Inositol-1,2-cyclic-phosphate 2- Inositol phosphohydrolase 

SUBSTRATE SPECIFICITY AND REGULATION OF ACTIVITY BY PHOSPHOLIPIDS, METAL ION CHELATORS, AND INOSITOL 2-PHOSPHATE*

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Glycerophosphoinositol (GroPIns) is a major inositol phosphate in many cell types. In this study we have determined the optimal conditions (pH 8.0 and 0.5 mM MnCl₂) for the metabolism of this molecule in an extract from human placenta, and we show that the major product is inositol (1)-phosphate (Ins(1)P). The enzyme activity that catalyzes this reaction is contained in the same protein designated previously as inositol-(1,2)-cyclic-phosphate 2-inositolphosphohydrolase (cyclic hydrolase), a phosphodiesterase that catalyzes the conversion of inositol-(1,2)-cyclic phosphate (cIns(1,2)P) to Ins(1)P. In addition, the enzyme also catalyzes the production of Ins(1)P from inositol (1)-methylphosphate. All of these substrates, (cIns(1,2)P, GroPIns, and inositol (1)-methylphosphate), contain a phosphodiester bond at the 1-position of the inositol ring. Additional phosphate groups on the 4- or 5-positions of the inositol ring prevent hydrolysis by cyclic hydrolase. The Kₘ values for GroPIns is 0.67 mM, and the Vₘ is 5 μmol/min/mg of protein. GroPIns competitively inhibits cIns(1,2)P hydrolysis with a Kᵢ equal to its Kₘ as a substrate. Hydrolysis of GroPIns and cIns(1,2)P is stimulated by MnCl₂, phosphatidylserine, and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA). However, whereas cIns(1,2)P hydrolysis is increased 5-8-fold by phosphatidylserine and EGTA only a 2-fold increase of GroPIns hydrolysis occurs under the same conditions. Hydrolysis of both GroPIns and cIns(1,2)P is inhibited by Ins(2)P; the ID₅₀ values are 12 and 1 μM, respectively. There are significant quantities of GroPIns and Ins(2)P in 3T3 cells, indicating that these compounds that alter cIns(1,2)P hydrolysis activity may modulate intracellular levels of cIns(1,2)P. Finally, we present evidence suggesting that the substrate specificity of this enzyme is altered during cell transformation.

The metabolism of phosphatidylinositol (PtdIns) is via

phosphatidylinositol-specific phospholipase C, which catalyzes the conversion of PtdIns to diacylglycerol and either inositol (1,2)-cyclic phosphate (cIns(1,2)P) or inositol (1)-phosphate (Ins(1)P). The phospholipase C-mediated degradation of PtdIns during signal transduction may occur in part to modulate protein kinase C activity by generating the activator diacylglycerol (1). However, diacylglycerol may arise from other sources (2) whereas the majority of cIns(1,2)P is derived from PtdIns (3). In fact, the major phosphoinositol product formed from PtdIns hydrolysis is cIns(1,2)P (4).

cIns(1,2)P was first detected in cells by Koch and Diringer (5). Recently many laboratories have isolated the higher phosphorylated forms of the inositol cyclic phosphate derivatives in addition to cIns(1,2)P (6-12). The inositol cyclic phosphates have different kinetic production and degradation compared with their noncyclic counterparts (12, 13). Additionally, inositol (1,2)-cyclic-(4,5)-trisphosphate (cIns(1,2,4,5)P₃) induces a membrane conductance response when injected into limbus photoreceptors (14) which is different from that induced by inositol (1,4,5)-trisphosphate (Ins(1,4,5)P₃). This suggests that the cyclic bond may be recognized by specific receptors distinct from the Ins(1,4,5)P₃ receptor (15).

cIns(1,2)P is converted to Ins(1)P by inositol-cyclic-(1,2)-phosphate 2-phosphohydrolase (cyclic hydrolase). This enzyme was discovered by Dawson and Clarke (16, 17) and was later isolated and characterized (18). Recently we found that this enzyme is a member of the annexin/lipocortin family of proteins, namely annexin III (19), and that it is activated by the same phospholipids that bind to the annexins in a cation-dependent manner (for a review of annexins, see Refs. 20 and 21; for annexin III, see 22-24). Since this enzyme controls the cellular levels of cIns(1,2)P, its further characterization is likely to give clues to the function of cyclic phosphates in vivo.

