Retinoic Acid-Induced Granulocytic Differentiation of HL60 Human
Promyelocytic Leukemia Cells Is Preceded by Downregulation of Autonomous
Generation of Inositol Lipid-Derived Second Messengers

By Emilio Porfiri, A. Victor Hoffbrand, and R. Gitendra Wickremasinghe

Inositol phosphates (InsPs) and diacylglycerol (DAG) are second messengers derived via the breakdown of inositol phospholipids, and which play important signalling roles in the regulation of proliferation of some cell types. We have studied the operation of this pathway during the early stages of retinoic acid (RA)-induced granulocytic differentiation of HL60 myeloid leukemia cells. The autonomous breakdown of inositol lipids that occurred in HL60 cells labeled with [3H]inositol was completely abolished following 48 hours of RA treatment. The rate of influx of "Ca2+ was also significantly decreased at 48 hours, consistent with the role of inositol lipid-derived second messengers in regulating Ca2+ entry into cells. The downregulation of inositol lipid metabolism clearly preceded the onset of reduced proliferation induced by RA treatment, and was therefore not a consequence of decreased cell growth. The generation of InsPs in RA-treated cells was reactivated by the fluoroaluminate ion, a direct activator of guanine nucleotide-binding protein(s) (G proteins) that regulate the inositol lipid signalling pathway. Subtle alterations to a regulatory mechanism may therefore mediate the RA-induced downregulation of this pathway. The data are consistent with the hypothesis that the autonomous generation of inositol lipid-derived second messengers may contribute to the continuous proliferation of HL60 cells, and that the RA-induced downregulation of this pathway may, in turn, play a role in signalling the cessation of proliferation that precedes granulocytic differentiation.

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MATERIALS AND METHODS

Cell cultures. Procedures for the maintenance of HL60 cells, determination of viable cell counts, and the estimation of [3H]thymidine incorporation have been described. In all of the experiments described here, cells were diluted to 0.2 x 10^6 cells/mL with fresh medium 24 hours before the commencement of the incubation.

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tion with the radiolabel to ensure that the cultures were in exponential phase.

**[3H]inositol-labeling experiments.** To measure the relative steady state levels of inositol phosphates and inositol lipids, exponentially growing HL60 cells (0.5 × 10⁷/mL) were labeled for 48 hours with 5 μCi/mL of myo-[2-3H]inositol (20 Ci/mmol) in inositol-free medium.22 RA (100 nmol/L) was added to the cultures 6, 24, or 48 hours before the end of the labeling period.21,22 This protocol ensures that cell cultures were labeled for a constant period (48 hours) that we have previously established was sufficient to label all inositol lipids and inositol phosphates studied here to isotopic equilibrium.23 At the end of the labeling period, the distribution of radiolabel in water-soluble [3H]inositol-containing compounds was determined by chromatography on Dowex A1-X8 (BDH, Poole, UK) columns.21,22 Total radioactivity incorporated in each inositol phosphate species was expressed as a percentage of total radiolabel in inositol lipids. We stress that Dowex column chromatography resolves inositol phosphates carrying different numbers of phosphate groups, but does not distinguish between positional isomers. The resolved species are therefore designated as InsP₁, inositol monophosphate; InsP₂, inositol bisphosphate; InsP₃, inositol trisphosphate; and InsP₄, inositol tetrakisphosphate. The total incorporation of [3H]inositol into inositol lipids was quantified by scintillation counting of cellular lipids extracted as described.21 Radiolabel in individual inositol lipids was determined following their resolution by thin-layer chromatography (TLC).21

**[3H]glycerol labeling experiments.** The relative rates of inositol phosphate generation in RA-treated and untreated cells were estimated following addition of LiCl to the cell cultures. LiCl inhibits the sequential degradation of InsP₄ and InsP₃ to free inositol by inhibiting the InsP₄ and InsP₃ phosphatases. Therefore, the addition of LiCl to cells whose inositol lipids have been labeled with [3H]inositol results in the accumulation of [3H]-labeled inositol phosphates, mainly [3H]InsP₃, when inositol lipid hydrolysis is occurring.19,23 HL60 cells were labeled with 5 μCi/mL myo-[2-3H]inositol for 48 hours and exposed to RA for varying periods. LiCl (10 mmol/L) was added to half of the cultures 1 hour before the end of the labeling period, the distribution of the radiolabel in individual neutral glycerolipids was determined by TLC.21 The radioactivity incorporated into each species was quantitated and expressed as a percentage of total radiolabel in glycerolipids.

