A novel target of lithium therapy

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Abstract Phosphatases converting 3′-phosphoadenosine 5′-phosphate (PAP) into adenosine 5′-phosphate are of fundamental importance in living cells as the accumulation of PAP is toxic to several cellular systems. These enzymes are lithium-sensitive and we have characterized a human PAP phosphatase as a potential target of lithium therapy. A cDNA encoding a human enzyme was identified by data base screening, expressed in *Escherichia coli* and the 33 kDa protein purified to homogeneity. The enzyme exhibits high affinity for PAP ($K_m < 1$ μM) and is sensitive to subtherapeutic concentrations of lithium ($IC_{50} = 0.3$ mM). The human enzyme also hydrolyzes inositol-1,4-bisphosphate with high affinity ($K_m = 0.4$ μM), therefore it can be considered as a dual specificity enzyme with high affinity (μM range) for both PAP and inositol-1,4-bisphosphate. Hydrolysis of inositol-1,4-bisphosphate was also inhibited by lithium ($IC_{50} = 0.6$ mM). Thus, we present experimental evidence for a novel target of lithium therapy, which could explain some of the side effects of this therapy.

Key words: 3′-Phosphoadenosine 5′-phosphate; Phosphatase; Inositol-1,4-bisphosphate; Lithium; Human

1. Introduction

3′(2′)-Phosphoadenosine 5′-phosphate (PAP) phosphatases (EC 3.1.3.7) [1] are enzymes present in bacteria, yeast, plant and animal cells [2–6]. They constitute a branch of a superfamily of magnesium-dependent lithium-sensitive phosphatases, which also includes fructose-1,6-bisphosphate 1-phosphatase, inositol-polyphosphate 1-phosphatase and inositol monophosphatase enzymes [7,8]. PAP phosphatases specifically catalyze the hydrolysis of the 3′-phosphate from PAP, thereby recycling PAP to adenosine 5′-phosphate (AMP) and inorganic phosphate [3]. PAP is a potent inhibitor of a variety of enzymes that use 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as cosubstrate, mainly PAPS reductases and sulfotransferases [9–13]. PAP acts as a competitive inhibitor with respect to PAPS because of their close structural similarity. Additionally, PAP mimics the monomers of a polyribonucleotide chain and this fact might explain the observed toxic effect of PAP on RNA processing enzymes [14]. Therefore, in order to prevent the inhibition of both PAPS-utilizing and RNA processing enzymes, the activity of PAP phosphatases is required [4,15]. Thus, PAP phosphatases play a crucial role in the maintenance of sulfation reactions, the assimilation of inorganic sulfate in *Saccharomyces cerevisiae* and probably in *Escherichia coli*, as well as in RNA processing metabolism [16].

The *S. cerevisiae* Hal2 protein was the first PAP phosphatase to be biochemically characterized [3]. The PAP phosphatase activity of Hal2p is crucial for the function of yeast PAPS reductase, which is a key enzyme in the yeast sulfate assimilation pathway that leads to the synthesis of methionine [17]. Indeed, *hal2* cells are auxotrophic for methionine [17,18]. We took advantage of this phenotype to clone a rat PAP phosphatase (RnPIP) by functional complementation of the auxotrophy for methionine of *hal2* cells with a rat cDNA library [5]. A mouse homologue of RnPIP was independently reported [6]. In spite of the high sequence identity of the mouse and rat enzymes, there exists a discrepancy regarding the substrate specificity of the two mammalian enzymes investigated. RnPIP exhibits high affinity both for PAP and inositol-1,4-bisphosphate [5], whereas for the mouse homologue only PAP can be considered a physiological substrate [6]. The mammalian PAP phosphatases are lithium-sensitive enzymes [5,6], and therefore, the human enzyme could represent a novel target of lithium therapy, in addition to the well-known inositol phosphatases [19]. Therefore, in the present work, we identified and characterized a human PAP phosphatase. The human enzyme shows a dual specificity, acting both on PAP and inositol-1,4-bisphosphate, and it is very sensitive to lithium. Finally, we discuss that, in contrast to what has been proposed [6], methionine supplementation in lithium therapy cannot overcome lithium toxicity (as occurs in yeast) [18] because of the dramatic differences in methionine metabolism between yeast and humans.

2. Materials and methods

2.1. Chemicals

All non-radioactive organic compounds were obtained from Sigma. Radioactive compounds were purchased from NEN Life Science Products.

