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Down-Regulation of P_{2U}-Purinergic Nucleotide Receptor Messenger RNA Expression During *In Vitro* Differentiation of Human Myeloid Leukocytes by Phorbol Esters or Inflammatory Activators

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SUMMARY

HL-60 human promyelocytic leukocytes express G protein-coupled P_{2U}-purinergic nucleotide receptors ($P_{2U}R$ or $P2Y_2R$) that activate inositol phospholipid hydrolysis and Ca^{2+} mobilization in response to ATP or UTP. We examined the expression of functional $P_{2U}R$ and $P_{2U}R$ mRNA levels during *in vitro* differentiation of HL-60 cells by dibutyryl-cAMP (Bt_2cAMP), which induces a granulocyte/neutrophil phenotype, or by phorbol-12-myristate-13-acetate (PMA), which induces a monocyte/macrophage phenotype. Both $P_{2U}R$ function and $P_{2U}R$ mRNA levels were only modestly attenuated during granulocytic differentiation by Bt_2cAMP. In contrast, $P_{2U}R$ function, as assayed by either Ca^{2+} mobilization or inositol trisphosphate generation, was greatly reduced in PMA-differentiated cells. This inhibition of $P_{2U}R$ function was strongly correlated with PMA-induced decreases in $P_{2U}R$ mRNA levels, as assayed by Northern blot analysis or reverse transcription-polymerase chain reaction-based quantification. Although PMA induced an early, transient up-regulation of $P_{2U}R$ mRNA, this was rapidly followed by a sustained decrease in $P_{2U}R$ mRNA to a level 5–10-fold lower than that in undifferentiated HL-60 cells. The half-life of the $P_{2U}R$ transcript in HL-60 cells was ~60 min, and this was not affected by acute exposure (≤4 hr) to Bt_2cAMP or PMA. PMA down-regulated $P_{2U}R$ mRNA in THP-1 monocytes and HL-60 granulocytes but not in A431 human epithelial cells or human keratinocytes. $P_{2U}R$ mRNA was also down-regulated in THP-1 monocytes differentiated into inflammatory macrophages by γ-interferon and endotoxin. These data indicate that myeloid leukocytes possess tissue-specific mechanisms for the rapid modulation of $P_{2U}R$ expression and function during differentiation and inflammatory activation.

Extracellular ATP elicits functional responses in many cell types by activating P_2-purinergic nucleotide receptors (1); these include both G protein-coupled nucleotide receptors (collectively termed the P_{2Y} class) and ionotropic ATP-gated channel receptors (termed the P_{2X} class). Each of these major classes comprises a number of pharmacologically and genetically distinct receptor subtypes; cDNAs or genes encoding at least six different P_{2Y} class receptors (2) and seven different P_{2X} class receptors (3) have been recently cloned. Despite the growing number of distinct ATP receptor subtypes with redundant signaling properties, few studies have addressed issues regarding the factors that regulate the cell-specific expression of these receptor subtypes.

The P_{2Y} receptor family includes the $P_{2U}R$ (also termed $P2Y_2$ by recommended IUPHAR nomenclature), a subtype for which ATP and UTP are equipotent agonists. In the presence of micromolar ATP or UTP, $P_{2U}R$ activate PI-PLC effector enzymes, rapid mobilization of $InsP_3$-sensitive Ca^{2+} stores, and enhanced Ca^{2+} influx (2, 4, 5). Depending on the cell type, $P_{2U}R$ can activate PI-PLC enzymes via the mediation of either the G_i or G_q family of G proteins (1, 2). Cloned DNAs encoding $P_{2U}R$ have been isolated from several species and sources (2), including murine neuroblastoma cells (6), human epithelial cells (7), and rat genomic DNA (8). All of these cDNAs are highly homologous. Northern blot analysis and functional studies have indicated that $P_{2U}R$ are expressed in a wide range of tissues. However, the organization and promoter sequences of $P_{2U}R$ genes have not been reported, and little is known concerning the regulation of $P_{2U}R$ expression.

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ABBREVIATIONS: $P_{2U}R$, P_{2U} receptor(s); Bt_2cAMP, dibutyryl-cAMP; PMA, phorbol-12-myristate-13-acetate; FPR, formyl peptide receptor; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; PLC, phospholipase C; $InsP_2$, inositol bisphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; $InsP_3$, inositol trisphosphate; BSA, bovine serum albumin; IFN, interferon; RT, reverse-transcription (or -transcriptase); PCR, polymerase chain reaction; PK, protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PI, phosphatidylinositol.