**Estimation of the rate of autonomous inositol phosphate generation.** The relative rates of inositol phosphate generation in RA-treated and untreated cells were estimated following addition of LiCl to the cell cultures. LiCl inhibits the sequential degradation of InsP₄ and InsP₃ to free inositol by inhibiting the InsP₄ and InsP₃ phosphatases. Therefore, the addition of LiCl to cells whose inositol lipids have been labeled with [3H]inositol results in the accumulation of [3H]-labeled inositol phosphates, mainly [3H]InsP₃, when inositol lipid hydrolysis is occurring.19,23 HL60 cells were labeled with 5 μCi/mL myo-[2-3H]inositol for 48 hours and exposed to RA for varying periods. LiCl (10 mmol/L) was added to half of the cultures 1 hour before the end of the incubation with radiolabel. Inositol lipids and inositol phosphates were extracted as described. Water-soluble inositol phosphates were resolved by anion exchange chromatography on Dowex A1-X8 columns and the radiolabel in each inositol phosphate was expressed as a percentage of total radiolabel in inositol lipids. The difference in the level of [3H]InsP between the control cultures and those exposed to LiCl provides a semi-quantitative measure of the level of autonomous inositol lipid breakdown.19,22 The reproducibility of separation of inositol phosphate species was verified by chromatography of authentic radiolabeled standards (Amersham International, Amersham, UK).

**HL60 cell fractionation.** HL60 cell fractions were prepared from parallel cultures of untreated and RA-treated cells. The cells were harvested by centrifugation and washed twice with Hank’s balanced salts solution (HBSS). The cell pellet was then resuspended (10⁷ cells/mL) in an ice-cold lysis buffer (5 mmol/L Tris-HCl, pH 6.8, 1 mmol/L EGTA, 10 mmol/L benzamidine, 1 μg/mL leupeptin, 500 μmol/L phenylmethylsulfonyl fluoride). After 30 minutes on ice, the cells were disrupted by 50 strokes in a Dounce homogenizer (Jencons, London, UK) followed by the addition of sucrose to 0.25 mol/L. Nuclei and remaining intact cells were removed by centrifugation at 700g for 15 minutes. The resulting post-nuclear cell fractions were stored in aliquots at -20°C.

**PI₃,PLC assay.** PI₃,PLC activity was estimated by incubating HL60 cell extracts (5 μg protein) with 50 μmol/L [3H]PIP₂ (3 counts/minute/pmol) in a final volume of 50 μL containing 20 mmol/L Tris-HCl, pH 6.8, and 0.1% sodium cholate. The free Ca²⁺ concentration was buffered at 250 pmol/L using a Ca²⁺/EGTA buffer calibrated using a Ca²⁺ electrode. The assay was terminated and water-soluble radiolabel was quantitated as described.19 These conditions were optimal for PI₃,PLC activity. Under the conditions described, the assay was independent of added guanine nucleotides and therefore enabled us to quantitate absolute levels of PI₃,PLC in control and treated HL60 cells. The assay was linear over a 60-minute incubation period and at protein concentrations up to 15 μg per assay. Column fractionation on Dowex A1-X8 columns of the [3H]-labeled compounds recovered in the aqueous phase demonstrated that [3H]InsP₃ represented more than 95% of the cleavage products.