Previously cIns(1,2)P was the only known substrate for this enzyme; we determined earlier that it does not hydrolyze inositol (1,2)-cyclic-(4)-bisphosphate (cIns(1,2,4)P₄), cIns(1,2,4,5)P₅, inositol (2)-phosphate (Ins(2)P), Ins(1)P, or cyclic nucleotides. We also demonstrated that Ins(2)P is a potent inhibitor of the enzyme (18). We now report that this enzyme is the major activity in human placenta which catalyzes the production of Ins(1)P from glycerophosphoinositol (GroPIns). Additionally, we compare the activation by phosphatidylinositol-(4,5)-bisphosphate; MeIns(1)P, inositol (1)-methylphosphate; GroPIns(4)P, glycerophosphorylinositol (4)-phosphate; GroPIns(4,5)P₅, glycero-phosphorylinositol (4,5)-bisphosphate; GroPIns(3)P, glycero-phosphorylinositol (3)-phosphate; HEPES, 4-(2-hydroxyethyl) 1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HPLC, high pressure liquid chromatography.

phatidylserine (PS) and EGTA and inhibition by Ins(2)P of cyclic hydrolyase activity toward GroPIns versus clns(1,2)P.

**EXPERIMENTAL PROCEDURES**

**Preparation of Inositol Phosphates**—[3H]PtdIns, [3H]phosphatidylinositol (4)-phosphate (PtdIns(4)P), and [3H]phosphatidylinositol (4,5)-phosphate (PtdIns(4,5)P) were purchased from New England Nuclear. [3H]PtdIns was purchased from Amer sham Corp. clns(1,2)P was prepared from [3H]PtdIns and [3H]PtdIns as described (18) except that 12.5 units of type III phosphatidylinositol-specific phospholipase C of Bacillus cereus (Sigma)/2.4 μmol of PtdIns was used for the cleavage of phospholipase C. Reaction mixtures contained 50 mM HEPES, pH 7.2, in 2 ml and were incubated for 2 h before extraction. The aqueous phase of the extraction was applied directly to a 2.0-ml AG 1-X8 Dowex formate (Bio-Rad) column.

[3H]Melin(1)P was prepared by methanolysis of [3H]Ins(1,2)P. Acetyl chloride (0.01 M, 7.5 μl of acetyl chloride/10 ml of methanol) was added to methanol to produce acetic methanol. One μmol of clns(1,2)P was evaporated to dryness and then incubated with 500 μl of the acidic methanol for 10 min at room temperature. The reaction was diluted with 3.5 ml of water, and the Ins(1)P and MeIns(1)P were separated on a 1-ml AG 1-X8 Dowex formate column (Melin(1)P 16 μl of 25 mM ammonium formate, pH 6.5) and Ins(1)P with 8 μl of 150 mM ammonium formate (pH 6.5).

GroPIns, glycerophosphorylinositol (4)-phosphate (GroPIns(4)P), and glycerophosphorylinositol (4,5)-bisphosphate (GroPIns(4,5)P) were prepared by deacylation of [3H]PtdIns, [3H]PtdIns(4)P, and [3H]PtdIns(4,5)P, respectively (26). [3H]Glycerophosphorylinositol (4)-phosphate (GroPIns(4)P) was prepared by phosphorylating PtdIns with [γ-32P]ATP (1 μM, 10 μCi) using immunoprecipitated PtdIns-3-kinase (27). TLC was used to separate the PtdIns(3)P and contaminating PtdIns(4)P from unreacted ATP 50 min). Unlabeled Ins(2)P was purchased from Sigma.

clns(1,2)P was evaporated to dryness and then incubated with 500 μl of water-saturated phenol to the plate. The phenol (97%2). The PtdIns(3)P was then desalloyated (26), and GroPIns(3)P was purified on a Whatman Partisil 10 SAX HPLC column (50–400 mM ammonium formate (pH 3.5) over 30 min followed by 790–960 mM ammonium formate (pH 3.5) over 10 min).

