Molecular Pharmacology Fast Forward. Published on May 6, 2008 as doi:10.1124/mol.107.044628
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MOL #44628

Cellular energetic status supervises the synthesis of bis-diphosphoinositol tetrakisphosphate ("InsP8"), independently of AMP-activated protein kinase.

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Molecular Pharmacology Fast Forward. Published on May 6, 2008 as DOI: 10.1124/mol.107.044628
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Running title: Bioenergetic balance and inositol pyrophosphates.

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Number of pages: 30 Number of tables: 1 Number of figures: 7
Number of references: 38 Words in the *Abstract:* 253
Words in the *Introduction*: 633 Words in the *Discussion:* 1230

Abbreviations: Inositol phosphates are designated according to IUPAC nomenclature, i.e., $InsP_5$; inositol pentakisphosphate, $InsP_6$; inositol hexakisphosphate, PP-$InsP_5$, diphosphoinositol pentakisphosphate ("$InsP_7$"); bis-diphosphoinositol tetrakisphosphate ("$InsP_8$"). AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; DN, dominant negative; EPAC, exchange protein directly activated by cyclic AMP, ACC, AcetylCoA carboxylase, AMPK, AMP-activated protein kinase, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, HEK, human embryo kidney, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MEFs, mouse embryo fibroblasts; PP-$InsP_4$, diphosphoinositol tetrakisphosphate; PPIP5K, PP-$InsP_5$ kinase (E.C. 2.7.4.24), ZMP, 5-amino-4-imidazolecarboxamide riboside monophosphate.

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ABSTRACT

Cells aggressively defend adenosine nucleotide homeostasis; intracellular bio-sensors detect variations in energetic status and communicate with other cellular networks to initiate adaptive responses. Here, we demonstrate some new elements to this communication process, and we show that this networking is compromised by off-target, bioenergetic effects of some popular pharmacological tools. Treatment of cells with AICAR, so as to simulate elevated AMP levels, reduced the synthesis of bis-diphosphoinositol tetrakisphosphate ($[PP]_2$-$InsP_4$), an intracellular signal that phosphorylates proteins in a kinase-independent reaction. This was a selective effect; levels of other inositol phosphates were unaffected by AICAR. By genetically-manipulating cellular AMPK activity, we showed that it did not mediate these effects of AICAR. Instead, we conclude that the simulation of deteriorating adenosine nucleotide balance itself inhibited $[PP]_2$-$InsP_4$ synthesis. This conclusion is consistent with our demonstrating that oligomycin elevated cellular [AMP] and selectively inhibited $[PP]_2$-$InsP_4$ synthesis, without affecting other inositol phosphates. Additionally, we report that the acute increases in $[PP]_2$-$InsP_4$ levels normally seen during hyperosmotic stress were attenuated by PD184352. The latter is typically considered an exquisitely specific MEK inhibitor, but siRNA against MEK or ERK revealed that this MAP kinase pathway was not involved. Instead, we demonstrate that $[PP]_2$-$InsP_4$ synthesis was inhibited by PD184352 through its non-specific effects upon cellular energy balance. Two other MEK inhibitors, U0126 and PD98059, had similar off-target effects. We conclude that the levels and hence the signaling strength of $[PP]_2$-$InsP_4$ is supervised by cellular adenosine nucleotide balance, signifying a new link between signaling and bioenergetic networks.

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INTRODUCTION

The inositol pyrophosphates (e.g., PP-InsP$_5$ ("InsP$_7$") and [PP]$_2$-InsP$_4$ ("InsP$_8$")) are a specialized subgroup of the inositol phosphate signaling family. The inositol pyrophosphates regulate a diverse range of physiological processes, including apoptosis, vesicle trafficking, transcription, and DNA repair (see Bennett et al., (2006) for a review). Recent evidence indicates that their "high-energy" pyrophosphate groups are deployed to directly phosphorylate a selected group of proteins through a mechanism that is independent of protein kinase activity (Bhandari et al., 2007;Saiardi et al., 2004). The degree of phosphorylation of these target proteins is proportional to the concentration of the inositol pyrophosphates (Saiardi et al., 2004). Thus, there is now a general anticipation that stimulus-dependent fluctuations in the cellular levels of inositol pyrophosphates might act as a signaling mechanism that directly controls protein function by altering the extent to which it is phosphorylated (Lee et al., 2007;Nagata et al., 2005). Consequently, there is great interest in understanding the intracellular and extracellular factors that determine the cellular levels of inositol pyrophosphates. Our laboratory has made progress in this area by demonstrating that the [PP]$_2$-InsP$_4$ concentration in mammalian cells is strongly elevated by either a thermal challenge, or by hyperosmotic stress (Choi et al., 2007;Choi et al., 2005;Pesesse et al., 2004). We have attributed this phenomenon to stress-dependent activation of the kinases (PPIP5K) (Choi et al., 2007) that phosphorylate PP-InsP$_5$ to [PP]$_2$-InsP$_4$; we (Choi et al., 2007) and others (Fridy et al., 2007) recently cloned these proteins.