The specificity of the PLC for the different inositol lipid species was determined by using as substrate a total lipid extract from HL60 cells labeled for 48 hours with 5 μCi/mL myo-[2-3H]inositol. The lipid extract was prepared as described previously.23 The total phospholipid content and individual phospholipid species were determined by the estimation of lipid-bound phosphate.23 The distribution of [3H]inositol radiolabel among inositol lipid species was determined by TLC on silica gel plates.23 The radiolabeled lipid extract was used as a substrate in the PLC assay described above, giving a final phospholipid concentration of 0.5 mmol/L and estimated PI₃,PLC, PIP₂, and PIP₃ concentrations of 40 μmol/L, 0.68 μmol/L, and 0.8 μmol/L, respectively. The inositol phospholipids were labeled at a specific activity of 8.5 (±1) x 10⁶ counts/minute/mmol and 200,000 counts/min were used in each PLC reaction. Fifty micrograms of enzyme protein were used per assay, and incubations were performed at 37°C for 30 minutes. After stopping the reaction,19 water-soluble inositol phosphates generated by PLC action were resolved and quantitated on Dowex columns.

**Inositol phosphate phosphatase assays.** The rate of hydrolysis of InSP₃ by HL60 cell fractions was measured using [3H]Ins (1,4,5)-P₃, as substrate and InSP₃ phosphatase was assayed using [3H]Ins (1)-P₃. These assays were essentially as described.21,23

**Measurement of Ca²⁺ influx.** Cells were washed twice in HEPES-buffered HBSS (pH 7.4) without Ca²⁺ and resuspended at 10⁷ cells/mL in the same medium. Aliquots (100 μL) were dispensed into Eppendorf microfuge tubes and equilibrated at 37°C for 5 minutes before addition of 10 nmol ⁴⁵Ca²⁺. The incubation was continued for the indicated times and the uptake of ⁴⁵Ca²⁺ stopped by the addition of ice-cold HEPES-buffered HBSS containing 1 mmol/L Ca²⁺. The cells were rapidly centrifuged at 4°C, washed twice in the same solution, dissolved in 100 μL 1% Triton X-100 (Sigma, Poole, UK), and the ⁴⁵Ca²⁺ uptake quantitated by scintillation counting. Blank values determined by the addition of ⁴⁵Ca²⁺ to the cell suspensions after addition of ice-cold HBSS were subtracted from all determinations. Negligible loss of ⁴⁵Ca²⁺ from cells occurred during the washing procedures.

**Materials.** All radiolabeled compounds were from Amersham International UK. RA was from Sigma. The sources of other reagents have been described.21,22

**RESULTS**

**Kinetics of the response of HL60 cells to RA.** Under our culture conditions, untreated HL60 cells proliferated expo-
in exponentially in liquid culture and resumed growth after redilution at day 4 (Fig 1). Cells treated with 100 nmol/L RA proliferated at the same rate as control cells for 4 days following RA addition. However, the cells did not resume growth after redilution on day 4. In agreement with the observations of others, we confirmed that RA-induced cessation of cell division was accompanied by granulocytic differentiation. Morphologic examination of Giemsa-stained cytospin preparations showed that untreated HL60 cells retained a blast-like phenotype throughout all the experiments, whereas the RA-treated cells gradually acquired a differentiated morphology, exhibiting a metamyelocytic (35%) or granulocytic (9%) morphology and ability to reduce NBT after 5 days of treatment (not shown). Uninduced HL60 cells were negative when stained with monoclonal antibodies against CD68 or CD14 markers. However, 7 days after RA addition 95% of the cells were positive for CD68 and 25% for CD14. Only 4% of the treated cells were CD68 positive and less than 1% CD14 positive after 2 days of RA treatment. (K.E. Nye, G. Riley, L.W. Poulter, E. Porfiri, A.V. Hoffbrand, R.G. Wickremasinghe, in preparation), indicating that cellular differentiation, as evidenced by the expression of these markers of mature myeloid cells, had barely commenced at this time.

Operation of the inositol lipid pathway in HL60 cells.

