Transcriptional Regulation and Increased Functional Expression of the Inositol Trisphosphate Receptor in Retinoic Acid-treated HL-60 Cells*

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The inositol trisphosphate (InsP₃) receptor is an essential regulator of intracellular calcium in many cells including chemoattractant- and cytokine-stimulated neutrophils and differentiated promyelocytic leukemia (HL-60) cells. We examined the expression and function of the InsP₃ receptor and the transcriptional regulation of the InsP₃ receptor gene in HL-60 cells and in HL-60 cells treated for 1-5 days with 1 μM retinoic acid. Radioligand binding studies using membranes from control and retinoic acid-treated HL-60 cells showed that the Bₘₐₓ of InsP₃ receptor increased progressively from 0.24 to 0.69 pmol/mg protein during 5 days retinoic acid treatment with no change in Kₐ (19 nM). During this period, maximal InsP₃-stimulated Ca²⁺ mobilization increased 2-3-fold. InsP₃ receptor mRNA was present at low levels in HL-60 cells but was increased significantly after treatment with retinoic acid, reaching maximal levels of approximately 4-fold greater than untreated cells after 4 days treatment with retinoic acid. Nuclear run-on assays indicated that the elevated steady state level of InsP₃ receptor mRNA in retinoic acid-treated HL-60 cells was primarily the result of enhanced transcription of the InsP₃ receptor gene. Furthermore, the transcriptional enhancing effect of retinoic acid was seen in the presence of cycloheximide, suggesting that the InsP₃ receptor gene is directly regulated by retinoic acid. The studies also demonstrate that the InsP₃ receptor mRNA is rapidly degraded in HL-60 cells by a mechanism that also requires protein synthesis.

The host protective function of neutrophils is intricately linked to the phosphoinositide signal transduction cascade (1). Detection of invasive microorganisms including bacteria involves cell surface receptors for bacterial peptides, cytokines, activated complement components, and proinflammatory arachidonate metabolites. A common signaling pathway for these activated receptors is the generation of inositol 1,4,5-trisphosphates (InsP₃)³ and the mobilization of intracellular calcium stores (2-4). InsP₃ has been shown to bind to a limited number of specific intracellular receptors within neutrophils causing the rapid efflux of Ca²⁺ from storage vesicles into the cytoplasm (5, 6). Elevated cytoplasmic calcium participates in enzymatic activation, degranulation, and secretion of bacteriolytic enzymes (7). The InsP₃ receptor is thus a crucial link in stimulus-response coupling in these cells.

Neutrophil precursors become competent to combat infection only after they undergo cellular differentiation. The morbidity and mortality associated with acute promyelocytic leukemias are not so much caused by the deleterious effects of these immature cells per se but rather the conspicuous lack of functions associated with mature granulocytes. All-trans-retinoic acid (Tretinoin) has been used therapeutically to stimulate myeloid cell differentiation in a limited number of patients with acute promyelocytic leukemia (8, 9). These patients attained complete remission and their bone marrow cultures were shown to achieve morphological maturation without significant hypoplasia. For these and other reasons, it is important to understand the underlying biochemical mechanisms of normal and chemically induced neutrophilic differentiation.

The HL-60 cell is a human cell line derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia (10). HL-60 cells resemble promyelocytes and are capable of undergoing monocytic or granulocytic differentiation under defined culture conditions (11). Retinoic acid and Me₂SO stimulate HL-60 cell differentiation along the neutrophilic lineage and this process has been associated with enhanced expression of several key regulatory proteins including the InsP₃ receptor (12), the formyl peptide receptor (13), the granulocyte/macrophage colony-stimulating factor receptor (14, 15), and the Mac-1 integrin adherence protein α- and β-subunits (CD11b and CD18, respectively) (16, 17). In the case of the Mac-1 integrin adherence protein, it has been demonstrated that the increased expression of CD11b in retinoic acid-treated HL-60 cells involves post-transcriptional regulation of mRNA stability, whereas the increased expression of CD18 primarily involves enhanced gene transcription (16, 17). In the present study, we examined the kinetics of induction of the functional InsP₃ receptor protein and its encoding mRNA in retinoic acid-treated HL-60 cells and we present evidence that the increased steady state level of functional InsP₃ receptor protein and mRNA is a result of enhanced transcription of the InsP₃ receptor gene. These studies suggest that the effect of retinoic acid on InsP₃ receptor gene transcription appears to be direct rather than secondary to the synthesis of other regulators.