[3H]Ins(2)P and [3H]Ins(2)P were prepared by hydrolyzing the appropriately labeled clns(1,2)P with 0.5 M HCl for 5 min at 80 °C. The reaction was neutralized with NaOH, and the Ins(2)P was separated from containing Ins(1)P on a Partisil 10 SAX HPLC column (isocratic 5 mM ammonium formate (pH 3.5) for 10 min followed by isocratic 30 mM ammonium formate (pH 3.5) for 10 min and then a gradient of 30–60 mM ammonium formate (pH 3.5) over 50 min). Unlabeled Ins(2)P was purchased from Sigma.

**Purification of the clns(1,2)P Hydrolase**—This procedure was modified slightly from that described previously (18) by substituting one run on a DEAE-HPLC industrial sized Bio-Rad column for the multiple DEAE columns (step 1) and omitting gel filtration 2 (step 3) (19).

**Assays**—Assays using either clns(1,2)P, GroPIns, or MeIns(1)P as a substrate were performed as described previously for clns(1,2)P (18) unless otherwise noted. All of these substrates are resistant to alkaline phosphatase; therefore assays contained alkaline phosphatase to convert the Ins(1)P product to inositol.

The assays using GroPIns(3)P, GroPIns(4)P GroPIns(4,5)P, and Ins(2)P as substrates were performed with the same and 50 times more enzyme (5 and 250 ng, respectively) than that typically used with clns(1,2)P as substrate. The conditions of the assay were as described for clns(1,2)P hydrolysis in the absence of alkaline phosphatase. Ins(1)P and GroPIns were separated on a Partisil 10 SAX HPLC column (as described above for GroPIns(3)P purification) with the exception of Ins(2)P-containing reactions that were applied to a 0.5-ml Dowex column under conditions in which product ([3H]inositol) flows through the column while the substrate ([3H]Ins(2)P) is retained.

**Isolation of Water-soluble Products of PtdIns Metabolism**—NIH 3T3 and Swiss mouse 3T3 cells were maintained as monolayers on 6-cm tissue culture dishes in Dulbecco’s modified Eagle’s medium, 10% calf serum, 10 units of penicillin/ml, and 10 μg of streptomycin/ml until approximately 90% confluent. At this point the medium was switched to the inositol-free medium (otherwise the same as above) with 0.5 μCi/ml [3H]inositol. Fresh medium of the same composition was replaced at 48 h. After another 48 h the cells were washed twice with phosphate-buffered saline, and 3C-labeled internal standard (either clns(1,2)P or Ins(1)P) was added to the plate, and cells were lysed by adding 250 μl of water-saturated phenol to the plate. The phenol extract was put into a 1.5-ml Eppendorf tube, and plates were washed with another 250-μl portion of phenol which was placed into a separate Eppendorf tube. The plates were then rinsed with two 500-μl portions of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and added to the two phenol samples. Chloroform (250 μl) was added to each tube, and the extracts were mixed vigorously with a Vortex for 1 min, organic and aqueous phases were separated by centrifugation (35,000 × g for 5 min), and the water phase (top) was washed with 500 μl of chloroform. The inositol phosphates were then separated on a Partisil 10 SAX HPLC column (isocratic 5 mM ammonium formate (pH 3.5) for 1 min, isocratic 30 mM ammonium formate (pH 3.5) for 10 min, 30–60 mM ammonium formate (pH 3.5) over 50 min, 60–903 M ammonium formate (pH 3.5) over 60 min).