In earlier studies (Choi et al., 2005;Pesesse et al., 2004), we demonstrated that this enhanced synthesis of [PP]$_2$-InsP$_4$ during hyperosmotic or thermal stress was

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attenuated when cells were treated with either of the two MEK inhibitors, PD98059 or U0126. These results led us to propose that PPIP5K activity is stimulated by the MEK/ERK kinase cascade (Pesesse et al., 2004;Choi et al., 2005). However, it has emerged that PD98059 and U0126, at concentrations used by us and by other laboratories, have an unexpected "off-target" effect upon adenosine nucleotide homeostasis (Dokladda et al., 2005;Yung et al., 2004). These two MEK inhibitors bring about a 2-3 fold increase in the cellular [AMP]/[ATP] ratio in HEK cells (Dokladda et al., 2005). Cells have bioenergetic sensing modules that are quite sensitive to such changes in cellular adenosine nucleotide levels (Hardie and Hawley, 2001). The most ubiquitous and well-characterized of these entities is the AMP-activated protein kinase (AMPK), a heterotrimeric protein complex containing a catalytic α-subunit and regulatory β- and γ-subunits (Hardie and Hawley, 2001). An increase in the [AMP]/[ATP] ratio activates AMPK directly, and also initiates a conformational change in AMPK that permits it to be phosphorylated and further activated by the tumor-suppressing Ser/Thr-kinase, LKB1 (Hardie and Hawley, 2001). Dokladda et al (2005) have reported that the bioenergetic stress brought about by PD98059 and U0126 causes a 2-fold increase in the degree of AMPK phosphorylation. This can influence cellular biochemistry and physiology in a number of ways. When activated, AMPK inhibits ATP-consuming anabolic processes (protein synthesis, gluconeogenesis, fatty acid synthesis) and activates ATP-generating, catabolic pathways (glycolysis, fatty acid oxidation) (Hardie and Hawley, 2001). AMPK achieves these effects both by direct phosphorylation of target proteins and also by regulating gene expression (Hardie and Hawley, 2001).

In view of the non-specific effects of PD98059 and U0126 upon AMPK (see above), we have now used a molecular approach to reinvestigate the mechanism by which MEK inhibitors affect cellular $[PP]_2$-$InsP_4$ synthesis. We show here that this particular action of the inhibitors is independent of MEK. Yet, surprisingly, inhibition of $[PP]_2$-$InsP_4$ synthesis by the MEK inhibitors is also independent of the concurrent activation of

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AMPK. We therefore investigated whether alterations in cellular adenosine nucleotide balance by itself can regulate $[PP]_2$-$InsP_4$ synthesis. Our data provide evidence of a novel link between an intracellular signal and the cellular energy-sensing apparatus.

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MATERIALS AND METHODS

Cell culture

DDT_1-MF_2 hamster vas deferens smooth muscle cells and HEK cells were cultured in "high-glucose" (25 mM) medium (GIBCO BRL, MD). Mouse embryo fibroblasts (MEFs) were kindly provided by Dr. Leif Ellisen (Harvard Medical School, Boston, Massachusetts, USA). These cells were seeded in either 100 mm dishes (5×10^5 cells/dish) or 60 mm dishes (2.5×10^5 cells/dish) and cultured at 37 ^{0}C in Dulbecco modified Eagle medium with 25 mM glucose, supplemented with 10% FBS (Hyclone, Utah),100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO BRL, MD). Where indicated, cells were radiolabled with [^{3}H]inositol (PerkinElmer, Boston, MA) as described previously (Choi et al., 2005).

Measurements of cellular levels of inositol phosphates and adenosine nucleotides

Cellular levels of individual [^{3}H]inositol phosphates were determined by HPLC separation of PCA-quenched cell extracts as previously described (Choi et al., 2005). The HPLC eluate was divided into 1 ml fractions which were individually mixed with scintillant and counted using a PerkinElmer liquid scintillation counter. For assays of adenosine nucleotides, PCA-quenched extracts (Choi et al., 2005) were resolved by HPLC using a 0.46 x 25 cm Vydac 3021C HPLC column (Grace-Vydac, Hesperia, CA) (Zakaria and Brown, 1981). The [ATP] was directly measured from the absorbance at 260 nm. The region of the chromatogram containing AMP was saved, and [AMP] was quantified from the decrease in absorbance at 264 nm upon its metabolism to inosine following the addition of 5-nucleotidase plus adenosine deaminase (Sigma) (Belfield and Goldberg, 1969).

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Molecular Constructs and Transfections

The cDNAs encoding dominant negative (DN) hemagglutinin-tagged, full-length forms of AMPK-α1 and -α2 were kindly provided by Dr. K.-L. Guan (University of Michigan, USA). The vectors were as described previously (Inoki et al., 2003). Transfection of the cells were performed with 2μg of constructs, mixed with FuGene6 (Roche, IN), in antibiotic-free, 10% DMEM medium for 16 hour. For the controls, 4 μg of pcDNA3 was used. Cells were typically analyzed 24 hr post-transfection. The cDNA for GFP was used to determine transfection efficiency (70-80%). The siRNA control (siCTL-Nontargeting Pool) and the siRNA oligonucleotides to knockdown human AMPK-α1, AMPK-α2, ERK1, ERK2, MEK1, MEK2 were all purchased from Dharmacon (Denver, CO). Cells were transfected at 30% confluency using 10-20 nM of each construct over a 16 hour period using Lipofectamine2000 (Invitrogen, CA). Transfection efficiency (70%) was determined using BLOCK-iT fluorescent Oligo (Invitrogen, CA). Finally, cells were serum-starved for 16 hour prior to the initiation of the experiment.

Enzyme assays

PPIP5K activity was purified from rat brain as previously described (Pesesse et al., 2004). Recombinant PPIP5K types 1 and 2 were prepared as previously described (Choi et al., 2007). Enzyme activity was assayed for 20 min at 37 ^{0}C in 100 μl of buffer containing 20 mM HEPES (pH 7.2), 10 mM NaF, 4 mM ATP, 6 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mg/ml BSA, and 2 μM PP-[^{3}H]$InsP_5$ (3000 d.p.m.). The PP-$InsP_5$ was prepared as previously described (Choi et al., 2007). Reactions were quenched with perchloric acid, and then neutralized and analyzed by HPLC as previously described (Choi et al., 2007).

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Western analysis.