EXPERIMENTAL PROCEDURES

Cell Culture—HL-60 cells were obtained from the American Type Culture Collection and cultured between passages 20 and 60 in RPMI 1640 media (Life Technologies, Grand Island, NY), 10% fetal bovine serum, 5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Retinoic acid (1 μM, all-trans, Sigma) was added to cultures starting at a cell density of 2-3 × 10⁶/ml and incubaions were continued for 1-5 days after which cells were harvested by centrifugation, washed in phosphate-buffered saline, and analyzed for various activities. Morphological assessment of retinoic acid-
induced neutrophilic differentiation was performed by Wright's staining of cytocentrifuged cells. Differentiation was confirmed biochemically by assessing nitro blue tetrazolium reduction and total cellular lysozyme activity as described (12, 18).

Preparation of Membranes—Untreated and retinoic acid-treated HL-60 cells were harvested by centrifugation, washed in phosphate-buffered saline, and lysed by N2 cavitation. Membrane-enriched fractions were prepared by differential centrifugation as described (12). The final membrane pellet was resuspended at 5-10 mg of protein/ml in 50 mM Tris, pH 8.2, 1 mM EDTA buffer and stored at -70 °C (un aliquots, 3ld). Marker enzyme activities (the plasma membrane (5'-nucleotidase) and microsomes (glucose 6-phosphatase)) were assayed in control and retinoic acid-treated cell membrane preparations and were shown to be similarly enriched 3-4-fold compared to the respective total cell cavitites.

Inositol 1,4,5-Trisphosphate Binding Assay—[^3H]Ins(1,4,5)P3 binding to membranes derived from HL-60 cells and from HL-60 cells treated for 1-5 days with 1 μM retinoic acid was performed essentially as described (12). Membranes (0.2-0.5 mg of protein in 50 μl) were added to 50 mM Tris, pH 8.2, 1 mM EDTA buffer containing from 3 to 60 nM[^3H]Ins(1,4,5)P3 (Du Pont-New England Nuclear, 17 Ci/mmol). Reaction mixtures of 0.5 ml final volume were incubated with shaking at 4 °C for 20 min, a time at which steady state InsP3 binding is achieved. Samples were rapidly diluted with cold Tris-EDTA buffer and filtered under vacuum through presoaked Whatman GF/B filters. Nonspecific binding, as determined by including 10 μM Ins(1,4,5)P3 (Boehringer Mannheim) in the reaction mixture, was consistently less than 10% of total binding except at 60 nM labeled ligand, in which case, it amounted to 15% of the total binding. Efficiency of GF/B filtration, as assessed independently by measurement of radioactivity of[^3H]Insositol-containing membranes from control and retinoic acid-treated HL-60 cells, was determined to be >95%. Binding data were analyzed by the ELIP/EDBA LIGAND program.