**RESULTS**

Mouse 3T3 fibroblasts labeled with [3H]inositol contain a major compound (approximately 50% of the total water-soluble radioactivity) that comigrates with [3C]-labeled GroPIns as shown in Fig. 1. The second major peak that elutes approximately 5 min after GroPIns is clns(1,2)P based on its cochromatography with a 3C-labeled internal clns(1,2)P standard, conversion to Ins(1)P and Ins(2)P upon acid treatment, and conversion to Ins(1)P when incubated with cyclic hydrolyase. The GroPIns is not an artifact of our process of extracting inositol phosphates from cells since similar amounts of GroPIns are found when mouse fibroblasts are harvested as described under “Experimental Procedures,” by extraction with trichloroacetic acid, or by extraction with boiling water as described by Hughes et al. (12) (data not shown). Furthermore, when we add [3H]PtdIns to cells prior to extraction none of it is converted to GroPIns. GroPIns levels range from 0.1 to 0.3 mM in mouse 3T3 cells grown under various conditions. The levels of GroPIns do not change dramatically during short term stimulation of cells (5, 6, 10), suggesting that it does not turn over rapidly. Considering the fact that this compound is a major inositol phosphate in many cells (5, 6, 10), its metabolism is important for replenishment of inositol stores. We initially investigated GroPIns metabolism by incubating [3H]GroPIns with a crude human placental homogenate under a variety of conditions. The release of inositol plus Ins(1)P was determined using Dowex formate chromatography to separate the substrate and products. Breakdown of GroPIns occurred most rapidly at pH 8.0 in the presence of 0.5 mM MnCl2. Mg2+ and Ca2+ did not stimulate GroPIns hydrolysis, and breakdown was completely inhibited by EDTA.

FIG. 1. Identification of GroPIns in mouse fibroblast cell extracts. A [3H]inositol-labeled extract of NIH 3T3 cells was prepared as described under “Experimental Procedures.” 25% of the extract from a confluent 60-mm dish of cells was mixed with [3C]-labeled GroPIns and chromatographed through a Partisil SAX HPLC column as described under “Experimental Procedures.” Closed circles, 3C-labeled internal standard of GroPIns; open circles, tritium.

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1 Levels were calculated from cells grown for 1 week in [3H]inositol at which time GroPIns was assumed to be labeled to equilibrium compared with PtdIns. The concentration calculation assumes that the 3T3 cells have a cellular volume of 4 pl.
The major product in the crude extracts was Ins(1)P, and the condition for optimal hydrolysis was identical to that for cIns(1,2)P hydrolase. We therefore incubated purified cyclic hydrolase with GroPIns and found that it was converted to Ins(1)P (Fig. 2). In this experiment, approximately one-third of the GroPIns was converted to Ins(1)P. No cIns(1,2,3)P was detected, suggesting that this product is not a reaction intermediate under the conditions used.

This latter result prompted us to investigate further the substrate specificity of this enzyme. We found that [3H]MeIns(1)P is also hydrolyzed to Ins(1)P by cyclic hydrolase (data not shown). In contrast, we were unable to detect hydrolysis of GroPIns(4)P, GroPIns(3)P, or GroPIns(4,5)P in the presence of cIns(1,2)P hydrolase. Thus it appears that as we found previously for the substrate, cIns(1,2,3)P addition of phosphates groups to the 4- and 5-positions of inositol eliminates the ability of GroPIns to serve as a substrate for the enzyme. Phosphorylation in the 3-position of inositol also prevents hydrolysis.

When fractions from anion exchange chromatography of a placental supernatant fraction were assayed for GroPIns hydrolase activity, there was one peak of activity which comigrated with that of placental supernatant fraction were assayed for GroPIns hydrolase. We therefore incubated purified cyclic hydrolase with GroPIns and found that it was converted to Ins(1)P. These data indicate that cyclic hydrolase is the major enzyme catalyzing the hydrolysis of GroPIns in human placenta.

The effect of varying GroPIns levels on cyclic hydrolase activity is shown in Fig. 4. The enzyme has a relatively poor affinity for GroPIns with an apparent $K_m$ of 0.7 mM. The $V_m$ in this experiment was 5 pmol of GroPIns hydrolyzed/min/mg compared with 25 pmol of cIns(1,2)P hydrolyzed/min/mg using the same enzyme preparation. The same catalytic site is involved in the hydrolysis of both cIns(1,2)P and GroPIns since unlabeled GroPIns competitively inhibits cIns(1,2)P hydrolysis as shown in Fig. 5. The calculated $K_i$ of GroPIns is 0.67 mM.