We and others have previously reported that $P_{2U}R$ are expressed in most myeloid leukocytes, including neutrophils, monocytes, macrophages, and the myeloid progenitor cells in marrow (9–12). Myeloid leukocytes provide a useful model for studying developmental regulation of ATP receptor expression because these cells are continuously replenished throughout adult life during two major stages of development and differentiation. Myeloid progenitor cells differentiate in the bone marrow over the course of several days to yield the circulating blood granulocytes and monocytes. However, blood monocytes and tissue macrophages (which are derived from monocytes) exist as only partially differentiated, quiescent cells until activated by immune or inflammatory stimuli. Thus, inflammatory activation of monocytes/macrophages represents the second stage of myeloid differentiation, which is characterized by major changes in gene expression and the induction, or repression, of signaling proteins involved in regulation of the inflammatory response (13). This raises the possibility that ATP receptors might also act as inducible or repressible signaling proteins whose expression can be regulated at the transcriptional/translational levels during myeloid differentiation and inflammatory activation.

In this study, we investigated the regulation of $P_{2U}R$ expression in HL-60 cells, a human promyelocyte line derived from a patient with M3 acute myelogenous leukemia. These progenitor cells have been extensively used as an *in vitro* model for myeloid differentiation (14). When cultured in the presence of dibutyryl cAMP or DMSO, these cells assume a granulocyte/neutrophil phenotype. In contrast, treatment with phorbol esters induces HL-60 cells to acquire many phenotypic features characteristic of inflammatory monocytes and macrophages. These studies indicate that the expression of both $P_{2U}R$ mRNA and functional $P_{2U}R$ is rapidly and significantly modulated during these programs of *in vitro* myeloid differentiation.

Materials and Methods

Cell culture. The HL-60 and THP-1 human leukocyte cell lines (American Type Culture Collection, Rockville, MD) were routinely maintained in Iscove's modified minimal essential medium medium (GIBCO, Grand Island, NY) with 10% iron-supplemented calf serum (Hyclone Laboratories, Logan, UT) in a humidified atmosphere of 92.5% air/7.5% CO_2, at densities between 3×10^5 and 1×10^6/ml. For granulocytic differentiation, HL-60 cells were transferred to serum-free Iscove's medium supplemented with transferrin, insulin, selenium, 2 mM glutamine, 1 mg/ml BSA, 100 units/ml penicillin, and 100 μg/ml streptomycin for 24 hr before induction with 500 μM Bt_2cAMP. Where indicated, HL-60 cells were also treated with 100 nM PMA or 5 μg/ml actinomycin D (Boehringer-Mannheim Biochemicals, Indianapolis, IN) added directly to serum-containing growth medium. In certain experiments, THP-1 promonocytes were differentiated into inflammatory macrophages by treatment with 1000 units/ml recombinant human IFN-γ (Genentech, South San Francisco, CA) and/or 1 μg/ml bacterial LPS (*Escherichia coli* 0111:B4; List Biomedicals, Campbell, CA) for 24–48 hr. A431 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 2 mM glutamine at 5% CO_2. Human keratinocyte primary cultures, prepared from foreskin samples, were generously provided by Drs. Richard Eckert and Jean Welter (Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH). These cells were passaged three times, allowed to reach 70% confluence, and then treated with PMA or actinomycin D as indicated.

Measurement of cytosolic $[Ca^{2+}]$. Adherent HL-60 cells from PMA-treated cultures were removed by washing in Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution, followed by incubation in this solution with 300 μM EDTA for 10 min at 37°. Nonadherent, suspended cells were removed from growth medium through centrifugation. All cell types were then washed and resuspended at 1×10^6/ml in a basal salt solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM Na-HEPES, pH 7.5, 5 mM glucose, and 1 mg/ml BSA. Cells were incubated with 500 nM Fura-2-acetoxymethyl ester ester (Molecular Probes, Eugene, OR) for 40 min at 37°, centrifuged, resuspended in fresh medium at 3.3×10^6/ml, and then incubated for an additional 10 min at 37°. Cells were stored on ice for ≤4 hr during measurements. Fura-2-loaded cells were assayed at 1.1×10^6/ml in 1.5 ml in a stirred quartz cuvette at 37°. Fura-2 fluorescence was measured using 339 nm excitation and 500 nm emission. Where indicated, cells were incubated with 100 nM PMA and/or 300 nM staurosporine for 15 min at 37° before assay. Cells were lysed with 20 μg/ml digitonin for calibration as described previously (4).

