Characterization of a Selective Inhibitor of Inositol Hexakisphosphate Kinases

USE IN DEFINING BIOLOGICAL ROLES AND METABOLIC RELATIONSHIPS OF INOSITOL PYR Phosphates

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Inositol hexakisphosphate kinases (IP6Ks) phosphorylate inositol hexakisphosphate (InsP6) to yield 5-diphosphoinositol pentakisphosphate (5-[PP]-InsP5 or InsP7). In this study, we report the characterization of a selective inhibitor, [(trifluoromethy)lbenzyl] N6-(p-nitrobenzyl)purine (TNP), for these enzymes. TNP dose-dependently and selectively inhibited the activity of IP6K in vitro and inhibited InsP7 and InsP8 synthesis in vivo without affecting levels of other inositol phosphates. TNP did not inhibit either human or yeast Vip/PPIP5K, a newly described InsP6/InsP7 1/3-kinase. Overexpression of IP6K1, -2, or -3 in cells rescued TNP inhibition of InsP7 synthesis. TNP had no effect on the activity of a large number of protein kinases, suggesting that it is selective for IP6Ks. TNP reversibly reduced InsP7/InsP8 levels. TNP in combination with genetic studies was used to implicate the involvement of two pathways for synthesis of InsP8 in yeast. TNP induced a fragmented vacuole phenotype in yeast, consistent with inhibition of Kcs1, a Saccharomyces cerevisiae IP6K. In addition, it also inhibited insulin release from Min6 cells in a dose-dependent manner further implicating InsP7 in this process. TNP thus provides a means of selectively and rapidly modulating cellular InsP7 levels, providing a new and versatile tool to study the biological function and metabolic relationships of inositol pyrophosphates.

Inositol(1,4,5)trisphosphate (Ins(1,4,5)P3)2 is the cytosolic product of inositol phospholipid-specific phospholipase C and serves multiple biological functions. In higher eukaryotes, it regulates Ca2+ release from intracellular stores via binding to Ins(1,4,5)P3-specific receptors located in the endoplasmic reticulum. In addition, in Saccharomyces cerevisiae and all other eukaryotes that have been studied, Ins(1,4,5)P3 also undergoes complex metabolism to generate a series of inositol polyphosphates with diverse functions (1, 2). Inositol hexakisphosphate (InsP6) can be synthesized in inositol phospholipid-specific phospholipase C/Ins(1,4,5)P3-mediated pathways in most, if not all, eukaryotes. InsP6 is further metabolized by the inositol hexakisphosphate kinases (IP6Ks) which add a pyrophosphate moiety at the 5-position to generate 5-[PP]-InsP5 or 5-InsP7. Dictyostelium discoideum and S. cerevisiae, each possess one IP6K gene product designated Kcs1 in yeast (3, 4). In mammals, three IP6Ks have been identified (5, 6). In addition, a second InsP6/5-InsP7 kinase, designated Vip/PPIP5K, has been identified in yeast (7) and mammalian cells (8, 9). This kinase is distinct from IP6K/Kcs1 in that it phosphorylates the 1/3-position of InsP6 and 5-[PP]-InsP5 (10).

Insights into the biological functions of inositol pyrophosphates have come from genetic studies in yeast and by manipulating expression of inositol polyphosphate kinases in mammalian cells. More recently, yeast mutants failing to synthesize inositol pyrophosphate molecules have been found to be impaired in several cellular functions, such as DNA repair, chromatin remodeling, and telomere length maintenance (11–14). Production of 1/3-InsP5 is necessary for regulating cellular phosphate starvation responses through the inhibition of the cyclin/cyclin-dependent kinase (7, 10, 15). In D. discoideum, InsP7 has been reported to be important in chemotaxis (3). In mammals, overexpression of the IP6K1 increases insulin release from pancreatic β cells, whereas overexpression of IP6K2 increases apoptosis in a variety of cell lines (16, 17).

As a means to further study the roles of inositol pyrophosphate messengers, we set out to develop pharmacological tools that permit acute inhibition of specific inositol polyphosphate kinases. Here we report the characterization of an IP6K inhibitor, which appears to be selective in vitro and in vivo and its use in furthering our understanding of the role of InsP7 synthesis in yeast and in insulin secretion in mammalian cells.
Inhibitor for IP6Ks

EXPERIMENTAL PROCEDURES

Materials

IP3-3 kinase inhibitor (TNP; see “Results”) and thapsigargin (TG) were from Calbiochem. [2-3H]Insitol and [γ-32P]ATP were from GE Healthcare. HeLa cells were from ATCC. PEI-cellulose TLC sheets were from Merck. Reduced glutathione was from Sigma. Oligonucleotides were from the University of Dundee in-house DNA synthesis center.