Addition of 10 mmol/L LiCl for 1 hour to untreated, exponentially growing HL60 cells, which had been labeled to isotopic equilibrium with [3H]inositol, caused a significant accumulation of [3H]InsP, which showed an increase of 50% over the level observed in the absence of LiCl (Fig 2). These results were in agreement with our previous observations and suggest that autonomous inositol lipid hydrolysis occurs in proliferating HL60 cells. LiCl-induced [3H]InsP accumulation was not impaired in cells treated with 100 nmol/L RA for 6 hours. However, the relative increase of [3H]InsP declined between 6 and 24 hours of treatment and was almost completely abolished in cells treated with RA for 48 hours, suggesting that autonomous inositol lipid hydrolysis had virtually ceased at that time (Fig 2). Analysis of total radiolabel in all of the inositol phosphates also showed that 48 hours of treatment with RA resulted in

Fig 1. Effect of RA on HL60 cell proliferation. HL60 cells were incubated without (○) or with (●) 100 nmol/L RA. At day 5 (↓) the medium was replenished and the cultures were rediluted to 0.25 × 10^6 cells/mL. This figure is representative of three independent experiments. Cell viability remained above 95% in this and all other experiments.

Fig 2. The effect of pretreatment with RA on the accumulation of InsP following LiCl addition to HL60 cells. HL60 cells were labeled for 48 hours with [3H]inositol. RA (100 nmol/L) was added to the designated cultures 6, 24, and 48 hours before cessation of labeling. LiCl was added to one-half of the cultures 1 hour before extraction and quantitation of inositol phosphates. Relative [3H]InsP accumulation, following LiCl addition (●), is expressed as percentage (±SEM) of total radiolabel recovered in InsP in LiCl-untreated cells. The significance of the differences in relative InsP accumulation between LiCl-treated and untreated cultures was estimated by Student's t-test: (*), P < .001; (†) P < .01. 0, n = 8; 6 hours, n = 4; 24 hours, n = 6; 48 hours, n = 6.
complete shutdown of the generation of all of these species (not shown).

**Changes in the relative steady state levels of water-soluble inositol phosphates during RA treatment.** The relative steady state levels of the water-soluble inositol phosphate species, before and during RA treatment, were measured in cells labeled to isotopic equilibrium with [3H]inositol. No significant difference in the relative steady state levels of each inositol phosphate was found between control cells and cells treated with RA for 6 hours (Fig 3). However, the relative steady state levels of the inositol phosphates were significantly reduced in cells treated for 24 hours with RA and a further reduction was evident 48 hours after addition of the drug. The decrease in the levels of all the inositol phosphates 48 hours after RA addition was confirmed by HPLC analysis (K. Nye, G. Riley, E. Porfiri, and R.G. Wickremasinghe, unpublished data, 1991).

**Changes in the rate of "Ca" entry during RA treatment.** High performance liquid chromatography (HPLC) analysis of the radiolabeled inositol phosphates of [3H]inositol-labeled HL60 cells showed that all of the [3H]InsP, was the Ins (1,3,4,5)-P, isomer, and that the level of this species was markedly decreased following 48 hours of RA treatment (K. Nye, G. Riley, E. Porfiri, and R.G. Wickremasinghe, unpublished data). Because Ins (1,3,4,5)-P, is thought to regulate Ca2+ influx into cells15 we studied the rate of entry of "Ca" into HL60 cells following 48 hours of RA treatment. The uptake of "Ca" was clearly reduced in RA-treated cells (Fig 4). In a further experiment (performed using sextuplicate 15-minute incubations) the rate of entry (per 10⁶ cells) into untreated cells was 2.67 ± 0.05 pmol/15 min, whereas that in RA-treated cells was decreased by 24% to 2.04 ± 0.09 pmol/15 min (P < .001, n = 12). Statistically significant decreases varying from 50% to 10% were observed in four other experiments.

**Relative levels of DAG following RA treatment of HL60 cells.** The steady state levels of DAG relative to the other glycerolipids were studied in HL60 cells labeled to equilibrium with [3H]glycerol. A 10% decrease (P < .05, n = 10) in DAG level was observed in cells treated for 24 hours with RA, but no significant decline was detected following 48 hours of RA treatment (P > .05, n = 8).