Determination of Ca2+ Using fura-2 in Permeabilized HL-60 Cells—Untreated and retinoic acid-treated HL-60 cells were washed in phosphate-buffered saline and resuspended at 4 x 10^6/ml in an intracellular-like solution composed of 20 mM NaCl, 100 mM KCl, 1 mM MgCl2, and 30 mM HEPES, pH 7.3. Two ml of cells were treated with permeabilization buffer and placed in a 1-cm cuvette and then placed in a Varian RF-5000 spectrophotometer and maintained at 22 °C. Digitonin (25 μM) and the fluorescent Ca2+ indicator fura-2 (2 μM, Calbiochem) were added to the cell suspension. Oligomycin (10 μg/ml) and antimycin A (10 μM) were also included to inhibit mitochondrial function. After 5-10 min, ATP (3 mM) was added to initiate uptake of ambient Ca2+ into vesicular stores. Two min later, InsP3 (3 μM) or iomycin (1 μM) (free acid, Calbiochem) was added to initiate release of stored Ca2+. Excitation wavelengths were 340 and 380 nm and emission monitored at 505 nm. Ca2+ concentrations were calculated from 340/380 fluorescence ratios after subtraction of autofluorescence (19). Ca2+ concentrations were determined by comparison of fura-2 fluorescence data with Ca2+ measurements. poly(A) RNA Isolation, Northern Blotting, and Dot Blotting—Total cellular RNA was isolated from washed cells by extraction in guanidine thiocyanate according to published methods (20). Cells were homogenized in 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% lauryl sarcosine, and 0.1 mM β-mercaptoethanol by passing the mixture 6-8 times through a 23-gauge needle. The homogenate was layered onto CsCl (96 g/ml in 0.1 M EDTA, pH 7.8) cushion and centrifuged (35,000 rpm at 15 °C) for 16 h. The RNA pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 7.2, and then precipitated by adding 0.1 volume of 3 M sodium acetate, pH 6, and 2.5 volumes of ice-cold ethanol. The RNA precipitate was washed with ethanol and resuspended in diethylpyrocarbonate-treated water, heated at 65 °C for 5 min, cooled, diluted with an equal volume of 2 x binding buffer (20 mM Tris, 1 M NaCl, 2 mM EDTA, 0.2% SDS, pH 7.5), and then poly(A) RNA selected using oligo(dT)-cellulose (Boehringer Mannheim) affinity column chromatography according to procedures recommended by the manufacturer. For Northern blot analyses, 2 μg of poly(A) RNA were blotted and transferred through a solution of polyethyleneimine and onto Gene Screen Plus nylon membranes (Du Pont-New England Nuclear). For dot blot analyses, serial dilutions of poly(A) RNA were applied to Gene Screen Plus membranes using a Bio-Rad apparatus and protocols. Blots were prehybridized at 65 °C for 1-2 h in a solution of 0.9 M NaCl, 90 mM Na6 citrate, 0.1% SDS, 10% dextran sulfate, and 2 x sodium denatured salmon sperm DNA and then hybridized at 65 °C for 12-16 h in buffer containing 0.1-0.5 x 10^6 cpm/ml (about 10^8 cpm/μg) of random hexamer primed [32P]dCTP-labeled (Du Pont-New England Nuclear, 3000 Ci/mmol) PC26 cDNA encoding part of the InsP3 receptor. Radiolabeling reactions utilized the Pharmacia Octoblotting Kit. The 2-3 kb PC26 cDNA clone was isolated by EcoRI and HindIII restriction enzyme digestion of the pcC118/PC26 plasmid. This plasmid was used for expression and isolation of a cDNA clone for mouse β-actin (22) and was end labeled using [y-32P]ATP (Du Pont-New England Nuclear, 6000 Ci/mmol) and T4 polynucleotide kinase as described by Sambrook et al. (23).

Nuclear Run-on Assay—Untreated and retinoic acid-treated HL-60 cells were washed and nuclei isolated by lysis in reticulocyte lysate presocratic buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2) containing 0.5% Nonidet P-40. The nuclei were washed twice in standard buffer, resuspended at a DNA concentration of 1 mg/ml in a solution of 40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM MgCl2 and 0.1 mM EDTA, and used immediately or stored at -70 °C. Assays were carried out essentially as described by McKnight and Palmiter (24). Reaction volumes of 0.2 ml contained nuclei (200 μg of DNA), 30% glycerol, 2.5 mM dithiothreitol, 1 mM MgCl2, 0.25 mM GTP and CTP, 0.5 mM ATP, and 100 μCi of [α-32P]UTP (Du Pont-New England Nuclear, 800 Ci/mmol). Nuclei were incubated for 45 min at 26 °C and then RNA extracted by homogenization in RNAse-free solution and isolated as described above. Approximately 2-3 x 10^4 dpm/ml (final concentration) of labeled total nuclear RNA was added to polyethylene bags containing hybridization solution and Gene Screen Plus membranes (Du Pont-New England Nuclear) to which heat-denatured target plasmids (50-150 ng) had been slot blotted in 0.1 x SSC and prehybridized as described above. Hybridization was allowed to proceed for 36 h at 65 °C before membranes were washed and exposed for autoradiography. Target plasmids included pUC118/PC26 (for InsP3 receptor mRNA detection) and pH322/β-actin (containing the complete coding sequence of β-actin) (25). The specificity of the procedure was demonstrated by the ability of labeled PC26 cDNA probes to hybridize to InsP3 receptor cDNA sequences but not to β-actin cDNA targets.