2.2. Expression of HsPIP in E. coli and protein purification

The open reading frame (ORF) of *HsPIP* was amplified by PCR using the following primers: ORF5′: 5′-ATT TAT **GAA TTC** *ATG*

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Abbreviations: AMP, adenosine 5′-phosphate; 3′-AMP, adenosine 3′-phosphate; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; HPLC, high-pressure liquid chromatography; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EST, expressed sequence tag

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GCT TCC AGT AAC ACT-3′ and ORF3′: 5′-ATT TAT **CTC GAG** *TTA* AGG AAC AAG TGC ATT-3′ (restriction sites are in bold face and start and stop codons are italicized). The resulting product was digested with *Eco*RI and *Xho*I and ligated into the pET-28a histidine-tagged expression vector (Novagen). The HsPIP/pET-28a vector was confirmed by sequencing, transformed into the *E. coli* strain BL21 (DE3) and the protein was expressed and purified using Ni^{2+} chromatography, as recommended by the manufacturer.

2.3. Colorimetric enzyme assay

PAP phosphatase and inositol-polyphosphate 1-phosphatase colorimetric assays were basically performed as described [3]. Briefly, a standard assay was conducted in a 100 μl mixture containing 50 mM Tris–HCl pH 7.5, 1 mM magnesium chloride, and the indicated amount of protein and substrate. After 30 min incubation at 30°C, the inorganic phosphate released was quantified by the malachite green method as described [20]. Under these conditions, the enzyme activity was linear with protein quantity (up to 3 μg) and reaction time (up to 1 h).

2.4. High-pressure liquid chromatography (HPLC) analysis of the hydrolysis of PAP and [^{3}H]inositol-1,4-bisphosphate

Analysis of reaction products and determination of K_m values for PAP and inositol-1,4-bisphosphate hydrolysis were carried out by HPLC. In the case of PAP, the conditions for the HPLC analysis and detection by ultraviolet absorption of the reaction product have been described previously [3]. Inositol-1,4-bisphosphate hydrolysis was assayed in a reaction mixture containing 0.2 M potassium *N,N*-bis(2-hydroxyethyl)glycine (bicine) (pH 8.0), 1 mM $MgCl_2$, and the indicated amount of inositol-1,4-bisphosphate and/or [^{3}H]inositol-1,4-bisphosphate (10 Ci/mmol). At the indicated time, 10 μl of reaction mixture was injected into a 4.6×10 mm Guard Cartridge 10 μm SAX column linked to a 4.6×250 mm Partisil 10 μm SAX column (pS Phase Sep), equilibrated in Milli Q water and maintained at 22°C with a flow rate of 1 ml/min. Inositol phosphates were eluted with a linear gradient of $(NH_4)H_2PO_4$, pH 3.7, according to [21]. [^{3}H]Inositol phosphates were detected with a RadioFlow detector LB509 (EGG Berthold) using Optiflow as scintillation cocktail at a flow rate of 3 ml/min.

3. Results

3.1. Cloning of HsPIP

RnPIP is a rat enzyme representative of the animal gene family of PAP phosphatases [5,6]. Expressed sequence tags (ESTs) encoding proteins with high sequence identity to *RnPIP* are present in the nematode *Caenorhabditis elegans*, *Drosophila* and humans. We have screened the human data base of ESTs to obtain a candidate cDNA encoding the human version of *RnPIP*. Data base screening identified a cDNA from a human germinal center B-cells library (I.M.A.G.E. Consortium Clone ID 1240760) [22] with 90% sequence identity to *RnPIP*. Sequencing of this cDNA reveals a 927 bp ORF that putatively encodes a protein of 308 amino acid residues with a calculated molecular mass of 33.4 kDa (Fig. 1). We named this gene *HsPIP* and the corresponding protein HsPIP (see below).