Additionally, we find that unlabeled cIns(1,2,3)P inhibits hydrolysis of labeled GroPIns. However, whether the inhibition is competitive cannot be determined since cIns(1,2,3)P is hydrolyzed at least 5 times faster than GroPIns (lower $K_m$, higher $V_m$), causing the concentration of cIns(1,2,3)P to fall rapidly during the experiment. In spite of this, we do observe 50% inhibition of hydrolysis of 500 pmol GroPIns in the presence of an initial concentration of 400 pmol cIns(1,2,3)P.

The Effect of PS, EGTA, and Ins(2,3)P on cIns(1,2,3)P and GroPIns Hydrolysis—We demonstrated previously that cIns(1,2,3)P hydrolysis is influenced by Ins(2,3)P, various metal ions (18), and acidic phospholipids (19). Since we have also found that EGTA stimulates hydrolysis of cIns(1,2,3)P in the presence of MgCl$_2$, as shown in Fig. 6A, it is difficult to determine how Ca$^{2+}$ affects the phospholipid effect on activity. The enzyme is half-maximally stimulated at 5 mM EGTA (data not shown). Both PS and EGTA increase the $V_m$ 5-fold since cIns(1,2,3)P when the divalent metal ion present is Mg$^{2+}$. When Mn$^{2+}$ instead of Mg$^{2+}$ is used, phospholipids cause a decrease in the cleavage of cIns(1,2,3)P above the maximal
conditions and symbols are the same as in Dowex formate column as accomplished previously for cIns(l,2)P. Alkaline phosphatase was omitted to avoid destroying the open squares, 500 pM GroPIns; this is suggested in Fig. 5. The Ins(l)P product was separated from GroPIns on a hydrolase was used per assay, and the substrate was GroPIns (1,500 ml PS (suspended in solution by sonicating at 100 watts for 30 min). levels seen with MgCl₂ plus lipid or that seen with MnCl₂ alone (19). The activation of GroPIns hydrolysis by PS and EGTA is much less than that observed for cIns(1,2)P hydrolysis (Fig. 6B). However, the activation by MnCl₂ is similar using either substrate. This suggests that stimulation of the enzyme by MnCl₂ has a different mechanism than stimulation by PS and EGTA.

In contrast to the activators of cIns(1,2)P hydrolyase, Ins(2)P is a potent inhibitor. Ins(2)P inhibits GroPIns hydrolysis with an average ID₅₀ of 12 µM (see Fig. 7). Previously, we found an ID₅₀ of 1 µM when cIns(1,2)P was used as the substrate (18). Although the kinetics of inhibition are complex, there is a noncompetitive component. This is suggested both by the different ID₅₀ values with respect to the different substrates used and by the apparent decrease in Vₘₐₓ in the presence of Ins(2)P as determined by either a Dixon plot or double-reciprocal plot (data not shown).

The inhibition of cIns(1,2)P hydrolyase by Ins(2)P is of potential physiological importance since we have found that NIH 3T3 cells contain significant quantities of Ins(2)P (1–5...

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**Fig. 6. Activation of cIns(1,2)P hydrolyase by EGTA, PS, and MnCl₂.** A. cIns(1,2)P hydrolyase (10 ng) and increasing amounts of cIns(1,2)P (900 cpm/nmol) were incubated in 25 µl of 5 mM MgCl₂, 50 mM Tris-HCl, pH 7.8, with activators for 10 min at 37 °C. Closed circles, no addition; open circles, 0.1 mM EGTA; closed squares, 25 µg/ml PS (suspended in solution by sonicating at 100 watts for 30 s); open squares, 0.375 mM MnCl₂ in the absence of MgCl₂. All conditions and symbols are the same as in A, but 100 ng of cIns(1,2)P hydrolyase was used per assay, and the substrate was GroPIns (1,500 cpm/nmol).