Methods

Cloning—The human IP6K1 and the catalytic fragment of IP3-3KA were cloned from IMAGE clones using the following primers: IP6K1, 5′-GACGATCATGGTCAAGCATTCAGCTGGTCTCAGTTCTG-3′ and 5′-GACGATCGCTACCTGTCGATGGTGCGATTTCG-3′; IP3-3KA, 5′-CATGGAATTCGCGAGCTGCGTGAGTCCGAT-3′ and 5′-CATGGAATTCAGTCTCGAGGCAGTCGTTTCTC-3′.

The primers include the recognition sequences for EcoRI and Sall, respectively. The genes were amplified using PCR using the Hi-fidelity PCR enzyme (Roche Applied Science). IP6K1 was cloned into the mammalian expression vector enhanced green fluorescence protein (Clontech), whereas the catalytic fragment of IP3-3KA was cloned into pGEX-6P-1 (for bacterial expression) as EcoRI-SalI fragments. Clones were sequenced in the in-house sequencing center.

Cell Culture—HeLa (human cervical cancer) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with fetal bovine serum (10%, v/v) at 37 °C in a humidified 5% CO2, 95% air environment. HeLa stable cell lines were generated by transfecting cells grown on 3.5-cm dishes with appropriate amounts of vector DNA of enhanced green fluorescence protein or enhanced green fluorescence protein-IP6K1 using the transfection reagent polyethyleneimine (10 μg/well) at ~70% confluence. We routinely achieved about 90% transfection efficiency by this method. After 48 h, the medium was replaced by similar medium as above but containing 0.8 mg/ml G418 sulfate. Stably expressing clones were selected after 3 weeks of growth in selection medium. Clones were identified by observing GFP fluorescence microscopically and by monitoring protein expression by Western blotting using anti-GFP antibody (Abcam).

Min6 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) containing heat-inactivated serum (15%, v/v) and 72 h after induction with isopropyl 1-thio-β-D-galactopyranoside and reduction of the bacterial growth temperature to 26 °C. The bacteria were harvested 18 h after induction with isopropyl 1-thio-β-D-galactopyranoside by centrifugation at 4000 rpm for 40 min at 4 °C. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μM β-mercaptoethanol, and complete protease inhibitor tablet from Roche Applied Science). Cells were ruptured by sonication (six 15-s bursts with 45 ± 6°C cooling between two bursts) on an Ultrasonic processor (VCX-750W) sonicator at 65% power. Subsequently, the sonicate was centrifuged at 18,590/40 for 40 min at 4 °C on an Avanti J-25 centrifuge. The supernatant was incubated with 1 ml of washed (with lysis buffer) glutathione-Sepharose 6B beads (Amersham Biosciences) for 2 h at 4 °C. Following incubation, the supernatant was aspirated off, and beads were washed with 50 ml of wash buffer (lysis buffer with no Triton X-100). Finally, the protein was eluted from beads with elution buffer (wash buffer plus 2 M reduced glutathione). Protein was electrophoresed on a Novex 4–12% BisTris gel in 1X MOPS running buffer (Invitrogen), and the gel was Coomassie-stained for verifying the purity of the protein. The protein was concentrated and stored at a final concentration of 2 mg/ml in wash buffer plus 50% glycerol (v/v) at −20 °C.

IP3-3K Assays—30 mM IP3-3KA was incubated with 0.1 μCi of [γ-32P]ATP and 5 μM Ins(1,4,5)P3 in assay buffer (20 mM Tris-HCl, pH 7.5, plus 100 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl2). 1 μl of the assay was spotted onto PEI-cellulose TLC sheets. Samples were chromatographed in 25 mM ammonium formate plus 1.0 M formic acid to separate out [32P]InsP4, [γ-32P]ATP, and any inorganic phosphate ([32P]Pi). The sheets

above lysate with 50 μl of protein G beads on a shaking nutator for 3 h at 4 °C. The lysate was aspirated off, and the beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of assay buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1 mM Na2OVO4, 1 mM sodium pyrophosphate, 1 mM NaF, 5 mM sodium β-glycerophosphate, and 1 mM β-mercaptoethanol) before electrophoresis for Western blot or activity assays. For activity assays, the beads were finally suspended in 200 μl of lysis buffer containing 5 μl of [2-3H]InsP6 (specific activity = 21.4 Ci/mmol, 5 μCi/0.5 ml) and appropriate concentrations of TNP or DMSO. Final concentration of DMSO was 1% (v/v). The reaction was started by the addition of ATP at a final concentration of 1 mM. Control reactions contained no ATP. Tubes were incubated at 37 °C on a shaking nutator for 1 h. Reactions were stopped by the addition of 300 μl of 1 mM EDTA. Reactions were centrifuged briefly, and the supernatant was transferred to a fresh tube. [2-3H]InsP6 was separated from [2-3H]InsP7 using HPLC (see below). [2-3H]InsP6 produced was estimated using the ratio of the DPM in the corresponding peak to the total DPM in both peaks. [2-3H]InsP7 was plotted against the corresponding TNP concentration for curve fitting (Sigma Plot software) and calculation of IC50 values.