Isotopic labeling of inositol phospholipids. HL-60 cells were removed from growth medium and resuspended at 1×10^6/ml in serum-free, inositol-free Iscove's medium supplemented with insulin, transferrin, selenium, 2 mg/ml BSA, 4 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 μCi/ml L-*myo*-[2-^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO) and incubated for 72 hr before experiments.

Measurement of inositol phosphate accumulation in intact HL-60 cells. Labeled cells were washed twice with basal salt solution. The cells were resuspended at 5×10^6/ml in this buffer, and 0.2-ml aliquots were preincubated for 5 min at 37° before the addition of nucleotide agonists. After 15 sec, the reactions were terminated, and the samples were processed for analysis of $InsP_2$ and $InsP_3$ content as described previously (5).

Measurement of [^{3}H]inositol phosphate production by isolated HL-60 cell membranes. Membranes from HL-60 cells were prepared as described previously (5). Briefly, cells were washed twice with an ice-cold buffer solution, resuspended in cold lysis buffer, and lysed by N_2 cavitation. EGTA-supplemented lysates were subjected to centrifugation, and final pellets were resuspended in cold EGTA-containing lysis buffer to yield stock membrane suspensions. Analysis of inositol polyphosphate production was performed exactly as reported previously (5). Briefly, aliquots of stock membrane suspensions were added to 37° assay buffer with the indicated concentrations of free Ca^{2+} and nucleotides. The reaction mixture was incubated at 37° for 5 min before organic extraction and analysis of [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ accumulation in the aqueous phase.

Northern blot analysis. Total RNA was extracted from cultured cells according to the method of Chomczynski and Sacchi (15). Poly(A)$^+$ RNA was selected on oligo(dT) cellulose columns, precipitated, dissolved, and quantified by UV spectrophotometry. Then, 3.0 μg of poly(A)$^+$ RNA/lane was electrophoresed on formaldehyde agarose gels. Gels were transferred to Nytran membrane by TurboBlotter rapid downward transfer (Schleicher & Schuell, Keene, NH) or by electroblotting in Tris/acetate/EDTA buffer (40 mM Tris/acetate, pH 8, 1 mM EDTA). The human $P_{2U}R$ cDNA probe (*SacI*/*Bgl*II 814-1369 fragment) or cDNA probes corresponding to the FPR, IL-1β, myeloperoxidase, and GAPDH gene products were random primer-labeled (Boehringer-Mannheim) with [α-^{32}P]dCTP (Amersham). Blots were hybridized with probes by incubation in Quik-Hyb solution (Stratagene) for 1 hr at 65°. The blots were then washed and processed by standard methods. Hybridization of ^{32}P probes to specific bands was quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Blots were stripped by boiling in 0.1× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.4) /0.1% sodium dodecyl sulfate before subsequent probing with other labeled cDNAs. The FPR cDNA was a generous gift from Dr. Daniel Perez (Department of Medicine, University of California, San Francisco, CA).

Semiquantitative RT-PCR. Total RNA was isolated by the above methods or by using a Qiagen (Studio City, CA) RNeasy total