Anti-GAPDH (mouse monoclonal) antibodies were purchased from Ambion (Austin, TX). Other antibodies were purchased from Cell Signaling (Danvers, MA). The dilution factor was 1:1000 to 1:2000 for the primary antibodies (in Tris-buffered saline containing 0.05% Tween-20 and 5% non-fat dry milk) and 1:5000 for the HRP-linked secondary antibodies (in Tris-buffered saline containing 10 ug/ml of BSA). Cells were lysed with M-PER Mammalian Protein Extraction Reagent (product #78501, Pierce, IL) supplemented with protease inhibitor cocktail (Roche Diagnositics, IN) and phosphatase inhibitor mixture (Sigma, MO). Lysates were cleared by centrifugation and protein concentration was quantified by using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of protein (40 μg) were applied to each lane of a NuPAGE 4-12% pre-cast gel (NOVEX, San Diego, CA). After transfer to PVDF membranes, samples were processed and visualized with ECL Western Blotting Reagents (Amersham Biosciences, Piscataway, NJ) as previously described (Choi et al., 2005). All of the Western data shown in this study are representative of at least three independent experiments. For some experiments, western blots were scanned (HP Scanjet 4470C, using Precisionscan Pro 3.1), converted to TIFF files, and then band intensities were quantified with ImageQuant (v5.1).

Other Materials

Oligomycin, PD98059 and AICAR were purchased from CalBiochem (San Diego, CA). The U0126 was supplied by Sigma (St. Louis, MO). PD184352 was kindly provided by Prof. P. Cohen at the University of Dundee, Scotland, UK.

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RESULTS

The effects of hyperosmotic stress and PD184352 upon inositol pyrophosphate synthesis.

In our earlier experiments (Choi et al., 2005;Pesesse et al., 2004), in which we used PD98059 and U0126 to inhibit MEK, we concluded that the MEK/ERK pathway activates PPIP5K activity following thermal or hyperosmotic stress. However, we have now revisited this conclusion, because these MEK inhibitors have been reported to have an additional, off-target effect which places cells under some bioenergetic stress (Dokladda et al., 2005). There is an alternate MEK inhibitor, PD184352, that reportedly does not have this non-specific effect, at least in HEK cells (Dokladda et al., 2005). We have now studied the effects of PD184352 upon inositol pyrophosphate turnover in DDT_1-MF_2 cells.

The levels of inositol pyrophosphates were recorded by anion-exchange HPLC analysis of cells labeled with [^{3}H]inositol through four cell generations (e.g. Fig. 1A). The levels of $[PP]_2$-[^{3}H]$InsP_4$ in control cells (open circles, Fig. 1A) were relatively low compared to the levels of the PP-[^{3}H]$InsP_5$ and [^{3}H]$InsP_6$ precursors. On the other hand, the estimated cellular concentration of $[PP]_2$-$InsP_4$ (0.2 to 0.3 μM, (Safrany et al., 1998)) is similar to that of another inositol phosphate signal, namely, Ins(1,4,5)P_3 (Irvine and Schell, 2001).

We (Pesesse et al., 2004;Choi et al., 2007) previously demonstrated that the rate of synthesis of $[PP]_2$-$InsP_4$ in mammalian cells is accelerated following the simulation of hyperosmotic stress by the addition of 0.2M sorbitol for 30 min. Similar effects were observed in the current study: levels of $[PP]_2$-$InsP_4$ increased several-fold without

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significantly affecting levels of the $InsP_6$ and PP-$InsP_5$ precursors (Fig. 1A). In our laboratory's earlier experiments (Pesesse et al., 2004), we demonstrated that the sorbitol-dependent increases in $[PP]_2$-$InsP_4$ levels were attenuated by the addition of either 10 μM U0126 or 50 μM PD98059. In the current study we found that 2 μM PD184352 also antagonized the effect of sorbitol upon $[PP]_2$-$InsP_4$ levels (Fig. 1C). None of these three MEK inhibitors affected the cellular levels of any of the other inositol phosphates (data not shown and (Pesesse et al., 2004)).

Pharmacological inhibition of MEK activates AMPK in DDT_1-MF_2 cells.

In an earlier study, Dokladda *et al* (2005) treated HEK cells with U0126 and PD98059, at concentrations (20 μM and 50 μM, respectively) which are typically used by other groups to inhibit MEK. Dokladda *et al* (2005) reported that these two MEK inhibitors had a non-specific effect upon cellular bioenergetic status that led to activation of AMPK. We have now investigated if the same phenomenon occurs in DDT_1-MF_2 smooth muscle cells. An increase in phosphorylation of AMPK at Thr-172 is considered to reflect an increase in AMPK activity (Hardie and Hawley, 2001). We found that 10 μM U0126 or 50 μM PD98059 each increased the degree of AMPK phosphorylation by 3 to 4-fold (Fig. 2). We additionally studied the phosphorylation status of acetyl CoA carboxylase, a downstream targets of AMPK (Hardie and Hawley, 2001). We found that U0126 and PD98059 brought about a several-fold increase in the degree of acetyl CoA carboxylase phosphorylation (Fig. 2).

Dokladda *et al* (2005) further reported that a third MEK inhibitor, PD184352, when used at a concentration of 2 to 4 μM, did not have these non-specific effects upon the AMPK signaling cascade in HEK cells. In fact, PD184352 has undergone clinical trials as an anti-cancer agent, and there is a general view that it is an exquisitely specific MEK inhibitor (Bain et al., 2007). In contrast to this consensus of opinion, we have found that

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as little as 2 μM PD184352 can increase the degree of phosphorylation of both AMPK and acetyl CoA carboxylase in DDT_1-MF_2 cells (Fig. 2). It is remarkable that three structurally distinct MEK inhibitors all have the same effect upon AMPK (Fig. 2).