**Fig. 7. Ins(2)P inhibition of GroPIns hydrolysis.** Hydrolyase (25 ng) was incubated with two different concentrations of GroPIns (3,000 cpm/nmol) at varying concentrations of Ins(2)P for 15 min at 37 °C under the standard assay conditions (see “Experimental Procedures”). Alkaline phosphatase was omitted to avoid destroying the inhibitor. The Ins(1)P product was separated from GroPIns on a Dowex formate column as accomplished previously for cIns(1,2)P (18). Each point is the average of triplicate assays. Open circles represent 528 µM GroPIns; closed circles represent 264 µM GroPIns.

**Fig. 8. Identification of Ins(2)P in mouse fibroblast cell extracts.** An extract of NIH 3T3 cells was prepared as described under “Experimental Procedures,” and half of it was treated with 10 µg of partially purified inositol monophosphatase (32) for 30 min at 37 °C. Inositol monophosphatase does not metabolize Ins(2)P but does metabolize Ins(1), Ins(3), and Ins(4) (25). Closed circles, no enzyme-treated control; open circles, monophosphatase-treated extract. The major signal peaks of the Partisil SAX HPLC chromatogram have been identified by chromatography with "C-labeled standards (data not shown).

**Fig. 9. cIns(1,2)P inhibits GroPIns hydrolysis of SRD extracts.** The SRD extract (10 µg) was assayed with 66 pM GroPIns (1,500 cpm/nmol) in the presence of varying concentrations of unlabeled cIns(1,2)P and GroPIns, respectively, used in order to conserve substrates.

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**TABLE I**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>cIns(1,2)P</th>
<th>GroPIns</th>
<th>Ratio*</th>
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</thead>
<tbody>
<tr>
<td>Human brain</td>
<td>4,000</td>
<td>370</td>
<td>0.09</td>
</tr>
<tr>
<td>Human placenta</td>
<td>680</td>
<td>200</td>
<td>0.3</td>
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<tr>
<td>Human neutrophil</td>
<td>12,000</td>
<td>2,400</td>
<td>0.2</td>
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<tr>
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<td>810</td>
<td>110</td>
<td>0.14</td>
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<tr>
<td>SM-3T3</td>
<td>2,000</td>
<td>500</td>
<td>0.25</td>
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<tr>
<td>SM-24</td>
<td>500</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>20</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>Balb/3T3</td>
<td>50</td>
<td>130</td>
<td>2.6</td>
</tr>
<tr>
<td>MH 7777 (rat hepatoma)</td>
<td>&lt;5</td>
<td>200</td>
<td>&gt;40</td>
</tr>
<tr>
<td>SRD (src-transformed)</td>
<td>&lt;5</td>
<td>3,100</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

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*The activity in this table is only an approximation of the activity since less than saturating concentrations of substrates (60 and 160 µM cIns(1,2)P and GroPIns, respectively) were used in order to conserve substrates.

*This is a cell line that was selected from the SM-3T3 cell line during chronic lithium treatment (Footnote 2 in text).
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µM) (Fig. 8). The second inositol monophosphate peak on the HPLC profile of NIH 3T3 cell extracts is Ins(2)P since it cochromatographs with a 1,4-C-labeled Ins(2)P internal standard (data not shown) and is resistant to hydrolysis by partially purified inositol monophosphatase under conditions in which cellular Ins(1)P and Ins(4)P are completely hydrolyzed as shown in Fig. 8. We have also determined that the Ins(2)P isolated from cells is not an artifact of nonenzymatic breakdown of clns(1,2)P during extraction of inositol phosphates since an internal standard of [14C]Ins(1,2)P added to monolayers of cells is not hydrolyzed to Ins(1)P and Ins(2)P (data not shown).