3.2. Purification of His-tagged HsPIP protein and biochemical characterization

The purification of HsPIP was carried out using an NH_2-

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   1  CGGCACGAGGCCATCCTTCTCAAAAGACTTATTGACAGTGCCAAAGCTCGGTACTGGACA   60
  61  CAACGAGGGACCTGGGTCTACGATAACGCGCTTTTGCTCCTCCTGAAGTGTCTTTGGTCC  120
 121  AACGTTGTTCCAGAGTGTACCATGGCTTCCAGTAACACTGTGTTGATGCGGTTGGTAGCC  180
                          M  A  S  S  N  T  V  L  M  R  L  V  A
 181  TCCGCATATTCTATTGCTCAAAAGGCAGGAATGATAGTCAGACGTGTTATTGCTGAAGGA  240
      S  A  Y  S  I  A  Q  K  A  G  M  I  V  R  R  V  I  A  E  G
 241  GACCTGGGTATTGTGGAGAAGACCTGTGCAACAGACCTGCAGACCAAAGCTGACCGATTG  300
      D  L  G  I  V  E  K  T  C  A  T  D  L  Q  T  K  A  D  R  L
 301  GCACAGATGAGCATATGTTCTTCATTGGCCCGGAAATTCCCCAAACTCACAATTATAGGG  360
      A  Q  M  S  I  C  S  S  L  A  R  K  F  P  K  L  T  I  I  G
 361  GAAGAGGATCTGCCTTCTGAGGAAGTGGATCAAGAGCTGATTGAAGACAGTCAGTGGGAA  420
      E  E  D  L  P  S  E  E  V  D  Q  E  L  I  E  D  S  Q  W  E
 421  GAAATACTGAAGCAACCATGCCCATCGCAGTACAGTGCTATTAAAGAAGAAGATCTCGTG  480
      E  I  L  K  Q  P  C  P  S  Q  Y  S  A  I  K  E  E  D  L  V
 481  GTCTGGGTTGATCCTCTGGATGGAACCAAGGAATATACCGAAGGTCTTCTTGACAATGTA  540
      V  W  V  D  P  L  D  G  T  K  E  Y  T  E  G  L  L  D  N  V
 541  ACAGTTCTTATTGGAATTGCTTATGAAGGAAAAGCCATAGCAGGAGTTATTAACCAGCCA  600
      T  V  L  I  G  I  A  Y  E  G  K  A  I  A  G  V  I  N  Q  P
 601  TATTACAACTATGAGGCAGGACCAGATGCTGTGTTGGGGAGGACAATCTGGGGAGTTTTA  660
      Y  Y  N  Y  E  A  G  P  D  A  V  L  G  R  T  I  W  G  V  L
 661  GGTTTAGGCGCCTTTGGGTTTCAGCTGAAAGAAGTCCCTGCTGGGAAACACATTATCACA  720
      G  L  G  A  F  G  F  Q  L  K  E  V  P  A  G  K  H  I  I  T
 721  ACTACTCGATCCCATAGCAACAAGTTGGTTACTGACTGTGTTGCTGCTATGAACCCCGAT  780
      T  T  R  S  H  S  N  K  L  V  T  D  C  V  A  A  M  N  P  D
 781  GCTGTGCTGCGAGTAGGAGGAGCAGGAAATAAGATTATTCAGCTGATTGAAGGCAAAGCC  840
      A  V  L  R  V  G  G  A  G  N  K  I  I  Q  L  I  E  G  K  A
 841  TCTGCTTATGTATTTGCAAGTCCTGGTTGTAAGAAGTGGGATACTTGTGCTCCAGAAGTT  900
      S  A  Y  V  F  A  S  P  G  C  K  K  W  D  T  C  A  P  E  V
 901  ATTTTACATGCTGTGGGAAGCAAGTTAACCGATATCCATGGGAATGTTCTTCAGTACCAC  960
      I  L  H  A  V  G  S  K  L  T  D  I  H  G  N  V  L  Q  Y  H
 961  AAGGATGTGAAGCATATGAACTCTGCAGGAGTCCTGGCCACACTGAGGAATTATGACTAC 1020
      K  D  V  K  H  M  N  S  A  G  V  L  A  T  L  R  N  Y  D  Y
1021  TATGCAAGCCGAGTTCCAGAATCTATTAAAAATGCACTTGTTCCTTAAAGGAAAGTTTCA 1080
      Y  A  S  R  V  P  E  S  I  K  N  A  L  V  P  *
1081  TTTGGCCGGGCGCGGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCAGGT 1140
1141  GGATCACTTGAGCTCAGGAGTTTGAGACCAGCCTGGGCAATATCGTGAGACCCCATCTCT 1200
1201  ACAAAAATACAAATTAACTGGGCATCCTGTCATGCGCCTGTCATCCCAGCTACTTGAGAG 1260
1261  GCTGAAGCAGAAGAATCTCTTGAGCCCGGAAGGCGGAGGTTGCAGTGAGCTGAGA      1315
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Fig. 1. The nucleotide sequence and the deduced amino acid sequence of the *HsPIP* cDNA. The amino acid sequence of the putative coding region is shown below the nucleotide sequence. The stop codon in frame with the ORF is indicated with an asterisk. The three conserved regions [8] involved in the coordination of phosphate and metal ions and nucleophilic water activation are indicated in bold face and underlined. The nucleotide sequence of the *HsPIP* gene has been deposited at the GenBank/EBI data base under the accession number AJ249339. The *HsPIP* cDNA clone was provided by the HGMP Resource Centre and it corresponds to the I.M.A.G.E. Consortium Clone ID 1240760.