RESULTS AND DISCUSSION

The HL-60 promyelocytic leukemia cell has proven useful in the study of cellular differentiation and signal transduction. When treated with retinoic acid or Me2SO, HL-60 cells cease to proliferate and begin to differentiate into cells which resemble peripheral blood neutrophils in both structure and function (11). Chemically differentiated HL-60 cells have many of the morphological and biochemical hallmarks of mature granulocytes (12, 26). Thus, chemically differentiated HL-60 cells respond to formyl peptides or other agonists with an immediate formation of Ins(1,4,5)P3, a rapid mobilization of intracellular Ca2+, and subsequent secretion of lyosomal enzymes (6, 27). It was of interest to us that, unlike Me2SO- or retinoic acid-treated HL-60 cells, untreated HL-60 cells were incapable of these responses. This refractoriness of signaling suggested that one or more of the proteins of the phosphoinositide signal transduction cascade may be lacking or expressed only at low levels in HL-60 cells and that the expression of these proteins may be increased during Me2SO or retinoic acid treatment.

Retinoic Acid Increases the Bmax and Ca2+ Mobilizing Activity of the InsP3 Receptor—Previously we have shown that HL-60 cells possess a high affinity InsP3-binding protein with properties which suggest that it is the authentic InsP3 receptor (12). The data in Table I show that the density of the InsP3 receptor in HL-60 cell membranes is increased progressively during 1-5 days treatment with 1 μM retinoic acid. Scatchard analyses of the data showed that specific[^3H]InsP3 binding to membranes from control and retinoic acid-treated HL-60

...of retinoic acid.
cells was to a single site with calculated apparent affinity ($K_0$) of 19 ± 2 nM. This dissociation constant is similar to the 21.7 nM value observed for the protein expressed from the cloned murine InsP$_3$ receptor cDNA (28). In contrast to the uncharged $K_0$ value for $[^3H]$InsP$_3$ binding during 1–5 days of retinoic acid treatment, the $B_{max}$ of $[^3H]$InsP$_3$ binding increased from a basal level of 0.24 to 0.30 pmol/mg ($p<0.05$) after 24 h. After 3 days of retinoic acid treatment, the $B_{max}$ had more than doubled to 0.50 pmol/mg and by 5 days the $B_{max}$ had nearly tripled (0.69 pmol/mg). The half-time for the observed increase in InsP$_3$ receptor was approximately 2.5 days.

