of inositol polyphosphates in DDT\(_1\) MF-2 cells. \(^{[3]}\text{H}\)Inositol-labelled cells were vehicle-treated (3\% ethanol, filled circles) or treated with W-7 (1mM, 60 minutes) (open circles), quenched, extracted and analyzed by HPLC as described in 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 \(^{[3]}\text{H}\)InsP\(_6\), PP-\(^{[3]}\text{H}\)InsP\(_5\) and \([\text{PP}]_2\)\(^{[3]}\text{H}\)InsP\(_4\). The ordinate is presented on a log\(_{10}\) scale, to allow visualisation of larger and smaller peaks in the same figure. The \(^{[3]}\text{H}\) d.p.m. in each peak for control (filled circles) and W-7-treated (open circles) cells were as follows: peak i, InsP\(_5\) = 103,400/16,210; peak ii, InsP\(_6\) = 40,750/6,684; peak iii, PP-InsP\(_5\) = 5877/366; peak iv, \([\text{PP}]_2\)\text{-InsP}_4 = 2256/41. The peak eluting at 24 minutes (3778/0) was an uncharacterised inositol pentakisphosphate.
Figure 5. Dose-dependent effects of W-7 (filled symbols) and chlorpromazine (open symbols) upon cellular levels of (a) PtdIns(4)P (circles) and PtdIns(4,5)P$_2$ (squares) and (b) ATP (circles) and combined HIPs and DIPs (squares). Cells were incubated for 60 min with the indicated concentrations of drug. Cells were quenched, processed and analyzed as described in Materials and Methods. Data are presented as percentages of vehicle-treated controls. Data are representative of 2-4 experiments.

Fig. 6. HPLC analysis of inositol polyphosphates in DDT$_1$ MF-2 cells. [$^3$H]Inositol-labelled cells were untreated (filled circles) or treated overnight with thapsigargin (10µM) (open circles), quenched, extracted and analyzed by HPLC as described in Materials and Methods. The ordinate is presented on a log$_{10}$ scale, to allow visualisation of larger and smaller peaks in the same figure. The [$^3$H] d.p.m. 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$_5$ = 2,116/3,872; peak iv, [PP]$_2$-InsP$_4$ = 93/99. The peak eluting at 23 minutes (474/450) was an uncharacterised inositol pentakisphosphate.
Figure 1
Figure 2
Figure 3

The graph shows the relationship between log([genistein]M) and % Control. The data points and trend line indicate a decrease in % Control as the log concentration of [genistein]M increases.
Figure 4
Figure 5

(a) % Control vs. \( \log[W-7/CPZ(\mu M)] \)

(b) % Control vs. \( \log[W-7/CPZ(\mu M)] \)
Figure 6