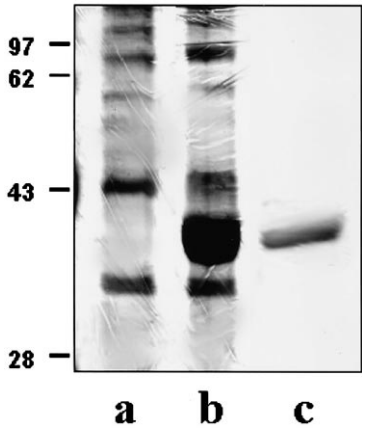

Fig. 2. Purification of His-tagged HsPIP protein. Equivalent amounts of crude extract from *E. coli* BL21 (DE3) cells expressing the HsPIP histidine-tagged fusion protein (lane a) before IPTG induction, (lane b) 3 h after induction and (lane c) 2 μg of purified protein after metal chelation chromatography. Samples were separated by SDS–PAGE and proteins visualized with Coomassie blue. Relative positions of the molecular weight markers are shown on the left.

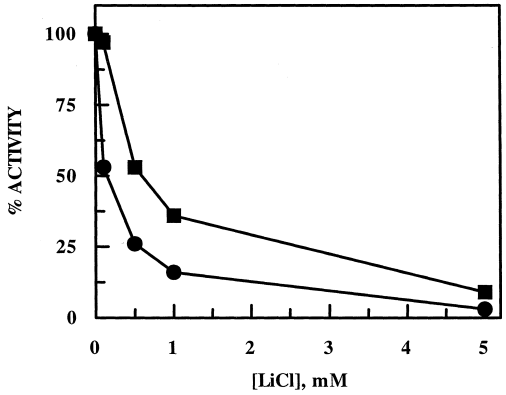

Fig. 3. Effect of lithium on the hydrolysis of PAP and inositol-1,4-bisphosphate. Increasing amounts of lithium were used in a standard phosphatase reaction with 1 μg of HsPIP and 0.1 mM PAP (black circle) or inositol-1,4-bisphosphate (black square) as substrate. The results are expressed as percent activity observed in the absence of lithium and are the average of at least two independent experiments, each performed in duplicate. S.D.s were less than 5%.

terminal histidine-tagged fusion protein prepared as described in Section 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis demonstrates that the fusion protein displays the expected apparent molecular mass of 33 kDa and is readily purified to homogeneity by Ni^{2+} chromatography (Fig. 2). The substrate specificity of the enzyme was determined in vitro by quantitating the release of inorganic phosphate. HsPIP catalyzes the dephosphorylation of both PAP and inositol-1,4-bisphosphate (Table 1), but not of the structurally related compounds adenosine 3′-phosphate (3′-AMP), inositol-1-phosphate and inositol-1,4,5-triphosphate (data not shown). The specific activity for PAP is approximately 5-fold higher than for inositol-1,4-bisphosphate (Table 1). Next, we examined the cation sensitivity of HsPIP. The phosphatase activity of HsPIP was strictly Mg^{2+}-dependent, with an optimal concentration of 1 mM (data not shown). The concentration of Li^+ that decreased the activity of the enzyme by 50% relative to a reaction without Li^+ (IC_{50}) was estimated at a substrate concentration of 0.1 mM PAP or 0.1 mM inositol-1,4-bisphosphate (Fig. 3). As this value that represents a saturating concentration of substrate (see below), the IC_{50} is very close to the K_i [23]. As expected, HsPIP was very sensitive to inhibition by Li^+ ($IC_{50} = 0.3$ mM for hydrolysis of PAP; $IC_{50} = 0.6$ mM for hydrolysis of inositol-1,4-bisphosphate). The effect of Li^+ is unique among the group of monovalent cations and HsPIP was not affected by high Na^+ concentration (up to 200 mM).