Knock-down of the MEK/ERK pathway by RNAi does not affect $[PP]_2$-$InsP_4$ synthesis

Is the effect of the MEK inhibitors upon stress-dependent stimulation of $[PP]_2$-$InsP_4$ synthesis by PPIP5K (Fig. 1B) caused by inhibition of MEK, or by their activation of AMPK (Fig. 2 and (Dokladda et al., 2005))? As a first step towards answering this question, we used siRNA to "knock-down" ERK1/2 expression by 80-90% in HEK cells (Fig. 3A). Despite the success of this knock-down, there was no impact upon the sorbitol-dependent increase in $[PP]_2$-$InsP_4$ levels (Fig. 3D). We also used siRNA to reduce MEK1/2 expression by over 70% (Fig. 3B), which was sufficient to prevent osmotic stress from enhancing the degree of phosphorylation of ERK (Fig. 3C). Nevertheless, this knock-down of MEK1/2 also had no impact upon the ability of osmotic stress to increase cellular levels of $[PP]_2$-$InsP_4$ (Fig. 3E). We therefore conclude that the MEK/ERK pathway does not regulate $[PP]_2$-$InsP_4$ synthesis by PPIP5K. This is an important conclusion because we now have to look to other signaling systems in order to explain how $[PP]_2$-$InsP_4$ synthesis is acutely activated by either hyperosmotic stress or by thermal challenges (Fig. 3 and see (Choi et al., 2007;Choi et al., 2005;Pesesse et al., 2004)). It should be noted that there was no direct effect of MEK inhibitors upon PPIP5K itself ((Pesesse et al., 2004) and Table 1).

$[PP]_2$-$InsP_4$ synthesis is inhibited by treating cells with AICAR.

We next investigated if the inhibition of stress-dependent $[PP]_2$-$InsP_4$ synthesis by MEK inhibitors might bear some relationship to their off-target effects upon AMPK (Fig. 2).

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For these experiments we used AICAR. Upon its uptake into cells, AICAR is metabolized to ZMP, an AMP-mimetic that causes AMPK activation (Hardie and Hawley, 2001). Others have shown that this AICAR treatment does not itself alter cellular levels of ATP or AMP (Fryer et al., 2002;Merrill et al., 1997;Luiken et al., 2003). In agreement with those earlier experiments, AICAR did not alter levels of either [ATP] (Fig. 4A) or [AMP] (Fig. 4B) in DDT_1-MF_2 cells incubated in iso-osmotic conditions.

The treatment of DDT_1-MF_2 cells with AICAR elicited a 3.6 ± 0.7 fold (n=4) increase in the degree of AMPK phosphorylation at Thr-172 (Fig. 4C provides a representative example). This AICAR treatment also reduced steady-state $[PP]_2$-$InsP_4$ levels by approximately 30% ($p<0.01$; Fig. 4D). No other inositol phosphates showed this response, including PP-$InsP_5$ (Fig. 4E and data not shown). Thus, $[PP]_2$-$InsP_4$ synthesis is specifically inhibited following AICAR treatment. This is a novel effect of AICAR that has important ramifications concerning how we interpret previous work with this compound. In control experiments, we found that AICAR did not have a direct effect upon PPIP5K (Table 1).

We further found that AICAR treatment strongly attenuated the elevation in $[PP]_2$-$InsP_4$ levels that occurs in response to hyperosmotic stress (Fig. 4D). We attribute this effect to the AICAR treatment simulating a rise in cellular [AMP]. There was no effect of AICAR upon actual AMP levels in sorbitol-treated cells (Fig. 4B). AICAR did cause ATP levels to rise in the sorbitol-treated cells (Fig. 4A). However, an increase in cellular [ATP] is not indicative of a general deterioration of adenosine nucleotide balance.

The inhibitory effect of AICAR upon $[PP]_2$-$InsP_4$ synthesis (Fig. 4) is not restricted to DDT_1-MF_2 cells. We have observed similar effects of AICAR in a human keratinocyte cell line, HaCaT (data not shown), the U2-OS osteosarcoma (data not shown) and

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MEFs (see below), although the degree to which AICAR inhibited $[PP]_2$-$InsP_4$ synthesis did vary between these different cell types. However, in HEK cells, AICAR is not phosphorylated to ZMP, and, therefore, AMPK is not activated (data not shown; also see (Marsin et al., 2000)). It was therefore a useful control experiment to verify that $[PP]_2$-$InsP_4$ levels in HEK cells were also not affected by AICAR treatment (measured as 10^3 x D.P.M. / D.P.M. lipid) in either non-stressed cells (no AICAR = 0.02 ± 0.003; + AICAR = 0.017 ± 0.003; $p>0.1$) or in cells subjected to osmotic stress (no AICAR = 0.28 ± 0.02; + AICAR = 0.39 ± 0.08; $p>0.1$). These data reinforce our proposal that it is not AICAR itself, but its metabolism to the AMP-mimetic, ZMP, that regulates $[PP]_2$-$InsP_4$ synthesis.

Down-regulation of cellular AMPK activity does not prevent AICAR from inhibiting $[PP]_2$-$InsP_4$ synthesis.

We next used a genetic approach to determine if AMPK regulates $[PP]_2$-$InsP_4$ synthesis in mammalian cells. The catalytic core of AMPK is its α-subunit; two α isoforms are expressed in mammalian cells (Hardie and Hawley, 2001). Therefore, we transiently overexpressed HA-tagged, full-length, dominant negative constructs of both α_1 (D159A; (Inoki et al., 2003)) and α_2 (D157A; (Inoki et al., 2003)) subunits of AMPK in DDT_1-MF_2 cells. Immunoblotting with anti-HA antibodies confirmed that these proteins were expressed (Fig. 5A). The constructs were also determined to be functional because they reduced the ability of AICAR to phosphorylate AMPK (Fig. 5A), and they attenuated the AMPK-dependent increase in acetylCoA carboxylase phosphorylation (Fig. 5C).