The increased $[^3H]$InsP$_3$ receptor $B_{max}$ in retinoic acid-treated HL-60 cell membranes may reflect $[^3H]$InsP$_3$ binding to nonfunctional receptor protein or to receptor protein not yet assembled properly into its target organelle. To address this point, the Ca$^{2+}$-mobilizing function of the InsP$_3$ receptor was assessed in untreated and retinoic acid-treated HL-60 cells which had been permeabilized with digitonin and incubated with fura-2, the fluorescent Ca$^{2+}$ indicator dye. The rationale for this series of experiments is based upon the hypothesis that if treated and untreated HL-60 cells have comparable intracellular Ca$^{2+}$ storage capacity, then the amount of Ca$^{2+}$ mobilized from these stores by a saturating concentration of InsP$_3$ should be a reflection of the InsP$_3$ receptor density. In the presence of ATP and mitochondrial inhibitors, digitonin-permeabilized HL-60 cells and retinoic acid-treated HL-60 cells lowered the ambient Ca$^{2+}$ concentration from 1–2 μM to 297 ± 14 nM (n = 12, mean ± S.E.) and 305 ± 39 nM (n = 10, mean ± S.E.), respectively. This Ca$^{2+}$ was sequestered into a nonmitochondrial vesicular store since the mitochondrial inhibitors antimycin and oligomycin were included in the incubations and since 1 μM ionomycin released the entire ATP-dependent Ca$^{2+}$ store (Fig. 1B). Similar results were seen with both control and retinoic acid-treated HL-60 cells indicating that both cell types have similar capabilities to sequester Ca$^{2+}$ into intracellular nonmitochondrial vesicular stores and that the capacities of these stores are approximately equal in both cell types. Despite the comparable Ca$^{2+}$ storage capacity, the addition of 3 μM InsP$_3$ to the permeabilized cell suspension caused the release of much more Ca$^{2+}$ from intracellular stores of retinoic acid-treated cells than from stores of untreated cells (Fig. 1A). On average, HL-60 cells treated with 1 μM retinoic acid for 3 days released twice as much Ca$^{2+}$ in response to 3 μM InsP$_3$ than did untreated HL-60 cells. Ca$^{2+}$ released by 10 μM InsP$_3$ was no different from that released by 3 μM InsP$_3$, indicating that a saturating amount of InsP$_3$ was used in these experiments. The increased Ca$^{2+}$ mobilization by InsP$_3$ in retinoic acid-treated HL-60 cells increased with time of retinoic acid exposure: 2-day treatment with retinoic acid showing approximately 53% increase over untreated, 3-day treatment about a 2.2-fold increase, and 5-day treatment about a 2.7-fold increase (Table II).

In the presence of InsP$_3$, the steady state extraskeletal Ca$^{2+}$ level, which is dictated by the opposing rates of Ca$^{2+}$ uptake and release, is shifted to higher concentrations in retinoic acid-treated cells compared to untreated cells. The experiments in Fig. 1 and Table II indicate that Ca$^{2+}$ uptake is similar in both cell types but the extent and thus the rate of Ca$^{2+}$ release is increased in retinoic acid-treated cells. These results suggest that retinoic acid-treated HL-60 cells have a higher density of Ca$^{2+}$ mobilizing InsP$_3$ receptors, a suggestion consistent with the observed retinoic acid-dependent increase in $[^3H]$InsP$_3$ radioligand binding. Despite this, other factors including post-translational receptor modification cannot be discounted and may also contribute to the observed increased effectiveness of the InsP$_3$ response in retinoic acid-treated cells.

**Retinoic Acid Increases InsP$_3$ Receptor mRNA Levels**

Several possible mechanisms could account for the enhanced

### Table I

**Effect of retinoic acid treatment on $K_0$ and $B_{max}$ of $[^3H]$InsP$_3$ binding to HL-60 cell membranes**

<table>
<thead>
<tr>
<th>HL-60 cell treatment</th>
<th>$K_0$ ($nM$)</th>
<th>$B_{max}$ (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>17 ± 2</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>1-Day retinoic acid</td>
<td>19 ± 4</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>3-Day retinoic acid</td>
<td>19 ± 3</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>5-Day retinoic acid</td>
<td>21 ± 4</td>
<td>0.69 ± 0.04</td>
</tr>
</tbody>
</table>

### Table II

**Effect of retinoic acid treatment on InsP$_3$-stimulated Ca$^{2+}$ release from nonmitochondrial vesicular stores in digitonin-permeabilized HL-60 cells**