RNA kit. RNA (1.0 μg) was reverse-transcribed to cDNA in a 20-μl reaction volume containing 0.5 μg of oligo(dT) primer, 8 mM concentration of dNTPs, 40 units of RNasin (Boehringer-Mannheim), 10 mM $MgCl_2$, and 25 units of avian myeloblastosis virus RT (Boehringer-Mannheim) dissolved in a RT buffer (Promega, Madison, WI). The reactions were incubated for 1 hr at 42°, stopped by boiling for 2 min, and then diluted to 100 μl with sterile RNase-free water. Parallel aliquots of RNA samples (from control cells in each experiment) were subjected to mock RT reactions. These samples were incubated and prepared as described above, but no avian myeloblastosis virus RT was included. Diluted aliquots from the *bona fide* or mock RT reactions were then used as templates for PCR with primers specific to the human $P_{2U}R$ (sense, 5′-CTC TAC TTT GTC ACC ACC AGC GCG-3′; antisense, 5′-TTC TGC TCC TAC AGC CGA ATG TCC-3′), generating the predicted 632-bp product. Commercial primers to GAPDH, IL-1β, TNF-α, and the human FPR (Stratagene) were also used to generate 600-, 332-, 355-, and 410-bp products, respectively. $P_{2U}R$ and FPR reactions included 1.0 μM concentration of each primer, 0.8 mM concentration of dNTPs, 60 mM Tris·HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, and 1.25 units of *Taq* polymerase (Boehringer-Mannheim or United States Biochemical, Cleveland, OH) in a 50-μl reaction volume that was preincubated with 275 ng of TaqStart antibody 5–30 min at room temperature (Clonetech, Palo Alto, CA). TNF-α reactions contained the same components but with 2.5 mM $MgCl_2$. GAPDH and IL-1β reactions included 1.0 μM concentration of each primer, 0.8 mM concentration of dNTPs, 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and *Taq* polymerase pretreated as above. The PCR cycling protocols for each primer set were as follows: $P_{2U}R$ and IL-1β, 1 min at 94°, 2 min at 55°, and 4 min at 72°; GAPDH, 1 min at 94°, 2 min at 60°, and 2 min at 72°; FPR, 45 sec at 94°, 45 sec at 60°, and 1.5 min at 72°; and TNF-α, 45 sec at 94°, 45 sec at 54°, and 1.5 min at 72°. Each protocol was carried out for 35 cycles and included an initial 5-min denaturation at 94° and a final 7-min extension at 72°. [α-^{32}P]dCTP (0.1–0.4 μCi) was included in some PCRs to permit quantification of ^{32}P-labeled PCR products. These samples were quantified in duplicate where indicated. Ten microliters of each PCR product was electrophoresed on 1% agarose gels containing ethidium bromide. Gels were photographed, and each PCR product band was excised with the use of a razor blade and transferred to glass scintillation vials. Agarose slices of equal size were cut from unloaded lanes to measure background cpm. Slices were melted in 1.6 ml of water and counted for 1 min in 15 ml of scintillation fluid. Standard curves were generated using serial dilutions of the RT reactions as templates for PCR with each primer set. These curves were used to determine the linear range of the assay for each primer set and showed the assay to be sensitive to 2-fold changes in template level. Products from the original 20-μl RT reaction volumes were appropriately diluted into the final PCR volumes to ensure nonsaturation of the PCR amplification reactions: 1:50 or 1:100 dilutions were used for $P_{2U}R$ analysis, 1:500 dilutions for the GAPDH analysis, and 1:50 dilutions for the FPR, TNF-α, and IL-1β analyses. Primary data are presented as the amount of cpm of ^{32}P incorporated in each PCR product after subtraction of background radioactivity. Where indicated, data for $P_{2U}R$ mRNA levels have been normalized relative to the amount of amplified GAPDH product. The absolute amount of radioactivity associated with given PCR products varies among experiments due to the use of [^{32}P]dCTP preparations with different specific activities.

This RT-PCR method was sensitive to 2-fold changes in template concentration and could detect mRNA levels not easily assayed by Northern blot analysis. Because the coding region of the $P_{2U}R$ gene is not interrupted by introns (8), it was important to verify that PCR analysis of the RT RNA samples was not compromised by unintended amplification of contaminating genomic DNA. PCR amplification with $P_{2U}R$ primers of mock RT reactions (using RNA from uninduced HL-60 cells in the absence of reverse transcriptase) confirmed that the RNA isolated in most experiments (>80%) was free of significant genomic DNA contamination. If genomic contamination was detected in the RNA from control HL-60 cells, the experiment was excluded from further analysis. Although DNase treatment of such RNA was effective in removing DNA contamination, this procedure (in our hands) was complicated by variable, quantitative recovery of RNA.