If AMPK had been responsible for mediating the AICAR-dependent decrease in $[PP]_2$-$InsP_4$ levels, then the dominant negative constructs should have reversed this effect of

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AICAR. No such effect was observed, either in cells subjected to osmotic shock or in vehicle-treated controls (Fig. 5B). These data indicate that it is not AMPK that mediates the effects of AICAR upon $[PP]_2$-$InsP_4$ signaling. Note that the slight decrease in the levels of $[PP]_2$-$InsP_4$ after transfection with the dominant negative constructs (Fig. 5B) was not a statistically significant effect.

Finally, we examined the effects of AICAR upon $[PP]_2$-$InsP_4$ synthesis in cells in which AMPK was "knocked-down" by RNAi. For these experiments we required a cell-line that could satisfy three criteria: first, the sequences of the *AMPK* genes must be known, second, the endogenous AMPK had to be susceptible to activation by AICAR treatment, and third, the $[PP]_2$-$InsP_4$ pool had to be readily radiolabeled using [^{3}H]inositol. We found that MEF cells met all of these requirements. We transfected MEF cells with siRNA against both AMPK-α1 and AMPK-α2, thereby reducing total AMPK levels by 65% (Fig. 6A). This genetic maneuver substantially compromised the AMPK cascade, eliminating the phosphorylation of acetylCoA carboxylase normally observed in cells treated with AICAR (Fig. 6B). Controls showed that total acetylCoA carboxylase protein was not affected (Fig. 6B).

When MEF cells were osmotically-stressed with 0.2M sorbitol, levels of $[PP]_2$-$InsP_4$ were elevated approximately 16-fold (Fig. 6C). The degree of this effect was not affected by knock-down of AMPK (Fig. 6C,D). Treatment of these cells with AICAR attenuated the sorbitol-dependent increase in $[PP]_2$-$InsP_4$ levels by 40-50% (Fig. 6C,D). The degree of this effect of AICAR was not reduced when AMPK expression was down-regulated (Fig. 6C,D). In summary, these experiments with RNAi, and our use of dominant-negative constructs (see above), together lead us to conclude that AICAR reduces $[PP]_2$-$InsP_4$ synthesis by a novel mechanism that is independent of AMPK.

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$[PP]_2$-$InsP_4$ synthesis is sensitive to oligomycin treatment.

We have shown that AICAR and the MEK inhibitors, which are two classes of completely different drugs, nevertheless have in common the ability to inhibit $[PP]_2$-$InsP_4$ synthesis (see above). It is also notable that MEK inhibitors increase the degree of AMPK phosphorylation (Fig. 2), which is a phenomenon also elicited by AICAR treatment (Fig. 4). Since AMPK itself does not regulate $[PP]_2$-$InsP_4$ metabolism, it is our hypothesis that the cell's adenosine nucleotide balance supervises the degree of $[PP]_2$-$InsP_4$ synthesis. We have now used a third independent protocol to test this conclusion. We incubated DDT_1-MF_2 cells with the mitochondrial poison, oligomycin (5 μM for 60 min), in media containing high (25 mM) glucose, so as to facilitate glycolytic ATP production. Under these conditions, AMP levels were elevated approximately 4-fold (Fig. 7B), confirming that the cells were subjected to bioenergetic stress following oligomycin treatment, even though ATP levels were not significantly reduced (Fig. 7A).

Cellular levels of $InsP_5$, $InsP_6$ and PP-$InsP_5$ were not significantly affected by our oligomycin protocol (Fig. 7C, D and data not shown). However, there was a dramatic and specific decrease in $[PP]_2$-$InsP_4$ levels following oligomycin treatment (Fig. 7E). Note that oligomycin did not itself directly inhibit PPIP5K (Table 1). We also demonstrated that oligomycin imitated the ability of AICAR to attenuate sorbitol-dependent increases in $[PP]_2$-$InsP_4$ levels (Fig. 7E). These data confirm the selective sensitivity of $[PP]_2$-$InsP_4$ synthesis to bioenergetic stress.

DISCUSSION

There are several new conclusions in this study. First, we have demonstrated that PD184352, previously considered an exquisitely-specific MEK inhibitor (Bain et al.,

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2007), in fact has a significant off-target action that it shares with U0126 and PD98059: the ability to activate AMPK. Second, using a molecular approach, we have shown that U0126, PD98059 and PD184352 have an additional non-specific effect, namely, to reverse stress-dependent activation of PPIP5K activity. This leads us to retract our earlier conclusion (Choi et al., 2005;Pesesse et al., 2004), that the ERK/MEK pathway regulates PPIP5K. Third, we have discovered that $[PP]_2$-$InsP_4$ synthesis is inhibited by a AICAR, a drug that is frequently deployed in the belief that it selectively activates AMPK. Moreover, we demonstrate that this particular effect of AICAR upon $[PP]_2$-$InsP_4$ synthesis is not mediated by its canonical target, AMPK. Finally, by using RNA interferance, and three independent pharmacological tools - oligomycin, AICAR and MEK inhibitors - we have demonstrated that cellular levels of $[PP]_2$-$InsP_4$ are closely linked to cellular energy homeostasis. These data point to a novel means by which cellular energy homeostasis communicates with a cell signaling cascade. This is a phenomenon that is highly specific to $[PP]_2$-$InsP_4$; the other higher inositol phosphates inside cells, namely, PP-$InsP_5$, $InsP_6$ and $InsP_5$, do not show this response.