Expression and Purification of the Catalytic Fragment of IP3-3KA—The catalytic fragment of IP3-3KA was expressed as a GST fusion protein in the BL21 Escherichia coli strain. 1 liter of E. coli was grown to an OD of 1.0 at 37 °C. Protein expression was induced by the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and reduction of the bacterial growth temperature to 26 °C. The bacteria were harvested 18 h after induction with isopropyl 1-thio-β-D-galactopyranoside by centrifugation at 4000 rpm for 40 min at 4 °C. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μM β-mercaptoethanol, and complete protease inhibitor tablet from Roche Applied Science). Cells were ruptured by sonication (six 15-s bursts with 45 ± 6°C cooling between two bursts) on an Ultrasonic processor (VCX-750W) sonicator at 65% power. Subsequently, the sonicate was centrifuged at 18,590 × g for 40 min at 4 °C on an Avanti J-25 centrifuge. The supernatant was incubated with 1 ml of washed (with lysis buffer) glutathione-Sepharose 6B beads (Amersham Biosciences) for 2 h at 4 °C. Following incubation, the supernatant was aspirated off, and beads were washed with 50 ml of wash buffer (lysis buffer with no Triton X-100). Finally, the protein was eluted from beads with elution buffer (wash buffer plus 2 M reduced glutathione). Protein was electrophoresed on a Novex 4–12% BisTris gel in 1X MOPS running buffer (Invitrogen), and the gel was Coomassie-stained for verifying the purity of the protein. The protein was concentrated and stored at a final concentration of 2 mg/ml in wash buffer plus 50% glycerol (v/v) at −20 °C.
were dried and then scanned using a PhosphorImager. The \([^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4\) formed was measured by calculating density corresponding to \([^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4\) as a percentage of the total density of \(\gamma\)-ATP plus \([^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4\). Percentage of \([^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4\) produced was plotted against TNP concentration. Curve fitting to the data was done using the SigmaPlot software. \(K_m/IC_{SO}\) values were calculated from the equation provided by the software.

**Calculation of \(K_v\) Values**—\(K_v\) values were determined using the equation,

\[
K_v = IC_{SO}/(1 + ([\text{substrate}]/K_m)),
\]

where \(IC_{SO}\) represents the value determined from curve fit analysis, \(K_m\) is the value for ATP, and [substrate] is the concentration of ATP used in the IP3-3K or IP6K1 assay, respectively.

**Vip/PIP5K Assays**—100 ng of purified Homo sapiens Vip2 fragment (amino acids 1–365) or \(S\). cerevisiae Vip1 was preincubated with vehicle (DMSO) or 10 \(\mu\)M TNP for 30 min on ice. Following the pretreatment, \(\gamma\)-ATP and 100 \(\mu\)M \(\text{Ins}\text{P}_6\) was added to the enzyme, and the assay was carried out at 37 °C for 60 min as per the protocol reported earlier by Friddy et al. (28). \([^{32}\text{P}]\text{P}_\gamma\text{ATP}, [^{32}\text{P}]\text{ATP, } [^{32}\text{P}]\text{InsP}_7, \text{ and } [^{32}\text{P}]\text{InsP}_8\) formed during the assay were visualized after separation by PEI-cellulose thin layer chromatography in a tank containing 1.09 \(m\) \(\text{KH}_2\text{PO}_4, 0.72 \(m\) \(\text{K}_2\text{HPO}_4, \) and 2.13 \(m\) HCl and subsequent phosphorimaging.

**[2-3H]Inositol Labeling of Cells**—HeLa cells were seeded at 1 \times 10^6 cells on 60-mm plates in 3 ml of inositol-free medium supplemented with dialyzed fetal calf serum (10% (v/v)), supplemented with dialyzed fetal calf serum (10% (v/v)). After 24 h, Tris (10\(\times\)) supplemented conditions were applied for 16 h. Following the pretreatment, \(\gamma\)-ATP and 100 \(\mu\)M \(\text{Ins}\text{P}_6\) was added to the enzyme, and the assay was carried out at 37 °C for 60 min as per the protocol reported earlier by Friddy et al. (28). \([^{32}\text{P}]\text{P}_\gamma\text{ATP, [^{32}\text{P}]ATP, [^{32}\text{P}]InsP}_7, \text{ and [^{32}\text{P}]InsP}_8\) formed during the assay were visualized after separation by PEI-cellulose thin layer chromatography in a tank containing 1.09 \(m\) \(\text{KH}_2\text{PO}_4, 0.72 \(m\) \(\text{K}_2\text{HPO}_4, \) and 2.13 \(m\) HCl and subsequent phosphorimaging.