**Relative levels of inositol phospholipids following RA addition to HL60 cells.** PIP2 is a rare membrane lipid that is derived by the phosphorylation of an abundant precursor, phosphatidylinositol (PI), via the intermediate phosphatidylinositol (4)-phosphate (PIP). Typically, PIP and PIP2 constitute between 1% and 4% each of the total inositol lipids of eukaryotic cells.14 Total uptake of radiolabel into these species and their relative steady state levels were studied by TLC in untreated and RA-treated HL60 cells labeled for 48 hours with [3H]inositol. In the equilibrium labeling experiments, total radiolabel incorporated into inositol lipids was typically 60,000 ± 15,000 counts/min per 10⁶ cells and this uptake was not affected by RA treatment for 48 hours. In untreated cells, 1.79% of the total radioactivity in [3H]inositol lipids was recovered in PIP2, 1.31% in PIP, and 96.9% in PI. Following 48 hours of RA treatment, the distribution of radiolabel among the inositol phospholipids was: PIP2, 2.07%; PIP, 1.83%; and PI, 96.1%. These values were not significantly different from those observed in the control cells (P > .05, n = 7).

**Quantitation and partial characterization of PIP2-PLC during RA-induced differentiation.** The major enzymologic characteristics of PIP2-PLC were determined in post-nuclear fractions derived from untreated HL60 cells and

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**Fig 3.** Effect of RA treatment on relative steady state levels of inositol phosphate species in HL60 cells labeled to isotopic equilibrium with [3H]inositol. RA (100 nmol/L) was added to the designated cultures 6, 24, and 48 hours before inositol phosphate extraction and quantitation. Levels of each inositol phosphate in RA-treated cells are expressed as a percentage of the level of corresponding inositol phosphate in control cells. At the end of the 48-hour labeling period, in 10⁶ untreated cells, 2,000 ± 220 counts/min were recovered in InsP2, 240 ± 60 in InsP4, 540 ± 80 in InsP3, and 480 ± 80 in InsP1. (*) P < .001; (†) P < .02. n = 4 at 6 hours, n = 8 at 24 hours, n = 8 at 48 hours.
from cells treated for 48 hours with 100 nmol/L RA. Neither the $K_m$ nor the $V_{max}$ of the enzyme was significantly altered in the RA-treated cells compared with controls (Table 1). The $Ca^{2+}$ dependence of PIP$_2$-PLC was also identical in treated and untreated cell fractions (not shown). These results were confirmed using a second set of post-nuclear cell fractions.

We also studied the in vitro specificity of HL60 PLC for PIP$_2$ by using as substrate a lipid extract obtained from HL60 cells whose inositol lipids had been labeled to equilibrium with $[^3H]$inositol. Under these assay conditions, Dowex fractionation of water-soluble $[^3H]$inositol-containing compounds produced in the reaction showed that although PIP$_2$ represented less than 2% of the inositol lipids in the substrate, $[^3H]$InsP$_3$ constituted more than 60% of the products of the reaction catalyzed by extracts from both untreated and RA-treated cells (Fig 5). $[^3H]$InsP$_2$ and $[^3H]$InsP$_1$ constituted 8% ($\pm 2$) and 15% ($\pm 3$), respectively, of the $[^3H]$inositol-labeled products of the reaction in both untreated and RA-treated HL60 cell fractions (Fig 5).

### Table 1. $K_m$ and $V_{max}$ of PIP$_2$-PLC, InsP$_3$, and InsP Phosphatase in Untreated and RA-Treated (48-hour) HL60 Cells

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<tr>
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<th>PIP$_2$-PLC</th>
<th>InsP$_3$ Phosphatase</th>
<th>InsP Phosphatase</th>
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<td>$K_m$</td>
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<tr>
<td>Untreated cells</td>
<td>45 \mu mol/L</td>
<td>79 \mu mol/L</td>
<td>1 mmol/L</td>
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<tr>
<td>RA-treated cells</td>
<td>38 \mu mol/L</td>
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<td>1 mmol/L</td>
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<td>($P &gt; 0.05$, $n = 4$)</td>
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<tr>
<td>$V_{max}$*</td>
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<tr>
<td>Untreated cells</td>
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<td>6.2</td>
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<td>($P &gt; 0.05$, $n = 4$)</td>
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*Values in pmol/min/\mu g protein.