Results

Differential attenuation of $P_{2U}R$ functional activity during granulocytic versus monocytic differentiation of HL-60 cells. In previous studies (4), we reported that functional activities of $P_{2U}R$ were similar in both undifferentiated HL-60 promyelocytes and in HL-60 cells differentiated into granulocytes by treatment with Bt_2cAMP. In contrast, a very significant attenuation of $P_{2U}R$ functional activity was observed in HL-60 cells treated with PMA, an agent that induces differentiation along the monocyte/macrophage pathway. Fig. 1, A–C, shows a comparison of the potency of UTP as a Ca^{2+}-mobilizing agonist in undifferentiated HL-60 promyelocytes versus HL-60 cells treated with PMA for 2 days. Equivalent and maximal Ca^{2+} mobilization was observed when undifferentiated cells were stimulated with UTP concentrations of >3 μM (Fig. 1A). In cells treated with PMA for 48 hr, 3 μM UTP elicited no Ca^{2+} mobilization, and the response to 300 μM UTP, a normally supramaximal concentration, was greatly reduced (Fig. 1B). Concentration-response plots (Fig. 1C) indicated that both the potency and efficacy of UTP were significantly attenuated in PMA-differentiated HL-60 cells. Fig. 1C indicates that a 1-day treatment with PMA was also sufficient to induce a 2-log unit decrease in UTP potency. Because Ca^{2+} mobilization is secondary to the activation of PI-PLC-β, the primary effector enzyme for these G protein-coupled $P_{2U}R$, we also compared UTP-induced accumulation of inositol polyphosphates in undifferentiated and PMA-differentiated HL-60 cells (Fig. 1D). Consistent with the Ca^{2+} mobilization data, both the potency and efficacy of UTP as an activator of PI-PLC were greatly reduced in PMA-differentiated cells.

To determine whether G protein coupling to PI-PLC effector enzymes was also affected by chronic PMA treatment, ^{3}H-inositol-labeled membranes were prepared from control HL-60 cells or PMA-differentiated cells. The membranes were then stimulated with GTP alone or with UTP plus GTP to assay $P_{2U}R$ potentiation of G protein-dependent PLC enzymes. Other membrane samples were stimulated with GTPγS to maximally activate G protein-dependent PLC signaling independent of receptor/G protein coupling. Membranes from 24-hr PMA-treated cells produced 50% less $InsP_2/InsP_3$ in response to 30 μM UTP (plus GTP) than did membranes from untreated cells (Table 1). UTP-induced inositol polyphosphate accumulation was reduced by 82% in membranes isolated from 48-hr PMA-treated cells. However, 100 μM GTPγS elicited similar amounts of $InsP_3$ accumulation in membranes from control or PMA-treated cells. These data demonstrate that the G protein/PI-PLC-β coupling is normal in membranes from PMA-treated cells but that $P_{2U}R$-mediated activation of the relevant G protein(s) is greatly attenuated.

Acute activation of PKC by phorbol esters has been shown to inhibit the activation of PI-PLC by G protein-coupled receptors in undifferentiated HL-60 promyelocytes (4) and differentiated HL-60 granulocytes (16). Thus, it was important