**Extraction and Analysis of Inositol Polyphosphates by HPLC**—Stimulated or transfected cells were washed with ice-cold PBS and then lysed with 0.5 \(m\) trichloroacetic acid. Subsequently, cells were scraped, and the supernatant was separated from the debris by centrifugation in a cold microcentrifuge for 10 min at full speed. The supernatant was neutralized by the addition of 1 \(m\) \(\text{K}_2\text{CO}_3, and the inositol phosphates were extracted, as explained above.**

**Insulin Release Assay from Min6 Cells**—Medium was removed, and cells were washed 10 times with glucose-free KREBS (10 \(mm\) Hapes-NaOH, pH 7.4, 119 \(mm\) NaCl, 4.74 \(mm\) KCl, 2.54 \(mm\) \(\text{CaCl}_2, \) 1.19 \(mm\) MgCl2, 1.19 \(mm\) \(\text{KH}_2\text{PO}_4, \) 25 \(mm\) NaHCO3, and 1 \(mm\) EGTA) to remove traces of culture medium and serum. Cells were treated with various concentrations of TNP for 2 h in 200 \(\mu\)l of glucose-free KREBS under the same conditions of temperature and CO2 as under culturing conditions. Following treatment with TNP, the glucose-free KREBS was replaced with KREBS containing 2.5 \(mm\) glucose and TNP at the same concentrations. 5 min later, aliquots of cell supernatants were taken out and used for measurement of insulin release. Insulin released was measured using the rat/mouse insulin enzyme-linked immunosorbent assay kit (Linco Laboratoories), as per the manufacturer’s instructions. For each assay, a standard curve was generated using purified insulin provided in the kit. Aliquots of cell supernatants withdrawn during the assay were diluted 1:10 with glucose-free KREBS to give a final absorbance value within the linear range of the standard curve. The
amount of insulin released was calculated from the standard curve generated. Blanks were stimulated with glucose-free KREBS.

RESULTS

IP6Ks belong to the IPK superfamily of inositol polyphosphate kinases, also comprising IP3-3Ks and IPMKs by virtue of significant homology in their sequences. A common structural fold has been reported for IP3-3KA from mammals and Ipk2p from S. cerevisiae, where the N and C termini of the proteins coordinate ATP binding, and the middle of the protein forms the inositol phosphate binding domain (18, 19). Since IP6Ks share sequence homology in the ATP and inositol phosphate binding regions with the other members of the IPK family, it is possible that they share a similar structural fold. Alignment of the ATP and inositol phosphate (IP) binding regions from human IP3-3KA, IP6K1-3, Kcs1p, and Ipk2p are shown in Fig. 1. Although both IP3-3KA and Kcs1p contain N-terminal sequences (not shown) that do not exist in the IP6Ks or Ipk2p, all of the members of the IPK family show high levels of conservation in their ATP binding regions and IP binding regions but little sequence conservation elsewhere (18, 20). For example, each contains the conserved “GH” (residues 34 and 35 in IP6K1) and the “SLL” motifs (residues 334–336) important for ATP binding and the fully conserved “PXXXDKX” motif (residues 221–226) required for IP binding.

A family of molecules having a purine base with either a nitrobenzyl (TNP; Fig. S1A), aminobenzyl (TAP; Fig. S1B), or benzyl (TPB; Fig. S1C) side chain attached at the N-6 position and an (m-(trifluoromethyl)benzyl) attached at the N-2 group were characterized as inhibitors for IP3-3K from a panel of purine-based molecules. Of these, TNP was found to have the lowest Ki value (4.3 μM) for IP3-3K (21). We have now characterized this compound as a potential inhibitor for IP6Ks.

**TNP Is a Selective Inhibitor of the IP6Ks in Vitro and in Vivo**

To determine if IP6Ks and IP3-3Ks are inhibited by TNP, we performed in vitro activity assays with both enzymes in the presence of increasing concentrations of TNP. For this purpose, HA-tagged IP6K1 was expressed and immunoprecipitated from HeLa cells. The Western blot of the immunoprecipitated enzyme is shown in Fig. 2A. The immunoprecipitated enzyme was incubated with [γ-32P]ATP and Ins(1,4,5)P3, and production of [32P]Ins(1,3,4,5)P4 was monitored in the presence of increasing

**FIGURE 1. ClustalW alignment of the IPK family members.** The following proteins are aligned: hIP3-3KA, hIP6K1, hIP6K2, hIP6K3, Sckcs1, and Sckip2, where the prefix “h” represents H. sapiens and “Sc” represents S. cerevisiae. Accession numbers are as follows: IP3-3KA, NP_002211; IP6K1, NP_695005; IP6K2, NP_057375; IP6K3, NP_473452; Sckcs1, NP_010300; Sckip2p, NP_010458. Amino acids highlighted in black boxes indicate identical residues. Amino acids highlighted in gray boxes indicate the conserved nature of the amino acids (such as hydrophobicity, charge, etc.). The numbers indicate the amino acids from the corresponding protein shown in that line.
doses of TNP by PEI-TLC separation of ATP and Ins(1,3,4,5)P₄ and subsequent radioimaging (Fig. 2E). The formation of Ins(1,3,4,5)P₄ decreased with increasing TNP, and the IC₅₀ value calculated from curve fitting was around 18 μM from two different batches of protein purified (Fig. 2E and F). In this case, the IC₅₀ and Kᵢ values are equivalent due to the low ATP concentrations in the assay. The Kᵢ of 18 μM (Table 1) is somewhat higher than the values reported by other authors (21, 22).