Table 1
Biochemical characterization of HsPIP

Substrates	K_m	V_{max}
PAP	<1	1.1
Inositol-1,4-bisphosphate	0.4	0.2

The K_m value for inositol-1,4-bisphosphate of HsPIP is taken from Fig. 5. The K_m value for PAP was below the detection limit (1 μM) of the HPLC analysis. The K_m values are in μM. The V_{max} values are expressed as μmol Pi/min/mg. The results are the average of two independent experiments, each performed in duplicate. S.D.s were less than 5%.

3.3. Analysis of reaction products and determination of K_m values by HPLC

To determine the position of the phosphate liberated from PAP and inositol-1,4-bisphosphate, we have analyzed the reaction products of either PAP or inositol-1,4-bisphosphate hydrolysis by HPLC. HsPIP converts 3′-PAP to 5′-AMP by hydrolysis of the 3′-phosphate (Fig. 4A). 2′-PAP was also hydrolyzed by HsPIP, although with a reduced efficiency (30% with respect to 3′-PAP). Inositol-1,4-bisphosphate was converted to inositol-4-phosphate (Fig. 4B) by hydrolysis of the 1′-phosphate. To measure the affinity of HsPIP for these substrates, we determined the K_m values for PAP and inositol-1,4-bisphosphate (Table 1). The apparent K_m for tritium-labeled inositol-1,4-bisphosphate was 0.4 μM (Fig. 5). The K_m for PAP hydrolysis was below the detection limit (1 μM) of the HPLC analysis. However, these data indicate a high affinity both for the PAP and inositol-1,4-bisphosphate substrates.

4. Discussion

Enzyme nomenclature distinguishes 3′-5′-bisphosphate nucleotidases (EC 3.1.3.7), i.e. PAP phosphatases, and inositol-1,4-bisphosphate 1-phosphatase (EC 3.1.3.57). However, the mammalian PAP phosphatases HsPIP and the previously described rat homologue RnPIP [5] act with high affinity on both PAP and inositol-1,4-bisphosphate, and differ in this respect from the PAP phosphatases of yeast and plants [4,24,25]. We propose that the mammalian genes encode a novel class of true dual specificity enzymes and thus should receive a separate EC entry. The defining feature of this enzyme family is a high affinity for both substrates, as opposed to the *Arabidopsis* and yeast PAP phosphatases, which only show high affinity for PAP and K_m values for inositol-1,4-bisphosphate in the millimolar range [4,24,25]. Therefore, in addition to a role as PAP detoxifying enzymes, the mammalian enzymes could play a role in inositol recycling and phosphoinositide metabolism. Future studies should investigate the relative contribution of HsPIP and inositol-polyphosphate