<table>
<thead>
<tr>
<th>HL-60 cell treatment</th>
<th>Ca$^{2+}$ concentration +ATP</th>
<th>+InsP$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>297 ± 14</td>
<td>378 ± 20</td>
</tr>
<tr>
<td>2-Day retinoic acid</td>
<td>290 ± 41</td>
<td>414 ± 32</td>
</tr>
<tr>
<td>3-Day retinoic acid</td>
<td>305 ± 39</td>
<td>481 ± 48</td>
</tr>
<tr>
<td>5-Day retinoic acid</td>
<td>299 ± 46</td>
<td>538 ± 50</td>
</tr>
</tbody>
</table>

2. Effect of retinoic acid treatment on InsP$_3$-induced and ionomycin-induced Ca$^{2+}$ release from permeabilized HL-60 cells. Untreated or 3-day retinoic acid (RA)-treated HL-60 cells were permeabilized with digitonin and incubated in an intracellular-like solution containing fura-2 and mitochondrial inhibitors. The cells were exposed to 3 mM ATP and then either 5 μM InsP$_3$ or 1 μM ionomycin as indicated and changes in Ca$^{2+}$ concentrations were monitored using fura-2 fluorescence as described under "Experimental Procedures." Numbers in the ordinates are calculated Ca$^{2+}$ concentrations. The curves are each representative of 10 experiments.
expression and effectiveness of the InsP₃ receptor protein in retinoic acid-treated cells. To investigate the possibility that there may be greater levels of InsP₃ receptor mRNA in these cells, Northern blot analyses were performed. Using the PCD6 probe which is a partial (2 kb) cDNA clone of the murine InsP₃ receptor (29, 30), a single hybridizing species of approximately 10 kb was detected in Northern blots of poly(A⁺) RNA of both untreated and retinoic acid-treated HL-60 cells (Fig. 2A). Similarly sized InsP₃ receptor RNA transcripts have been observed in mouse and rat brain and the complete sequence of the cloned InsP₃ receptor cDNAs from these sources is 9871 base pairs (28, 31). To further support the contention that the 10-kb poly(A⁺) RNA species identified by the PCD6 probe in HL-60 cells is indeed the InsP₃ receptor mRNA, Northern blots were performed with a probe derived by polymerase chain reaction cloning of an InsP₃ receptor cDNA fragment from HL-60 cells. This polymerase chain reaction probe, shown by DNA sequencing to encode nucleotides 489 through 970 of the InsP₃ receptor cDNA, recognized an identically sized 10-kb mRNA on HL-60 cell Northern blots (not shown). Northern blots using the labeled PCD6 cDNA probe are shown in Fig. 2A. In untreated HL-60 cells there is a low level of InsP₃ receptor mRNA expression but this level increased substantially in cells treated for 24 h with 1 μM retinoic acid (Fig. 2A, lanes 0 and 1). The amount of the 10-kb InsP₃ receptor mRNA was further increased after 2, 3, and 4 days treatment with retinoic acid and then declined somewhat at 5 days (Fig. 2A, lanes 2–5). Throughout the course of treatment with retinoic acid, the level of β-actin mRNA was unchanged (Fig. 2B).

InsP₃ receptor mRNA levels in untreated and retinoic acid-treated HL-60 cells were quantitatively compared by poly(A⁺) RNA dot blot dilution analyses (Fig. 3). After 24 h treatment with 1 μM retinoic acid, InsP₃ receptor RNA transcripts increased 2-fold and after 4 days the mRNA was maximally increased to approximately 4-fold over untreated HL-60 cells. By this method, β-actin mRNA levels were indistinguishable in control and retinoic acid-treated HL-60 cells (not shown).