The in vitro data translate to an ~30-fold difference in the Kᵢ values for TNP between IP6K1 and IP3-3KA (Table 1), whereas the Kᵢ values for ATP were ~100-fold apart. From these data, the following conclusions were drawn. 1) When the ATP concentra-
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In each case, the total radioactivity under the corresponding peak (base line-subtracted) was divided by the squares extracted (see “Experimental Procedures”). Since the InsP6 peak did not change by more than 10% between all the data.

**TABLE 1**

| Name of enzyme | IP3-3KA | IPMK | IP6K1 |
|----------------|---------|------|-------|
| Substrate (μM) | IC50 (μM) | Ki (μM) | Km (M) |
| InsP3 | 0.01 | 0.01 | 0.01 |
| InsP4 | 10.8 | 10.8 | 10.8 |
| InsP5 | 18.0 | 18.0 | 18.0 |
| InsP6 | 17.9 | 17.9 | 17.9 |
| InsP7 | 17.9 | 17.9 | 17.9 |

**a** Values for IP3-3KA were determined in our laboratory and in other laboratories (22), and values for IPMK and IP6K1 have been reported elsewhere (5, 6, 23, 24).

**b** Values determined in this work.

**c** Not determined.

**d** Ki values were determined using the equation, Ki = IC50/1 + ([substrate]/Km).

We next tested if the decreases in cellular InsP7 caused by TNP treatment could be rescued by overexpression of IP6Ks. HeLa cells transformed with IP6K1 were labeled with [3H]inositol to equilibrium, and InsP7 levels were evaluated as a function of TNP concentration. Fig. 4A shows the HPLC separation of InsP6 and InsP7 isolated from cells overexpressing IP6K1 and control cells, treated with different concentrations of TNP. IP6K1-overexpressing cells had 2-fold more InsP7 than control cells (Fig. 4B). Following TNP treatment, InsP7 levels decreased in parallel in the two cell systems. As expected, IC50 values were not significantly different (2.0 and 1.2 μM), but at the IC50 value, IP6K1-overexpressing cells had InsP7 levels comparable with noninhibited control cells, and at 10 μM TNP, the residual InsP7 was ~50% of that in untreated controls. Hence, IP6K1 overexpression rescues InsP7 synthesis in the face of treatment with TNP. TNP had a similar effect on InsP7 levels in cells overexpressing IP6K2 and -3 as those overexpressing IP6K1 (Fig. S2). Since all three mammalian IP6Ks share significant homology with each other and all of them have similar Km values for ATP, the 90% reduction in InsP7 levels in cells overexpressing IP6K1 (Fig. 4B) or IP6K2 or -3 (Fig.
S2) strongly suggests that the effect of TNP on InsP₇ occurs via direct inhibition of one or all three kinases.

Previous studies reported an increase in basal calcium levels and a subsequent decrease in the amounts of ATP-stimulated calcium release in cells treated with TNP (21). Such an increase in intracellular calcium could conceivably lead to the observed decrease in InsP₆, through indirect mechanisms. To investigate this possibility, InsP₃ levels in equilibrium-labeled cells treated with TNP were compared with those in cells treated with TG, which increases intracellular basal levels of calcium by inhibiting the Ca²⁺ pumps on the ER (26). HPLC separation of the various inositol phosphates isolated from cells treated with TNP and TG are shown in Fig. 5A. Quantification of InsP₃, InsP₄, and InsP₇ as a percentage of InsP₆ shows that there are significant differences in InsP₇ levels between TG- and TNP-treated cells. TNP-treated cells showed a dramatic 90% reduction in InsP₇, whereas TG-treated cells showed only minor changes in InsP₇ (20–30%) when compared with InsP₇ levels in controls. There was no significant change in InsP₅ levels in TG-treated cells (94% of control), and this observation serves as a good negative control, since it has been comprehensively established that exposure to TG increases intracellular calcium without any change in Ins(1,4,5)P₃ levels. These data further support the hypothesis that decreases in InsP₇ levels following TNP treatment occur due to a specific inhibition of IP6Ks.

Additionally, we sought to determine the effects of TNP on the Vip/PPIP5K class of kinases (7–9). In vitro assays were conducted with an active fragment of H. sapiens Vip2 or S. cerevisiae Vip1, in the presence and absence of TNP. Vip2 produced either [PP]-InsP₇ (InsP₇) or [PP]₂-InsP₄ (InsP₈) when InsP₆ was used as the substrate for the reaction, and its activity was unaffected by the presence of TNP (Fig. 6). The activity of S. cerevisiae Vip1 was also unaffected when 5-[PP]-InsP₇ was used as a substrate (data not shown). These data demonstrate that Vip/ PPIP5K enzymes are not targets of TNP.