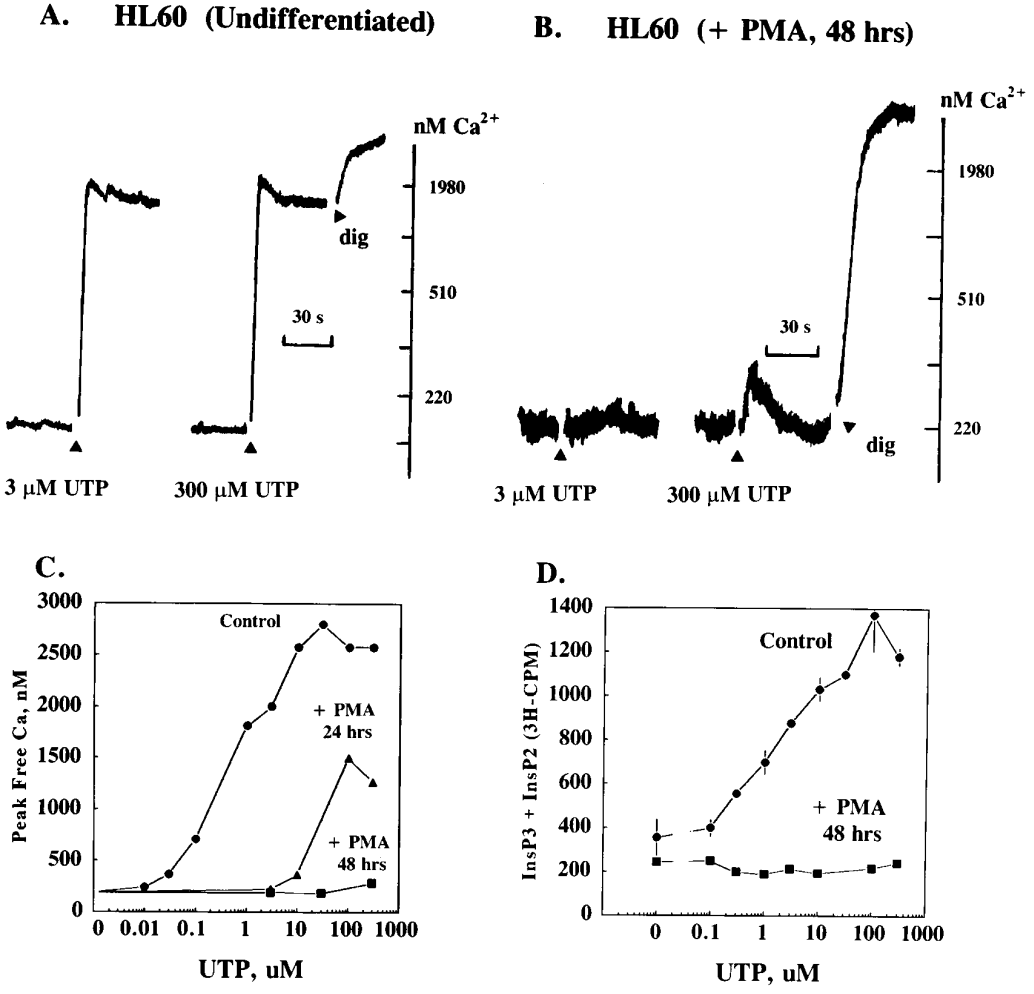


Fig. 1. UTP-induced Ca^{2+} mobilization and inositol polyphosphate generation in control and PMA-differentiated HL-60 cells. HL-60 cells were cultured in the (A) absence or (B) presence of 100 nM PMA for 48 hr. Fura-2-loaded cells were assayed in a fluorimeter for Ca^{2+} mobilization in response to the indicated concentrations of UTP. Where indicated, the cells were lysed by the addition of 20 μg/ml digitonin (*dig*) to permit calibration of the release of Fura-2. C, Concentration-response relationships describing UTP-induced Ca^{2+} mobilization in (circles) control HL-60 cells or cells treated with 100 nM PMA for (▲) 24 or (■) 48 hr. D, Concentration-response relationships describing UTP-induced $InsP_3$ production in (●) control and (■) 48-hr PMA-treated HL-60 cells. HL-60 cells were labeled with ^{3}H-inositol in serum-free medium as described in Materials and Methods for 24 hr and then cultured in the presence or absence of 100 nM PMA for an additional 48 hr. Cells were washed and resuspended in assay buffer (see Materials and Methods) and incubated with the indicated concentrations of UTP for 15 sec at 37° before the assay for $InsP_2$/$InsP_3$ production.

TABLE 1

PI-PLC activity in membranes isolated from control and PMA-differentiated HL-60 cells

HL-60 cells were labeled with [^{3}H]inositol in serum-free medium as described in Materials and Methods for 24 hr and then cultured in the presence or absence of 100 nM PMA for an additional 24 or 48 hr. Membranes were prepared as described in Materials and Methods, resuspended in basal medium, and assayed for $InsP_2$/$InsP_3$ production in response to a 5-min incubation in the presence of the indicated concentrations of calcium and nucleotides. Basal medium contained 100 mM KCl, 5 mM NaCl, 50 mM K-HEPES, pH 7.4, 3 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol. Data for control membranes represent the mean ± standard error of values (each assay performed in duplicate) from four experiments, each performed in duplicate. Data for PMA-treated cell membranes represent the average ± range of values (each assay performed in duplicate) from two experiments.

Assay conditions	Source of membranes: Control cells (n = 4)	1-day PMA-treated cells (n = 2)	2-day PMA-treated cells (n = 2)
	[^{3}H]$InsP_2$ and -$InsP_3$ (cpm/10^5 cpm ^{3}H-membranes)		
Basal medium	76 ± 32	80 ± 36	24 ± 24
200 nM free Ca^{2+}	151 ± 22	134 ± 66	68 ± 28
+5 μM GTP	145 ± 16	94 ± 10	152 ± 32
+5 μM GTP + 30 μM UTP	513 ± 98	260 ± 12	95 ± 20
+100 μM GTPγS	1559 ± 118	1752 ± 40	1324 ± 36