Retinoic Acid Increases the Transcription Rate of the InsP₃ Receptor Gene—The increased steady state level of InsP₃ receptor mRNA in retinoic acid-treated HL-60 cells might arise from increased transcription of the InsP₃ receptor gene, from enhanced stabilization of existing mRNA transcripts, or both. These mechanisms have been shown to be operative in elevating specific mRNAs in HL-60 cells (16, 17). In order to investigate the contributions of these mechanisms to the demonstrated increase in InsP₃ receptor mRNA, nuclear run-on assays were performed with untreated HL-60 cells and HL-60 cells treated for 4 days with 1 μM retinoic acid. Nuclear run-on assays allow a determination of the rates of specific mRNA synthesis which reflects transcriptional start rates at specific genes (24). The results of nuclear run-on experiments with nuclei from control and 4-day retinoic acid-treated HL-60 cells, [³²P]UTP-labeled nuclear RNA was purified from each group of nuclei and equal amounts of the [³²P]RNA were hybridized to Gene Screen Plus nylon membranes containing denatured plasmid probes for the InsP₃ receptor (pUC118/PCD6, 50 and 150 ng) or for β-actin (pBR322/β-actin, 50 ng).

Fig. 3. Dot blot analysis of InsP₃ receptor mRNA expression. Poly(A⁺) RNA (0.5–4 μg) was applied to Gene Screen Plus nylon membranes and hybridized with a radiolabeled probe for InsP₃ receptor mRNA (PCD6 probe). RNA was derived from HL-60 cells treated with 1 μM retinoic acid for the number of days (0–5) indicated on the ordinate.

Fig. 4. Nuclear run-on studies of control and retinoic acid-treated HL-60 cells. Transcription assays were performed using nuclei from control and 4-day retinoic acid-treated HL-60 cells. [³²P]UTP-labeled nuclear RNA was purified from each group of nuclei and equal amounts of the [³²P]RNA were hybridized to Gene Screen Plus nylon membranes containing denatured plasmid probes for the InsP₃ receptor (pUC118/PCD6, 50 and 150 ng) or for β-actin (pBR322/β-actin, 50 ng).

Fig. 2. Northern blot analysis of poly(A⁺) RNA from control and retinoic acid-treated HL-60 cells. Cells were incubated with 1 μM retinoic acid for the indicated number of days (0–5) and the size separated poly(A⁺) RNA was hybridized with radiolabeled probes for (A) InsP₃ receptor mRNA (PCD6 probe) or (B) β-actin mRNA. Migration of RNA molecular size standards is indicated.
increase in steady state mRNA levels, to the approximate tripling of the InsP₃ receptor density in prepared membranes, and to the 2–3-fold increase in InsP₃ receptor mRNA levels in untreated and retinoic acid-treated HL-60 cells. Poly(A⁺) RNA was prepared from HL-60 cells which were treated for 20 h with or without 1 μM retinoic acid (RA) and in the presence (+) and absence (−) of 2 μg/ml CHX.

Northern blots of poly(A⁺) RNA (2 μg) were sequentially hybridized with the InsP₃ receptor PCD6 probe (left-hand side) and the β-actin probe (right-hand side).

Cycloheximide Stabilizes InsP₃ Receptor mRNA—To investigate whether the observed transcriptional enhancing effect of retinoic acid is due to direct or indirect actions of retinoic acid on the InsP₃ receptor gene, mRNA induction was determined in the presence and absence of cycloheximide (CHX), an inhibitor of protein synthesis. If enhanced transcription of the InsP₃ receptor gene depends upon the retinoic acid-stimulated synthesis of a secondary transcription factor, then CHX should block any retinoic acid-dependent increase in InsP₃ receptor mRNA. The results of these experiments are shown in Fig. 5. In control studies, HL-60 cells treated for 20 h with 1 μM retinoic acid show an approximate 2-fold increase in InsP₃ receptor mRNA compared to untreated HL-60 cells (Fig. 5, first and third lanes). Surprisingly, CHX (3 μg/ml) in the presence and absence of retinoic acid significantly increased InsP₃ receptor mRNA levels above that seen with retinoic acid alone (second and fourth lanes). The latter results suggest that protein synthesis is required either to degrade InsP₃ receptor mRNA and/or to repress gene transcription in HL-60 cells. Similar results were seen with 10 μg/ml CHX.