Furthermore, we tested the effects of TNP on a panel of protein kinases. In vitro assays were performed in the presence of 1, 10, and 100 μM TNP, using protocols described earlier (27–29). None of the kinases in the panel were inhibited by TNP even at 100 μM. Since all experiments in this study were carried out at 10 μM concentrations, this observation underlines the fact that TNP is specific for the IPK family.

Since TNP is a purine analog and inhibits IP6Ks via binding to the ATP binding site, the effect of TNP should be reversible. The reversibility of TNP inhibition was monitored by treating HeLa cells with TNP and allowing them to recover. Fig. 7 shows the results of this experiment. Following treatment with TNP, InsP₇ levels fell, and when cells were allowed to recover for 2 h after the removal of TNP, InsP₇ levels returned close to control levels, showing that TNP inhibition of IP6Ks is reversible.

**TNP Is a Useful Pharmacological Tool for Examining the Metabolism and Biological Functions of Inositol Pyrophosphates**

Genetic and biochemical analyses demonstrate that InsP₈ is produced by the combined action of IP6K and Vip/PPIP5K activities. Two pathways have been proposed. In pathway I, InsP₈ is first pyrophosphorylated at the 5-position by IP6K and then at the 1/3-position by Vip/PPIP5K, whereas pathway II is the reciprocal order (Fig. 8A). Given the selective effects of TNP on the IP6K proteins, we postulated that the drug would be a useful probe for acutely monitoring the relative contributions of each InsP₈ biosynthetic pathway. TNP inhibition of pathway I would deplete both InsP₇ and InsP₈, whereas only InsP₈ levels would change if TNP were to act through pathway II. Since basal levels of InsP₈ in many cell types are near or below the detection limits, we first enhanced its production using hyperosmotic stress, as previously reported (30). To this end, HeLa cells labeled to equilibrium with [³H]inositol were stressed by treatment with 0.2 M sorbitol in the presence or absence of TNP, and inositol polyphosphates were separated and analyzed by HPLC. Sorbitol treatment caused a 5-fold increase
in the peak corresponding to InsP₈ (Figs. 8, B and C). TNP reduced the levels of both InsP₇ and InsP₈ in a dose-dependent manner with IC₅₀ values of ~3.0 and 1.0 μM. The extent of inhibition at the highest dose of TNP used was ~90%, consistent with the notion that pathway I accounts for the majority of InsP₇ synthesis under the equilibrium labeling conditions tested.

However, because previous studies have shown that loss of the inositol pyrophosphatase (Ddp1) unmasks or exposes pathway II (12, 31), which is otherwise undetectable by equilibrium radiolabeling methods, we tested TNP inhibition of InsP₇ metabolism in a variety of budding yeast strains. In WT cells, the basal level of InsP₇ is about 3% of that of InsP₆. Upon treatment of wild-type cells with TNP, we observed a loss of InsP₇ (Fig. 9A). Similarly, when Kcs1 is deleted, this peak of InsP₇ is lost, consistent with inhibition by TNP of pathway I. In further support of pathway I, we observed an ~3-fold increase in the level of InsP₇ in cells deficient for Vip1 (vip1Δ) and found that treatment with TNP or concomitantly deleting Kcs1 (vip1kcs1ΔΔ) ablated InsP₇ accumulation (Fig. 9B).

We confirmed that pathway II could be unmasked by analyzing strains deficient for Ddp1 (ddp1Δ) and observed a 3-fold increase in InsP₇ levels upon loss of the phosphatase (Fig. 9C). Treatment with TNP or co-deletion of Kcs1 (ddp1kcs1ΔΔ) further increased InsP₇ (presumably 3-InsP₇) levels 5–7-fold (15–21-fold as compared with wild-type cells) (Fig. 9C), consistent with a major flux of metabolism through pathway II, which could only be visualized by equilibrium labeling when Ddp1 was inactivated. Together, our experiments offer support for the presence of both pathway I and II in yeast.

In order to study the phenotypic effects of TNP on cellular functions proposed for [PP]-InsP₅, we utilized two biological systems. In S. cerevisiae, loss of the IP6K, Kcs1, results in aberrant vacuolar fragmentation and morphology (4, 32). Wild-type yeast strains were therefore treated with TNP for 2 h, and vacuoles were analyzed using the fluorescent probe, Cell Tracker CMAC (blue), which selectively stains the vacuolar lumen. Treatment with TNP rapidly caused the development of multi-fragmented vacuoles in wild type yeast (Fig. 9D (i)) compared with DMSO-treated controls (Fig. 9D (ii)), a result that is very similar to that which is observed in ipk2Δ and kcs1Δ strains (Figs. 9D, iii and iv). The fact that TNP mimics the vacuolar phenotype observed in these KO strains confirms the utility of TNP as a probe for the biological functions of inositol pyrophosphates and shows for the first time that a relatively acute loss of InsP₇ rapidly affects vacuolar dynamics.