to distinguish potential effects of chronic PMA treatment on $P_{2U}R$ expression from acute PKC-mediated inhibition of $P_{2U}R$ signal transduction. HL-60 cells were treated for 15 min or 18 hr with 100 nM PMA and assayed for UTP-stimulated Ca^{2+} mobilization in the presence or absence of the PKC inhibitor staurosporine (Fig. 2). Control cells responded with robust Ca^{2+} mobilization to 300 μM UTP (a supramaximal concentration), and these responses were not affected by acute staurosporine treatment. The response to 300 μM UTP was diminished in cells acutely treated with PMA, but staurosporine treatment restored Ca^{2+} mobilization to control levels. In contrast, cells chronically treated with PMA (18 hr) were unresponsive to this supramaximal concentration of UTP, and staurosporine restored only a minor fraction (15%) of the peak UTP-induced Ca^{2+} mobilization observed in control or acutely PMA-treated cells. This indicated that long term PMA treatment inhibited UTP-stimulated Ca^{2+} mobilization through a mechanism distinct from acute uncoupling of the receptor/G protein/PI-PLC signaling cascade.

Changes in P_{2U} receptor mRNA levels during granulocytic versus monocytic differentiation of HL-60 cells. To determine the effects of granulocytic or monocytic differentiating agents on $P_{2U}R$ mRNA expression, HL-60 cells were treated with Bt_2cAMP or PMA over a time course of 3 days and subjected to Northern blot analysis. These Northern blots were sequentially probed with cDNA probes for $P_{2U}R$, myeloperoxidase, IL-1β, FPR, and GAPDH. Hybridization with a probe to the carboxyl-terminal half of the human $P_{2U}R$ coding sequence revealed that a 2.3-kb $P_{2U}R$ mRNA is abundantly expressed in undifferentiated HL-60