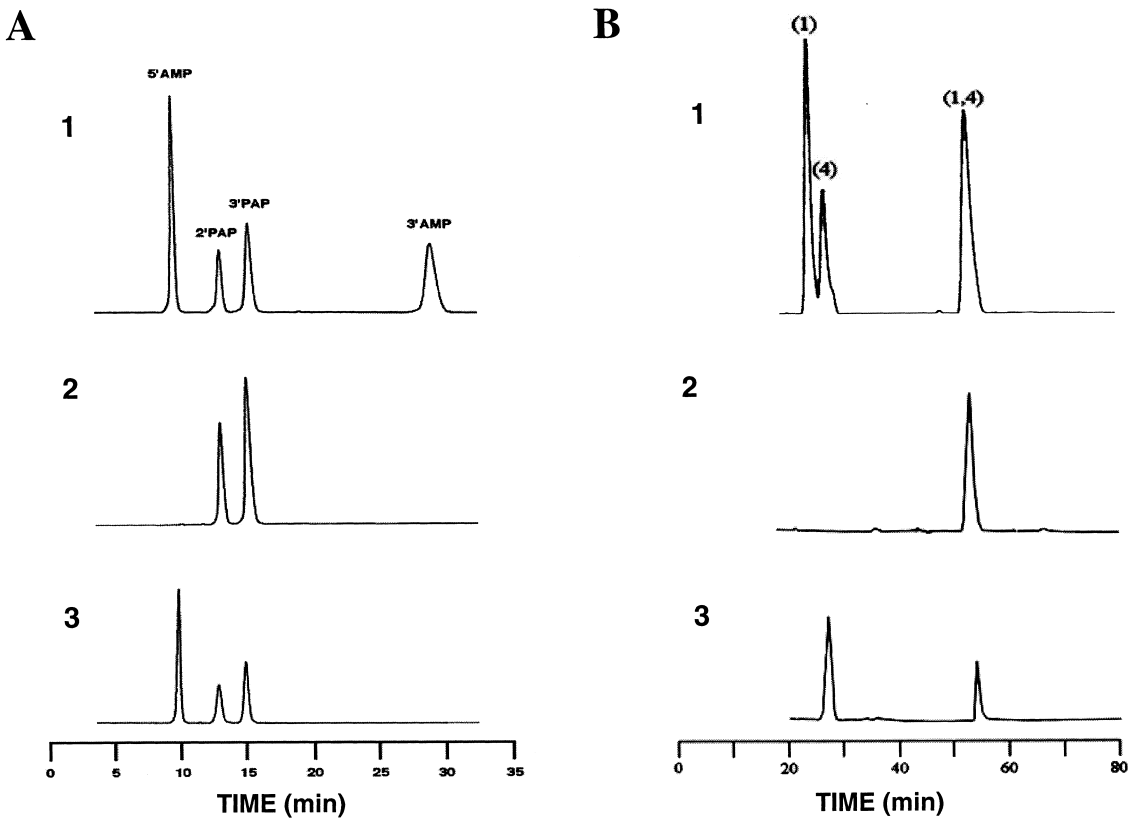

Fig. 4. Identification by HPLC analysis of the reaction product of either PAP or inositol-1,4-bisphosphate hydrolysis catalyzed by HsPIP. A: PAP hydrolysis. HsPIP (1 μg) was incubated for 1 h at 30°C in a buffer containing 0.2 M potassium bicine (pH 8.0), 1 mM $MgCl_2$ and 1 mM PAP as substrate (mixture of 3′-PAP and 2′-PAP). The conditions of the HPLC analysis have previously been described [3]. 1, Standards of AMP, 3′-(2′)PAP and 3′-AMP were analyzed to determine the retention time. 2, Reaction mixture at time zero. 3, Reaction mixture at 1 h. B: Inositol-1,4-bisphosphate hydrolysis. HsPIP (50 ng) was incubated for 5 min at 30°C in a buffer containing 0.2 M potassium bicine (pH 8.0), 1 mM $MgCl_2$, 10 μM inositol-1,4-bisphosphate and 0.1 μM [^{3}H]inositol-1,4-bisphosphate (10 Ci/mmol). The conditions of the HPLC analysis are described in the text. 1, Standards of [^{3}H]inositol-1-phosphate (1), [^{3}H]inositol-4-phosphate (4) and [^{3}H]inositol-1,4-bisphosphate (1,4) were analyzed to determine the reaction time. 2, Reaction mixture at time zero. 3, Reaction mixture at 10 min.

1-phosphatase to inositol-1,4-bisphosphate hydrolysis in different tissues.

Our results for the mammalian enzymes RnPIP [5] and HsPIP (this work) differ substantially from those reported in [6], as the K_m for hydrolysis of inositol-1,4-bisphosphate was at least 100-fold lower than the result reported for a murine homologue [6]. Our biochemical characterization of the HsPIP and RnPIP enzymes, together with a phylogenetic study [5] of the family of magnesium-dependent lithium-sensitive enzymes, points to a novel family of dual specificity enzymes. Instead, the results reported for a murine homologue indicate that PAP is the only physiological substrate [6]. We suggest that the different methodology used for determination of the hydrolysis of inositol-1,4-bisphosphate might account for this discrepancy, i.e. HPLC coupled to a Radio-Flow detector ([5], this work) versus Dowex chromatography [6]. We also disagree with the proposal of these authors for co-administration of lithium with methionine in patients treated for bipolar disorder. Based on the observation that methionine supplementation can suppress lithium toxicity in yeast [18], the authors [6] suggest a 'similar' strategy in humans. As we discuss below, this suggestion lacks a scientific basis and it could lead to an erroneous clinical practice.