We next used a second biological system developed from observations that alterations in InsP₇ influence the sensitivity of pancreatic β cells and insulinoma cell lines to glucose-stimulated insulin secretion (16). We therefore followed insulin release from Min6 pancreatoma cells stimulated with glucose. These cells showed a robust response to glucose that increased insulin release by about 5-fold compared with basal level, a response that was dose-dependently inhibited by TNP. Overexpression of IP6K1 significantly increased insulin secretion in this model and substantially protected these cells from the inhibitory effects of TNP, suggesting that TNP acts to inhibit secretion via its inhibition of one or more IP6Ks (Figs. 10, A and B). These results strongly support the conclusions of previous work implicating InsP₇, in insulin secretion and show that these effects are acute and not due to adaptive responses to sustained alterations in the cellular level of this compound.

**DISCUSSION**

In the present study, we show that a purine analogue, TNP, originally characterized as an IP3-3K inhibitor (21), is a relatively selective and reversible inhibitor of IP6K6s. The selectivity of TNP for Ip6Ks arises from two factors. Ki values given in Table 1 show that TNP has an absolute potency for IP6K1 ~70-fold greater than that for IP3-3K. Ki values, however, show...
that the affinities of these two enzymes for substrate, ATP, also differ markedly but in the reverse direction, with IP3-3Ks having a $K_m$ that is 100-fold lower than that of IP6Ks. Because TNP is an ATP site competitive inhibitor of these enzymes and, because cellular ATP levels are in the mM range, well above the $K_m$ for IP3-3Ks, these data predict in vivo selectivity of $\sim 1000$-fold. This prediction appears to be borne out by experiments analyzing inositol phosphate levels in $[^3H]$-inositol-labeled HeLa cells where TNP dose-dependently reduced Ins$_7$ and Ins$_8$ levels by at least 90% without significantly affecting any other inositol phosphate analyzed.

Of the several purine-based molecules that were tested for IP3-3K inhibition, in addition to TNP, two other structurally related molecules had IC$_{50}$ values of around 13.5 $\mu$M (Fig. 2, B and C) (21). It seems quite likely that these compounds would be similarly effective as inhibitors of IP6Ks. Apart from TNP and the analogues shown in Fig. 2, Adriamycin, a topoisomerase inhibitor, was reported to inhibit production of Ins(1,3,4,5)P$_4$ (33). In our hands, Adriamycin did not show any inhibition of IP3-3KA in vitro (data not shown), which may be compatible with speculation that its mode of inhibition was nonspecific. Several plant polyphenols have also been reported to be inhibitors of both IP6Ks and IPMKs (24). Although this inhibition might also extend to IP6Ks, most plant polyphenols have other cellular targets that would render them unsuitable for intracellular studies.

The mechanisms of Ins$_7$ generation in S. cerevisiae can help us elucidate similar pathways of Ins$_7$ generation in mammals. Yeast Kcs1p was initially presumed to be the only IP6K phosphorylating the monooester phosphate on the 5-position of the inositol ring to generate 5-[PP]-Ins$_5$. However, kcs1A cells retained low but detectable levels of an Ins$_7$. This led to the identification and cloning of Vip/PPIP5K, a novel Ins$_6$ kinase contributing to a novel
pool of cellular InsP₇ comprising two isomers that is masked in WT strains during metabolic labeling experiments by the Ddp1 phosphatase (7). A clue that a similar mechanism of InsP₇/InsP₈ synthesis existed in mammalian cells was provided by Pesesse et al. (30) and is seen in our data (Fig. 7), where sorbitol-induced increase in the InsP₈ peak was at the cost of the InsP₇ peak. This suggested that the enzymes involved in InsP₇ and InsP₈ synthesis were different. Two enzymes, Vip1/PPIP5K1 and Vip2/PPIP5K2 (8, 9), which are the human homologues of the yeast enzyme, have now been identified, which synthesize 1/3-[PP]-InsP₅. Hence, as shown in Fig. 8A, both IP6Ks and Vips are required to complete the bifurcating pathways from InsP₆ to InsP₈.