Quantitation of InsP$_1$ and InsP$_3$ phosphatase during RA-induced differentiation of HL60 cells. $Ins(1,4,5)$-P$_3$ and Ins$_{(1,3,4,5)}$-P$_4$ are degraded sequentially by a series of phosphatases, ultimately yielding free inositol. The $V_{max}$ and $K_m$ of InsP$_3$ and InsP phosphatase activities of HL60 cells were not significantly altered following 48 hours of RA treatment (Table 1). These results were confirmed on another batch of cell fractions derived from independent HL60 cultures (not shown).

The generation of inositol phosphates can be reactivated by the fluoroaluminate ion in HL60 cells treated with RA. In some cellular systems, membrane receptors are coupled to PIP$_2$-PLC by guanine-nucleotide binding proteins (G proteins). The binding of ligands to these receptors triggers the exchange of G protein-bound GDP for GTP and the active, GTP-bound form is presumed to activate PIP$_2$-PLC. In several cell types, the fluoroaluminate ion (AIF$_4^-$), has been shown to activate G proteins directly. Therefore, AIF$_4^-$ is able to activate PIP$_2$-PLC via binding to a G protein. To investigate whether RA-induced downregulation of inositol lipid breakdown could be reversed, we exposed 48-hour RA-treated and $[^3H]$inositol-labeled HL60 cells to AIF$_4^-$. In these cells, the addition of LiCl alone did not result in...
significant [3H]InsP accumulation (Fig 3). However, treatment with AlF₄⁻ (added as a mixture of 50 mmol/L NaF and 5 μmol/L AlCl₃) alone for 30 minutes led to a significant increase of [3H]InsP, which increased to 160% of the baseline levels (Fig 6). The simultaneous addition of LiCl and AlF₄⁻ did not significantly increase the yield of InsP and of inositol phosphates compared with the addition of AlF₄⁻ alone. This result was presumably due to the fact that the fluoride ion itself, like LiCl, is a potent inhibitor of InsP phosphatase (not shown).

Addition of AlF₄⁻ to untreated HL60 cells resulted in a 25% increase in [3H]InsP over baseline levels (Fig 6). However, this increase was comparable with that obtained when LiCl was added during 30 minutes of incubation (not shown), in contrast with the observations on RA-treated cells. Thus, it is likely that the increase in [3H]InsP observed upon AlF₄⁻ addition to control cells was attributable solely to the inhibition of InsP phosphatase by the fluoride ion rather than to an effect on a G protein. Therefore, the data suggest that the activation of G proteins by AlF₄⁻ did not result in an increase in the autonomous inositol lipid breakdown of control HL60 cells, but was able to activate this reaction in cells whose inositol lipid turnover has been downregulated following 48 hours of treatment with RA.

Abolition of HL60 cell proliferation by hydroxyurea is not accompanied by downregulation of inositol lipid hydrolysis. Hydroxyurea abolishes cell proliferation by selectively inhibiting the enzyme ribonucleoside diphosphate reductase and, consequently, DNA synthesis. HL60 cells exposed to 1 mmol/L hydroxyurea for 24 hours remained viable but did not proliferate. The ability of this drug to block HL60 proliferation completely and reversibly has been described previously. DNA synthesis, measured by [3H]thymidine incorporation, was abolished following 1 hour of incubation with the drug. We investigated the effects of hydroxyurea-induced cessation of proliferation on the operation of the inositol lipid pathway in HL60 cells labeled to equilibrium with [3H]inositol. Addition of LiCl for 1 hour to untreated cells led to significant [3H]InsP accumulation, which increased to 133% of baseline levels. In cells exposed to 1 mmol/L hydroxyurea for 24 hours, [3H]InsP accumulation following LiCl addition also increased to 130% of baseline levels ($P > .05, n = 4$), indicating that autonomous operation of inositol lipid hydrolysis was not affected by the drug-induced cessation of cellular proliferation.

**DISCUSSION**

We have investigated the changes in the breakdown of inositol lipids that occur during the early phase of RA-induced granulocytic differentiation of HL60 cells. In agreement with our earlier results, the evidence reported here suggests that autonomous (ie, exogenous growth factor-independent) PLC-mediated hydrolysis of inositol lipids occurs in these cells. Several lines of evidence suggest that this phenomenon may have regulatory consequences. An in vitro study of phosphoinositide-specific PLC(s) of HL60 cells showed that this activity displayed a marked preference for the hydrolysis of PIP₂ compared with the approximately 50-fold more abundant PI, in agreement with studies in other cell systems. Because only a small fraction of the PI of mammalian cells is able to give rise to PIP₂, it is unlikely that the PLC pathway of HL60 cells is involved in the metabolic turnover of inositol lipids.