$[PP]_2$-$InsP_4$ belongs to the pyrophosphorylated subgroup of the inositol phosphate signaling family; these inositol pyrophosphates regulate apoptosis, vesicle trafficking, transcription, and DNA repair (see (Bennett et al., 2006) for a review). To achieve these effects, inositol pyrophosphates competitively antagonize the functionally-significant binding of inositol lipids to certain target proteins (Luo et al., 2003;Ali et al., 1995). Inositol pyrophosphates may also act as allosteric regulators of protein function (Lee et al., 2008). Additionally, inositol pyrophosphates can directly phosphorylate proteins (Saiardi et al., 2004;Bhandari et al., 2007). In all of these cases, the signaling intensity of the inositol pyrophosphates is dictated by their intracellular concentrations (Saiardi et al., 2004;Luo et al., 2003;Ali et al., 1995). However, limited knowledge of the mechanisms that control cellular levels of the inositol pyrophosphates is hindering our insight into their roles as intracellular signals. This is why it is so important to

Molecular Pharmacology Fast Forward. Published on May 6, 2008 as DOI: 10.1124/mol.107.044628
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understand how inositol pyrophosphates turnover is regulated. Some insight into this issue has come from previous work from this laboratory which demonstrated that the rate of $[PP]_2$-$InsP_4$ synthesis is accelerated by either hyperosmotic stress (Pesesse et al., 2004) or by a thermal challenge (Choi et al., 2005). The work in the current study adds to our understanding of the biological regulation of inositol pyrophosphate turnover by showing that bioenergetic stress can inhibit $[PP]_2$-$InsP_4$ synthesis. This work also reveals new aspects of functional hierarchy (Figs. 4,7); the inhibition of $[PP]_2$-$InsP_4$ synthesis by bioenergetic stress (either caused by oligomycin or simulated by AICAR) is dominant over the enhanced synthesis of $[PP]_2$-$InsP_4$ that normally follows hyperosmotic stress (simulated by sorbitol).

What is the biological significance of $[PP]_2$-$InsP_4$ synthesis being sensitive to the bioenergetic health of the cell? ATP is consumed in order to sustain the ongoing metabolic flux through the kinase/phosphatase cycles that direct inositol pyrophosphate synthesis and degradation (Menniti et al., 1993). Thus, a decreased rate of synthesis of $[PP]_2$-$InsP_4$ in response to bioenergetic stress might help conserve cellular ATP reserves. Additionally, several of the cellular processes that are stimulated by inositol pyrophosphates are themselves substantial energy-consumers, including vesicle trafficking DNA repair and transcription. It may become expedient to reduce the energy investment in these processes, when the cell's energetic status is under stress. It is also tempting to speculate that decreases in $[PP]_2$-$InsP_4$ levels may, like AMPK, have additional cell-signaling effects which aid bioenergetic homeostasis; this might be a profitable direction for future research.

Another biologically important situation to which our data may be relevant is an earlier observation that receptor-dependent elevations in cAMP inhibits the cellular synthesis of $[PP]_2$-$InsP_4$ (Safrany and Shears, 1998). The mechanism behind this effect has never

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been established, although we have excluded both PKA (Safrany and Shears, 1998) and EPAC (data not shown) from being involved. It is therefore of interest that receptor-dependent increases in cAMP have been reported to activate AMPK in adipocytes (Daval et al., 2005;Yin et al., 2003). We have found a similar effect to occur in DDT_1-MF_2 cells (data not shown). However, cAMP is known not to activate AMPK directly (Henin et al., 1996;Carling et al., 1989). Others (Epperson et al., 2005) have speculated that, in some cell types, receptor-dependent cAMP turnover might generate sufficient AMP to activate AMPK. Additionally, the current study reveals that $[PP]_2$-$InsP_4$ synthesis is inhibited when cellular energy status is perturbed. Maybe this explains why increases in cellular [cAMP] are associated with reduced levels of $[PP]_2$-$InsP_4$.

We have shown that the synthesis of $[PP]_2$-$InsP_4$ by PPIP5K is inhibited by an elevation in cellular [AMP], which we simulated by using AICAR (Hardie and Hawley, 2001). The AMP-activated protein kinase (AMPK) (Hardie and Hawley, 2001) is typically credited with being the major cellular sensor of an elevated cellular AMP levels. However, we have found that AMPK does not mediate this effect of AICAR treatment upon PPIP5K activity. We therefore propose that, *in vivo*, PPIP5K is regulated by another protein that senses changes in AMP levels. There are at least 12 AMPK-related protein kinases that might be considered as candidates, were it not for the fact that all of these proteins have been reported to be insensitive to AMP, and none of them share the AMP-binding domain of AMPK (Al Hakim et al., 2005). However, the AMP-binding cystathionine-β-synthase (CBS) module that is present in AMPK also occurs in a large range of diverse proteins, including ATP-binding cassette (ABC) transporters, voltage-gated chloride channels and transporters, a variety of other transporter families, and a number of enzymes (Biemans-Oldehinkel et al., 2006). It is possible that one of these proteins might mediate an AMP-dependent attenuation of PP-$InsP_5$ kinase activity. Our study

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indicates that future work to delineate this regulatory pathway could be an important new direction in inositide research. As a result of the current study we should also consider that perturbation of $[PP]_2$-$InsP_4$ turnover in cells treated with AICAR might explain some of the biological effects of this widely-used pharmacological tool.

It is well established that a fundamental necessity for cell survival is the maintenance of tight energy homeostasis. This requires the presence of appropriate bio-sensors that first detect variations in energy balance and subsequently communicate this information to other cellular networks which then initiate adaptive responses. AMPK has been the primary focus of much of the attention that has been given to understanding how cells recognize and adapt to adenosine nucleotide imbalance. The current study offers $[PP]_2$-$InsP_4$ as providing a new means by which a signaling system can interface with cellular bioenergetic status. This new development can be significant because cellular energy-sensing machinery is potentially an exploitable target for cancer therapy (Swinnen et al., 2005;Sofer et al., 2005). Finally, our data raise the possibility of a new phenomenon associated with aging: attenuation of inositol pyrophosphate signaling, because of its hypersensitivity to the slight, but progressive decline in cellular adenosine nucleotide homeostasis that others have noted in fibroblasts derived from aging individuals (Miyoshi et al., 2006). Pharmacological or genetic intervention in the pathways of inositol pyrophosphate signaling may therefore ultimately prove to be of benefit to human health.