TNP treatment of radiolabeled HeLa cells brings the dual pathways into focus. TNP is 40-fold greater than the Ki for IP6Ks and should be similar to the expected cellular IC₅₀ because of its high Km for ATP. Although this concentration of TNP should be sufficient to wipe out all cellular InsP₇, 10% residual InsP₇ could be observed. This can be either due to incomplete inhibition of the cellular IP6K or due to the production of other InsP₇ isomers by the Vip/PPIP5K enzymes. Adding credence to the latter alternative are the following observations. 1) Vip/PPIP5K proteins contain the ATP-"GRASP" domains, which differ from the ATP binding regions of IPK synthesis existed in mammalian cells was provided by Pesesse et al. (30) and is seen in our data (Fig. 7), where sorbitol-induced increase in the InsP₈ peak was at the cost of the InsP₇ peak. This suggested that the enzymes involved in InsP₇ and InsP₈ synthesis were different. Two enzymes, Vip1/PPIP5K1 and Vip2/PPIP5K2 (8, 9), which are the human homologues of the yeast enzyme, have now been identified, which synthesize 1/3-[PP]-InsP₅. Hence, as shown in Fig. 8A, both IP6Ks and Vips are required to complete the bifurcating pathways from InsP₆ to InsP₈.
family members in both sequence and in structure; it is very unlikely that they will prove to be efficient targets of TNP, and this was confirmed by a direct in vitro assay (Fig. 6). 2) The InsP$_7$ isomers, which are the products of IP6Ks and Vip/PPIP5Ks (viz. 5-[PP]-InsP$_7$ and 1/3-[PP]-InsP$_7$ (10) respectively), would be likely to be indistinguishable by the gradients used in our HPLC separation.

Under the sorbitol-stimulated conditions that were required to visualize and accurately measure InsP$_7$ levels, pathway I appears to be the dominant pathway (Fig. 8A). However, pathway II could be revealed in yeast strains deficient for Ddp1 in which InsP$_7$ levels were greatly elevated. Under these circumstances, TNP actually further increased InsP$_7$ levels, establishing the occurrence of pathway II, albeit as a quantitatively minor component in wild type cells (Fig. 9). In mammalian cells, it is most likely that if all of the Dipps (four genes of which have been identified (34, 35)) are completely inhibited, the pool of InsP$_7$ generated by Vip/PPIP5K will be unmasked, in a manner analogous to that seen in ddp1Δ or kcs1ddp1ΔΔ cells in S. cerevisiae.

The concept of "pharmalogs," which are enzymes having similar affinities for inhibitors because they share structural similarities in substrate binding domains irrespective of whether they share sequence similarity, was introduced by Knight et al. (36), who characterized numerous inhibitors of the PIKK and PI 3-kinase family of enzymes. Our data suggest that this concept can be extended to the IPK family of inositol polyphosphate kinases. Although these concepts are valuable when considering the development of potent kinase inhibitors, for such compounds to prove useful as probes of biological function and ultimately, in some cases, as drugs, they must have a limited tendency toward off-target effects. Regarding kinases, a key problem is the existence of large families of genes encoding related enzymes. Protein kinases make up by far the largest group of kinases with ~500 representatives in the human genome. We therefore tested the selectivity of TNP against a panel of 70 protein serine/threonine and tyrosine kinases (Table S1). None were significantly inhibited at 10 μM, the largest dose used in the cell-based studies described here. This strongly supports the value of TNP as a highly selective pharmacological tool in future studies of the functions of inositol polyphosphates.

The vacuolar defect observed when wild type yeast were treated with TNP closely phenocopies yeast strains lacking either Kcs1p or Ipk2p, confirming the utility of this pan of 70 protein serine/threonine and tyrosine kinases (Table S1). None were significantly inhibited at 10 μM, the largest dose used in the cell-based studies described here. This strongly supports the value of TNP as a highly selective pharmacological tool in future studies of the functions of inositol polyphosphates. The vacuolar defect observed when wild type yeast were treated with TNP closely phenocopies yeast strains lacking either Kcs1p or Ipk2p, confirming the utility of this compound in studying the biological functions of inositol polyphosphates in a range of cell types and organisms. The acute effects of an inhibitor of IP6K clearly implicate a direct rather than an adaptive effect of loss of InsP$_7$, and should allow further studies of the dynamics of vacuole formation and turnover that would not be possible using genetic approaches. Similarly, inhibition of insulin release from Min6 cells confirms the broad utility of TNP, suggests that the function of InsP$_7$ in this process is likely to be direct, and offers the possibility to study the dynamics of the insulin secretory machinery in more detail.

The last few years have seen major advances in our understanding of the cellular and organismal functions of inositol polyphosphates fueled primarily by studies in genetically tractable organisms, especially yeast and most recently in mice. Overexpression and/or small interfering/short hairpin RNA-based knockdown of key enzymes has also been used to infer new functions, although the identification of the major molecular targets that account for these proposed roles, especially of the pyrophosphates, has not yet been realized. The availability of a selective, cell-penetrant inhibitor of IP6Ks greatly augments the available approaches to studying the significance of inositol pyrophosphate species and will complement genetic studies. TNP rapidly reduces InsP$_7$ levels in mammalian cells, allowing quantitative and dynamic manipulation of the pathway, and will contribute to pinpointing the molecular mechanisms it controls.

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