HPLC analysis of the [3H]inositol phosphates of [3H]-labeled HL60 cells (K. Nye, G. Riley, E. Porfiri, and R.G. Wickremasinghe, unpublished data) also suggests that the products of inositol lipid breakdown may have regulatory functions. All InsP₄ species detected represented the Ins(1,3,4,5)-P₄ isomer, which is known to play a role in regulating cellular Ca²⁺ levels. Indeed, significant decreases in the rate of entry of Ca²⁺ were detected 48 hours after RA addition, suggesting that the RA-induced decrease in the level of Ins(1,3,4,5)-P₄ may consequently modulate the rate of Ca²⁺ influx. The induction of HL60 cell granulocytic differentiation by treatment with 100 nmol/L RA resulted in the complete abrogation of inositol phosphate generation in the presence of LiCl. This result is strongly suggestive of the shutdown of PLC-mediated inositol lipid hydrolysis. However, we cannot completely rule out alternative explanations of the data because LiCl also inhibits cellular enzyme systems other than InsP and InsP₄ phosphatases, eg, adenylate cyclase. Nevertheless, relative steady state levels of all inositol phosphate species also declined in the same time frame, supporting the interpretation that RA treatment resulted in the progressive downregulation of PIP₂-PLC. Steady state levels of inositol phosphates have also been shown to decrease following the induction of differentiation of HL60 cells or Friend murine erythroleukemia cells by dimethyl sulfoxide. However, these studies did not attempt to dem-

![Fig 6. The effect of AlF₄⁻ on inositol lipid breakdown by HL60 cells. HL60 cells were either untreated or treated with 100 nmol/L RA for 48 hours. AlF₄⁻ (a mixture of 50 mmol/L NaF and 5 μmol/L AlCl₃) was added to one-half of each set of cultures (B), whereas the other half received no AlF₄⁻ (C). Thirty minutes later the cells were processed for determination of radiolabel in InsP. The significance of the difference between cultures not exposed and exposed to AlF₄⁻ was $P < .005 (n = 4)$.](image)
onstrate directly that the decreases in steady state levels were the result of decreased breakdown of inositol lipids.

The decrease in steady state levels of DAG following RA treatment was modest and transient compared with the decreases observed in the levels in the inositol phosphates. However, whereas the inositol phosphates are derived only from the breakdown of the inositol lipids, DAG is also a precursor for phospholipid biosynthesis. Therefore, it is plausible that any RA-induced changes in PIP2 derived DAG were masked by other cellular pools of DAG that are not involved in signalling.

Significantly, downregulation of inositol lipid breakdown and the decline in inositol phosphates and DAG levels occurred at least 48 hours before RA-induced cessation of HL60 cell proliferation, and also preceded the appearance of a more differentiated cell phenotype as evidenced by the expression of immunologic markers. Therefore, the downregulation of inositol lipid breakdown was not a consequence of the cessation of cell proliferation. This conclusion is reinforced by the observation that a block in HL60 cell proliferation induced by the DNA synthesis inhibitor hydroxyurea did not cause any change in either the rate of autonomous inositol lipid hydrolysis or in the steady state levels of inositol phosphates. On the contrary, given the crucial role of the inositol lipid signalling pathway in the transduction of mitotic signals in normal cells and in the continual proliferation of fibroblasts transformed by some oncogenes, the data here are consistent with but do not prove the hypothesis that autonomous operation of the inositol lipid signalling pathway may contribute to signalling the continuous proliferation of HL60 cells, and that the downregulation of this pathway may be part of the mechanism by which cell proliferation is halted following RA treatment. The observation that synthetic DAG analogs can inhibit dimethyl sulphoxide-induced differentiation of Friend erythroleukemia cells is consistent with this hypothesis.