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FOOTNOTES

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Molecular Pharmacology Fast Forward. Published on May 6, 2008 as DOI: 10.1124/mol.107.044628
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LEGENDS FOR FIGURES

Fig. 1. The effects of hyperosmotic stress and PD184352 upon $[PP]_2$-$InsP_4$ levels in DDT_1-MF_2 cells.

Panel A. [^{3}H]inositol-labeled DDT_1-MF_2 cells were incubated for 30 min with either vehicle (open symbols) or 0.2 M sorbitol (closed symbols). [^{3}H]Inositol phosphates were then extracted and analyzed by HPLC and the chromatogram shows the elution of $InsP_6$, PP-$InsP_5$ and $[PP]_2$-$InsP_4$ (see Methods). The structures of these compounds are depicted above each peak. Note that the placement of the 6-diphosphate group in $[PP]_2$-$InsP_4$ is only tentative, based on an analysis of this material in *Dictyostelium* (Laussmann et al., 1997); the structure of $[PP]_2$-$InsP_4$ has yet to be defined in mammalian cells. Panel B. Control cells were pre-treated for 30 min with either vehicle (circles) or 2 μM PD184352 (squares) and only the elution of $[PP]_2$-$InsP_4$ is shown. Panel C. Cells were incubated with either vehicle (circles) or 2 μM PD184352 (squares) for 30 min prior to the addition of sorbitol for a further 30 min. Only the elution of $[PP]_2$-$InsP_4$ is shown.

Fig. 2. MEK inhibitors activate AMPK.

DDT_1-MF_2 cells were treated for 60 min with either, 2 μM PD184352, 10 μM U0126, 50 μM PD98059, or with vehicle, and then samples were prepared for Western analysis of phosphorylated AMPK (P-AMPK), phosphorylated acetylCoA carboxylase (P-ACC) or total AMPK, as described under Methods. Densitometric analysis of three independent experiments revealed the following -fold increases in the degree of AMPK phosphorylation: 4.3 ± 1 by PD184352, 2.9 ± 0.8 by U0126 and 3.8 ± 0.6 by PD98059.

Fig. 3. Knock-down of MEK or ERK does not prevent hyperosmotic stress from stimulating $[PP]_2$-$InsP_4$ synthesis in HEK cells.

Molecular Pharmacology Fast Forward. Published on May 6, 2008 as DOI: 10.1124/mol.107.044628
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HEK cells were transfected with either control siRNA, or siRNA against MEK1/2, or siRNA against ERK1,2 as described under Methods. Panels A and B show immunoblots of cell lysates using anti-ERK and anti-MEK antibodies. Densitometric analysis (see Methods) of three experiments indicated that the degree of knock-down of ERK ranged from 80 to 90%, while MEK knock-down exceeded 70%. Panel C shows an immunoblot of anti-phospho-ERK in cells transfected with either control siRNA or siRNA against MEK. These cells were treated with either vehicle (labeled as "control"), or 50 μM PD98059 for 60 min, or 0.2M Sorbitol for 30 min (labeled as "Sor"), or both PD98059 plus sorbitol. $[PP]_2$-$InsP_4$ levels in [^{3}H]inositol-labeled cells were also analyzed in either control cells (closed circles) or in cells transfected with siRNA against either ERK1/2 (Panel C) or MEK1/2 (Panel D), all of which were treated with 0.2M sorbitol for 30 min.

Fig. 4. The effects of AICAR and osmotic stress upon inositol pyrophosphate turnover.

[^{3}H]inositol-labeled DDT_1-MF_2 cells were treated for 60 min with either 2 mM AICAR, or vehicle, prior to the addition of 0.2 M sorbitol or vehicle (for a further 30 min). In parallel experiments, cells were quenched, neutralized, and analyzed either for ATP (panel A) or AMP (panel B). Panel C shows a representative anti-phospho-AMPK immunoblot. Densitometric analysis of 4 experiments indicated the following -fold increases in AMPK phosphorylation: AICAR alone 3.6 ± 0.7, sorbitol + AICAR = 2.7 ± 0.3. Sorbitol by itself activated AMPK within 2-5 minutes (not shown), but this response was transient; in 5 of 8 experiments, the degree of AMPK phosphorylation gradually returned to baseline, usually by the 30 min timepoint. Shown is one of three experiments where the phospho-AMPK signal was still 2-fold elevated at the 30 min time-point. Also shown are the levels of $[PP]_2$-$InsP_4$ (panel D) and PP-$InsP_5$ (panel E). The latter data were obtained from 4-7 experiments (means ± standard errors). Asterisks depict effects of AICAR that are statistically significant (*$p < 0.05$, **$p < 0.01$).

Fig. 5. The effect of DN-AMPK upon $[PP]_2$-$InsP_4$ levels in DDT_1-MF_2 cells

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DDT_1-MF_2 cells were transfected for 48 hr. with DN hemagglutinin-tagged, AMPK-α1 plus DN AMPK-α2 constructs, or vector alone, as described in the Methods Section. Then, the cells were treated with vehicle, or 0.2 M sorbitol for 30 min, or 2 mM AICAR for 60 min followed by 30 min with 0.2 M sorbitol. Panel A: Cell lysates were analyzed for expression of the DN-constructs (using anti-hemagglutinin antibody), and phosphorylation of Thr-172 of AMPK, as described under Methods. In parallel (panel B), the level of $[PP]_2$-$InsP_4$ in [^{3}H]inositol-labeled DDT_1-MF_2 cells was measured by HPLC (vector = black bars; DN constructs = grey bars). Data are from three independent experiments. Panel C shows the degree of phosphorylation of acetyl CoA carboxylase.

Fig. 6. Knock-down of AMPK by siRNA does not affect the reduction in $[PP]_2$-$InsP_4$ levels brought about by treatment with AICAR.