Significantly, the data in Fig. 5 also indicate that even in the absence of CHX the InsP₃ receptor mRNA level is still elevated by retinoic acid. Scanning densitometry from two separate experiments show that in the presence of CHX, InsP₃ receptor mRNA is elevated 2.5-fold by retinoic acid treatment. These results suggest that protein synthesis is not required for the induction of InsP₃ receptor gene transcription by retinoic acid. This is consistent with the results from the nuclear run-on experiments and together may suggest that a retinoic acid-response element may be present in the regulatory region of the InsP₃ receptor gene. The dramatic accumulation of InsP₃ receptor mRNA in the presence of CHX indicates that continued protein synthesis is required either for the specific rapid degradation of the InsP₃ receptor mRNA or for the synthesis of a labile gene repressor. However, these processes are not responsive to retinoic acid. Control studies show that the levels of β-actin mRNA are unaffected by retinoic acid and unchanged or reduced somewhat in the presence of CHX (Fig. 5).

To examine the effect of inhibition of protein synthesis on the stability of InsP₃ receptor mRNA in the absence of transcription, HL-60 cells were treated for 20 h in the presence or absence of 3 μg/ml CHX and during the last 0, 1, or 3 h, actinomycin D (2 μg/ml) was included. Poly(A⁺) RNA was then prepared and analyzed for InsP₃ receptor mRNA levels by Northern blot. Data in Fig. 6 (lanes 1 and 3) show that when further transcription is inhibited by actinomycin D, InsP₃ receptor mRNA levels are relatively stable for 1 h but decay to nearly undetectable levels by 3 h. However, in the presence of the protein synthesis inhibitor CHX, InsP₃ receptor mRNA is stable for longer periods. Even after 3 h treatment with actinomycin D, InsP₃ receptor mRNA is still elevated. The results suggest that protein synthesis is required to degrade InsP₃ receptor mRNA, consistent with the hypothesis that a transient nuclease must be synthesized continuously in order to degrade InsP₃ receptor mRNA. The increased level of InsP₃ receptor mRNA in the presence of CHX is somewhat surprising since this superinduction effect of CHX is characteristic of several early response genes, including c-fos, c-myc, c-fosB, and zif268 (32–34). The relationship if any between the InsP₃ receptor gene and the genes for important regulatory transcription factors as suggested by these experimental results is not at all clear.

Conclusions—One of the principal reasons why leukemic promyelocytic cells and HL-60 cells are incapable of mounting an antibacterial response may be the relative functional deficiency of the phosphoinositide signal transduction cascade including the Ca²⁺-mobilizing action of the InsP₃ receptor. In studies with HL-60 cells, it has been shown that when treated with either retinoic acid or Me₂SO, the cells become competent to raise intracellular Ca²⁺ levels, generate superoxide, and secrete bacteriolytic enzymes in response to stimulation (27, 35). The results reported here show that retinoic acid treatment of HL-60 cells stimulates a functional increase in the InsP₃ receptor protein and that the basis for this increase may lie in a direct stimulation of InsP₃ receptor gene transcription. The analysis of InsP₃ receptor mRNA levels in HL-60 cells indicates that InsP₃ receptor gene transcription has properties that are characteristic of several early response genes. Thus, InsP₃ receptor gene transcription is induced by hormone and the resultant mRNA is rapidly degraded through a process that requires continued protein synthesis. InsP₃ receptor gene transcription may thus be a proximal event, or a reflection of proximal events critical to HL-60 cell granulocytic differentiation. The further study of InsP₃ receptor gene transcription may elucidate some of the molecular processes which determine the commitment to a differentiated state in myeloid cell development. The direct action of retinoic acid on the InsP₃ receptor gene as suggested by these studies...
would predict that a functional retinoic acid receptor response element would be found in the regulatory elements of this gene. In this regard, it has been demonstrated that the retinoic acid receptor α is expressed in HL-60 cells and is necessary for retinoic acid-stimulated HL-60 cell differentiation (35). The observations that the InsP₃ receptor is critical to neutrophil differentiation.

The observations that the InsP₃ receptor is critical to neutrophil differentiation may provide insight into the molecular mechanisms of normal and pathologically arrested myelocytic differentiation.

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