Comparison of GroPIns- and clns(1,2)P-hydrolyzing Activity in a Variety of Cell Extracts—The amount of GroPIns- and clns(1,2)P-hydrolyzing activity in a variety of cell extracts is shown in Table I. It is remarkable that the ratio of clns(1,2)P hydrolysis to GroPIns hydrolysis is dramatically different in the extracts from the src-transformed cell line (SRD) and the rat hepatoma compared with the other extracts.

Since the SRD extract has no detectable clns(1,2)P-hydrolyzing activity the GroPIns-hydrolyzing activity could result from a modified form of cyclic hydrolyase with altered substrate specificity or from a protein different from cyclic hydrolyase. Dawson and Hemington (28) described a microsomal GroPIns-hydrolyzing activity in rat kidney which may be distinct from cyclic hydrolyase. Although there is not yet a clear distinction between the two possibilities, the GroPIns-hydrolyzing activity of the SRD extract is inhibited by clns(1,2)P, as shown in Fig. 9. The K, of this inhibition is 100 µM, which is similar to the K, of cyclic hydrolyase for clns(1,2)P. In addition the SRD cell GroPIns-hydrolyzing activity is inhibited by Ins(2)P, as has been shown for placental cyclic hydrolyase (data not shown). These data suggest that cyclic hydrolyase is modified in SRD cell to alter its substrate specificity.

DISCUSSION

In this study we have shown that clns(1,2)P hydrolysis catalyzes the hydrolysis of GroPIns but not GroPIns derivatives phosphorylated on the 3-, 4-, or 5-position of the inositol ring. This enzyme is the major activity in human placenta which metabolizes GroPIns. The activation of this enzyme by PS and EGTA and inhibition by Ins(2)P are more effective when clns(1,2)P is the substrate. These enzymatic data together with the finding of significant amounts of these inositol phosphate-containing compounds in cell extracts suggest that anionic phospholipids, GroPIns, and Ins(2)P influence cyclic hydrolyase activity and suggest important regulatory mechanisms for the control (albeit indirect) of clns(1,2)P levels.

It is clear that the cellular level of clns(1,2)P varies tremendously among cell types, is determined by cyclic hydrolyase activity, and correlates with the density of cells at confluence.

It has also been shown that the ratio of GroPIns to "Ins(1)P" in Rat-1 and NIH 3T3 cells increases upon transformation (29). In the latter study the values presented for cellular concentrations of Ins(1)P included clns(1,2)P since acidic conditions, which destroy the cyclic bond, were used to isolate the inositol phosphates. We have found that >90% of the inositol monophosphate in the NIH 3T3 cells is in the cyclic form. Therefore, the relative amounts of clns(1,2)P and GroPIns may be of physiological importance and may be controlled by regulation of cyclic hydrolyase activity and substrate specificity.

* Levels were calculated from cells grown for 1 week in [3H]inositol at which time Ins(2)P was assumed to be labeled to equilibrium compared with PtdIns. The concentration calculation assumes that the 3T3 cells have a cellular volume of 4 pl.

In fact, the study comparing "normal" fibroblast and transformed fibroblast GroPIns/Ins(1)P ratios (29) correlates with the enzyme activity ratios that we have found (Table I). Nontransformed cells have a GroPIns- to clns(1,2)P-hydrolyzing activity ratio of 0.1–0.3, partially transformed cells have a 2.6 ratio, and the ratio in transformed cells increases to greater than 40. Alonso et al. (29) did not analyze cell lines that have been defined as nontransformed such as the Swiss mouse 3T3 cells or neutrophils. Rat-1, NIH 3T3, and Balb/3T3 cells have been characterized as partially transformed (30, 31). Partial transformation is defined as the ability of a cell line to be transformed by the ras or myc oncogene. Normal cells are resistant to transformation by either of these proteins alone (30).

Our data suggest that there is a modification of cyclic hydrolyase in vivo which alters its specificity for one substrate or the other, especially in transformed cells. This implies that metabolism of these two substrates is regulated, and this regulation may alter the cellular phenotype. We hope to determine whether the greatly increased GroPIns-hydrolyzing activity we observe in the transformed cells is a general phenomenon associated with transformation.

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