In yeast, PAP is generated as a side product of PAPS reductase, which is a key enzyme in the sulfate assimilation pathway that leads to the synthesis of methionine [17]. PAP accumulation has toxic effects on the cell because it inhibits PAPS reductase [10], and hence methionine biosynthesis, and RNA processing enzymes [14]. Lithium inhibits the yeast PAP phosphatase Hal2p [3], leading to the accumulation of PAP [26] and therefore, the inhibition of methionine biosynthesis and RNA processing. In the yeast context, it is obvious that methionine supplementation can alleviate lithium toxicity because methionine biosynthesis is inhibited by PAP [3,26]. Additionally, methionine supplementation prevents the accumulation of PAP in yeast (and therefore, the inhibition of RNA processing) by feed-back repression of the genes involved in sulfate assimilation [27]. Sulfur metabolism in humans differs dramatically from yeast [28]. For instance, the predicted PAP accumulation after lithium treatment in humans cannot have a deleterious effect on methionine biosynthesis (as in yeast) because humans do not have such a pathway. Instead, methionine is an essential amino acid that has to be ingested by a proper diet [28]. In humans, PAP accumulation could have a toxic effect on RNA processing and PAPS-utilizing enzymes [5], but there is no reason to hypothesize that methionine

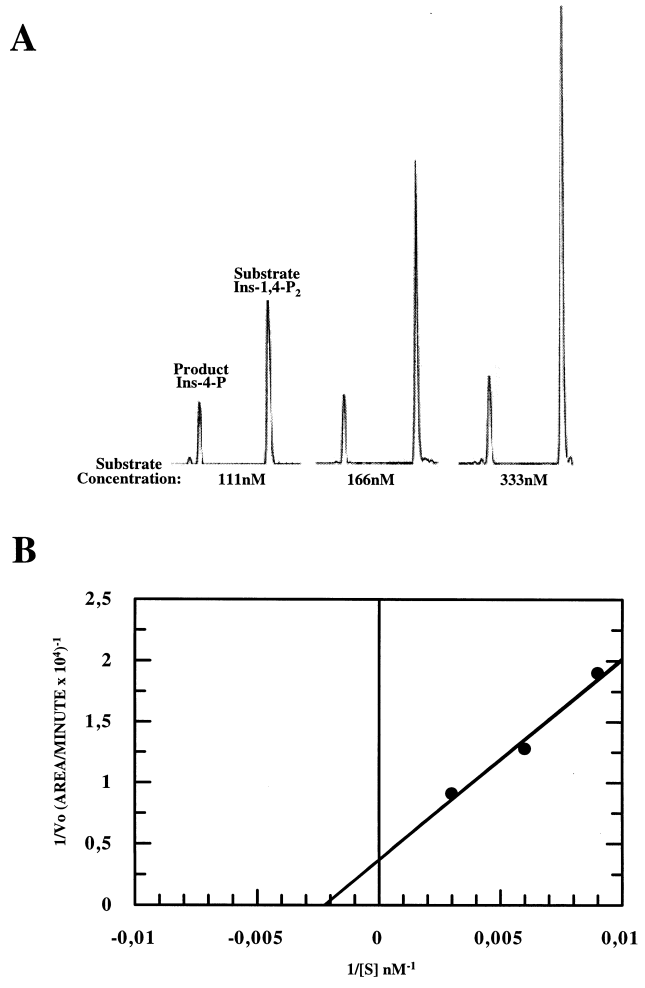

Fig. 5. Determination of the K_m value for inositol-1,4-bisphosphate hydrolysis. A: HPLC analysis was employed to measure reaction rates at substrate concentrations of 111, 166 and 333 nM [^{3}H]inositol-1,4-bisphosphate (10 Ci/mmol). Analysis of the reaction was performed by coupling the HPLC to a RadioFlow detector as indicated in Section 2. B: Double reciprocal plot of initial rate versus concentration of [^{3}H]inositol-1,4-bisphosphate. The graph represents the average of at least two independent experiments performed in duplicate. Regression analysis of the data was performed using the Kaleidagraph V.3.0.2 program (Abelbeck software). The regression coefficient was 0.982.

supplementation could prevent PAP accumulation and alleviate lithium toxicity.

The human HsPIP phosphatase described in the present work is a potential target of lithium therapy and it should be considered in future pharmacological studies. In particular, the predicted toxic accumulation of PAP during lithium treatment could explain some of the side effects of this therapy whose positive effects are probably related to the inhibition of inositol monophosphatase in hyperactive brain zones [29]. HsPIP will also be inhibited by lithium in the same concentration range as that associated with inositol monophosphatase inhibition. Therefore, the subsequent PAP accumulation could have a variety of deleterious effects because of the pleiotropic interference with RNA processing metabolism and sulfation reactions.

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