MEF cells were pretreated for 1 hr. with 2 mM AICAR followed by 30 min treatment with either vehicle or 0.2 M sorbitol as indicated. Cells were also transfected with 20 nM of either siCTL non-targeting pool or siRNA against AMPK-α1 plus AMPK-α2, as indicated. Panel A: Western analysis of total AMPK in cells. Panel B, Western analysis of phosphorylation of acetylCoA carboxylase (ACC); total ACC and GAPDH serve as loading controls. Panels C and D show cellular levels of $[PP]_2$-$InsP_4$.

Fig. 7. The effect of oligomycin upon the synthesis of inositol pyrophosphates in DDT_1-MF_2 cells.

Non-radiolabeled (panels A, B) or [^{3}H]inositol-labeled (panels C, D, E) DDT_1-MF_2 cells were treated for 1 hr with either vehicle or 0.2M sorbitol or 5 μM oligomycin prior to extraction and analysis of levels of either ATP (panel A), AMP (panel B), [^{3}H]$InsP_6$ (panel C), PP-[^{3}H]$InsP_5$ (panel D) or $[PP]_2$-[^{3}H]$InsP_4$ (panel E). Data are means ± standard errors from 4-5 experiments. Asterisks depict effects of oligomycin that are

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statistically significant (**$p < 0.01$).

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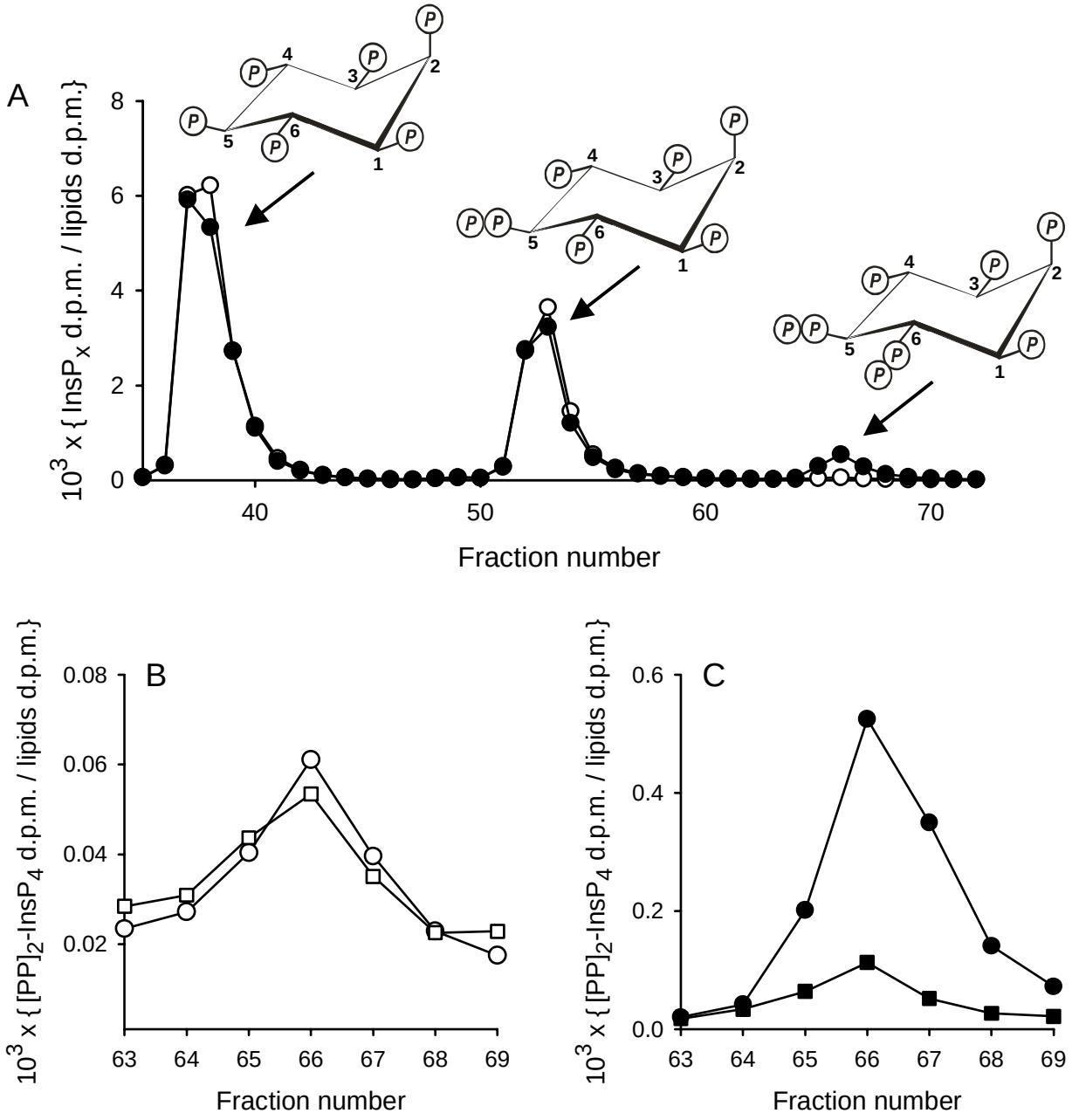
Table 1. Reagents deployed in the current study that do not directly affect PPIP5K activity *in vivo*.

Compound	Concentration
AMP	0.5, 1 and 2 mM
AICAR	2 and 4 mM
PD184352	2 μM
oligomycin	5 μM

All four reagents were tested using native PPIP5K (Pesesse et al., 2004) purified from rat brain, and recombinant PPIP5K types 1 and 2 (Choi et al., 2007), each incubated in 100 μl buffer as described in the Methods Section. Reactions were quenched, neutralized and analyzed by HPLC as described in the Methods Section. Each experiment was performed 2-3 times, and the kinase activity under the indicated test condition was less than 5% different from the corresponding control assays. The incubations that contained AMP were supplemented with 100 μM Ap_5A, an inhibitor of adenylate kinase.



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