The mechanism by which downregulation of inositol lipid-derived second messengers may secure the cessation of proliferation is unclear. However, the RA-induced differentiation of HL60 cells has been shown to be associated with the downregulation of expression of the c-myc proto-oncogene and of the recently described myeloblastin gene in the same timeframe as the downregulation of inositol lipid breakdown described here. The products of all of these genes are required for the proliferation of HL60 cells, because anti-sense oligonucleotides to each of their messenger RNAs (mRNAs) can block proliferation although several days elapsed between the addition of the anti-sense sequences and the eventual cessation of proliferation. The c-myc and c-fos proto-oncogenes appear to be positively regulated at least in part by the inositol lipid-derived second messengers generated consequent to growth factor stimulation of normal cells. While it is plausible that RA-induced downregulation of the inositol lipid signalling pathway may lead in turn to the downregulation of expression of genes important to cell proliferation, the data presented here do not allow us to draw firm conclusions on the putative mechanism(s) by which downregulation of inositol lipid breakdown may eventually secure the withdrawal of HL60 cells from the proliferative state. The alternative possibility that downregulation of expression of nuclear protooncogenes by the RA-RAR complex leads to downregulation of inositol lipid breakdown appears unlikely, because there is no evidence that the products of c-myc and c-fos genes can modulate inositol lipid turnover. In fact, transformation of fibroblasts by the v-myc and v-fos genes has been shown not to result in altered inositol lipid breakdown. In any event, these mechanisms are likely to involve complex interacting pathways, given the time interval between the shutdown of inositol lipid breakdown and the cessation of proliferation.

We have also attempted to elucidate the mechanism underlying RA-induced downregulation of inositol lipid breakdown. Because RA is a regulator of transcription, we asked whether the observed effect of RA on inositol lipid metabolism may be mediated via decreased levels of PIP2-PLC or increased levels of inositol phosphate phosphatases. We detected no changes in the levels of these key enzymes of the inositol lipid pathway that may account for the observed downregulation. Furthermore, RA treatment did not induce any change in levels of PIP or PIP2, eliminating the possibility that the supply of inositol lipid substrate for cleavage by PIP2-PLC via an alteration in the levels of PI kinase or PI kinase underlie the observed changes. Although we obtained no evidence that RA addition altered the levels of enzymes directly involved in inositol lipid metabolism, the observed effect of this agent on inositol lipid breakdown may be modulated via the increased expression of genes regulated positively by the RA-RAR complex and whose products negatively regulate this pathway in intact cells. On the other hand, these effects may be mediated via transcriptional repression of the expression of proteins that stimulate the inositol lipid signalling pathway. For example, the RA-RAR complex is known to repress transcription regulated via AP-1 sites.

A large body of evidence suggests that the PIP2-PLC of normal cells is linked to cell surface receptors via a G protein. Indeed, inositol lipid breakdown could be reactivated by the addition of ALF2, a direct activator of G proteins, to RA-treated cells. Thus, RA-treated HL60 cells express a functional system for inositol lipid breakdown, consistent with the measurements of PIP2-PLC in vitro. This observation suggests in turn that the autonomous breakdown of inositol lipids in HL60 cells may be mediated by a G-protein–linked mechanism and that this mechanism is inactivated following RA treatment. This interpretation is consistent with the observation that ALF2 addition to untreated cells did not increase the autonomous breakdown of inositol lipids. It is possible that HL60 cells secrete an autocrine growth factor or that they express a mutant growth factor receptor or other signalling protein that stimulates inositol lipid breakdown and that may be downregulated following RA treatment. Further investigations at the molecular level will be required to distinguish between these possibilities.

In conclusion, we have shown here that the autonomous breakdown of inositol lipids that occurs in HL60 promyelo-
cytic leukemia cells is downregulated following the induction of granulocytic differentiation by RA. Several features of inositol lipid hydrolysis in these cells suggested that this phenomenon is related to the well-known inositol lipid signalling pathway rather than to the metabolic turnover of cellular phospholipids and is regulated by a G-protein-linked mechanism. It would be of interest to establish whether similar changes in this important regulatory path-

way occur during differentiation of fresh blast cells from patients with acute myeloid leukemia.

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