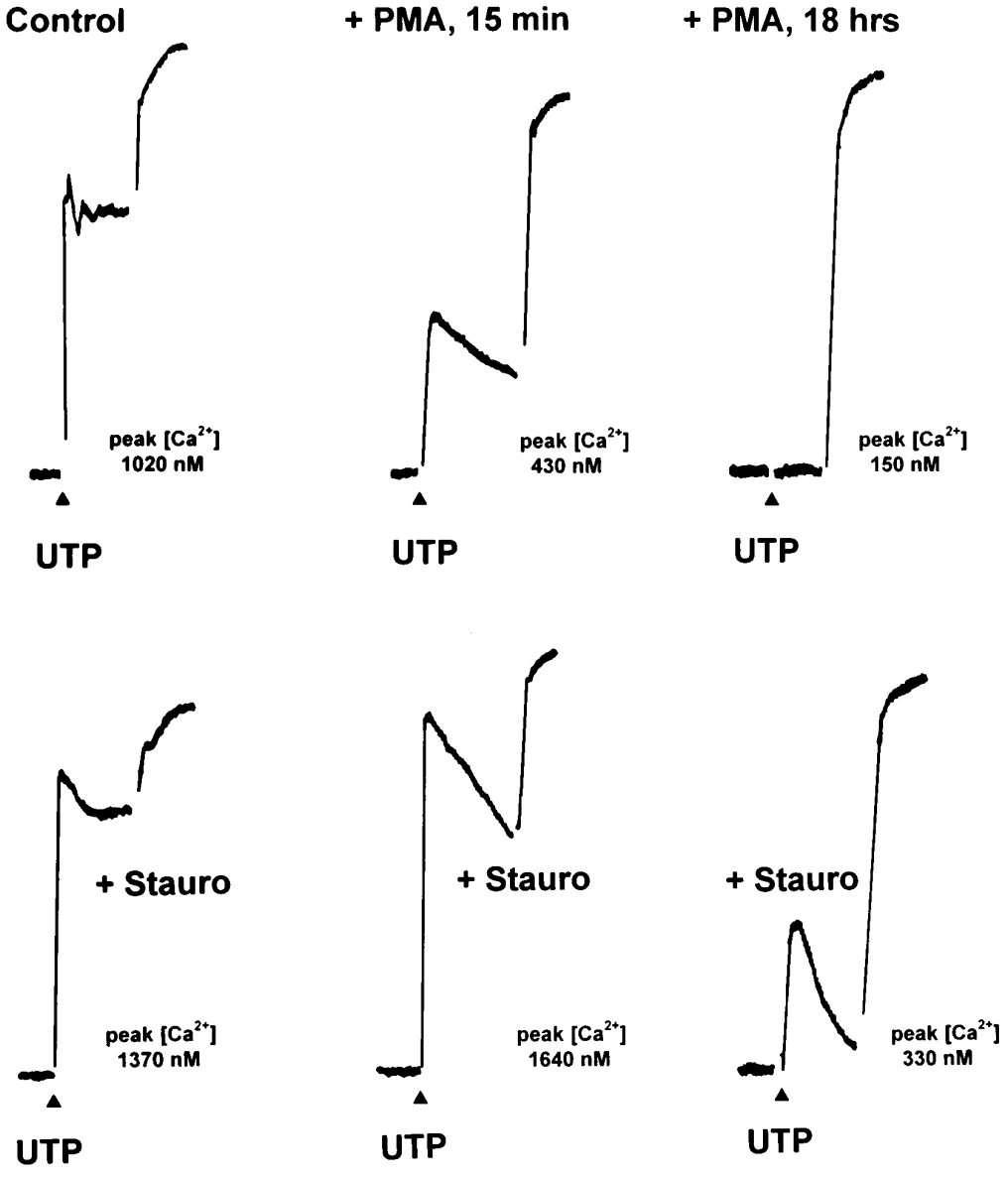


Fig. 2. UTP-induced Ca^{2+} mobilization in HL-60 cells after acute or chronic PMA treatment. HL-60 cells were cultured in the presence or absence of 100 nM PMA for 18 hr and Fura-2-loaded as described in the legend to Fig. 1. *Top*, Ca^{2+} mobilization in response to 300 μM UTP was measured in control cells, in control cells incubated with 100 nM PMA for 15 min before the assay, and in 18-hr PMA-treated cells. *Bottom*, UTP-induced Ca^{2+} mobilization was also measured in the cells treated with 300 nM staurosporine (+*Stauro*) for 5 min before the assay. After the cells were stimulated with UTP for ~60 sec, 20 μg/ml digitonin was added to release Fura-2 into the extracellular medium for calibration (this causes the steadily maintained increase in fluorescence at the end of each trace). The peak cytosolic [Ca^{2+}] triggered by UTP in each cell suspension is also indicated.

cells (Fig. 3A). $P_{2U}R$ mRNA levels were transiently up-regulated by 2.5-fold after a 2-hr treatment with Bt_2cAMP; this was followed by a return to preinduction $P_{2U}R$ mRNA levels after 8 hr and a gradual decline during the following 2 days of *in vitro* differentiation (Fig. 3A, *left top*). These relative changes in $P_{2U}R$ mRNA levels, as quantified by PhosphorImager analysis and normalized to GAPDH mRNA levels, are illustrated in Fig. 3B. We did not determine whether these effects of Bt_2cAMP on $P_{2U}R$ mRNA levels could be mimicked by physiological agents that increase cAMP. However, previous studies have indicated that most effects of Bt_2cAMP on HL-60 cell differentiation can be elicited when these cells are cotreated with prostaglandin E_2, which activates G_s-coupled prostaglandin receptors, and theophylline, which inhibits phosphodiesterase (17).

PMA similarly induced a transient 2.5-fold increase in $P_{2U}R$ mRNA after 2 hr (Fig. 3A, *top right*). However, in contrast to the Bt_2cAMP-treated cells, $P_{2U}R$ mRNA levels in PMA-induced cells declined to 40% of the preinduction levels after 4 hr and were nearly undetectable after 24 hr. This large and sustained down-regulation of the $P_{2U}R$ mRNA by PMA correlates with the loss of $P_{2U}R$ function in PMA-differentiated HL-60 cells and supports the hypothesis that reduced expression of $P_{2U}R$ at the cell surface accounts for most of the greatly diminished functional responses to UTP and ATP.

Bt_2cAMP- and PMA-induced differentiation also produced the expected changes in mRNA expression for three other myeloid marker genes. Consistent with previous reports (17, 18), myeloperoxidase mRNA was expressed at high levels in the early stages but not at the later stages of either granulocytic or monocyte/macrophage differentiation (data not shown). In contrast, the IL-1β mRNA was up-regulated during differentiation by PMA (Fig. 3A, *bottom right*) or Bt_2cAMP (data not shown). The FPR is expressed only at the later stages of myeloid cell development (8, 18a) and we

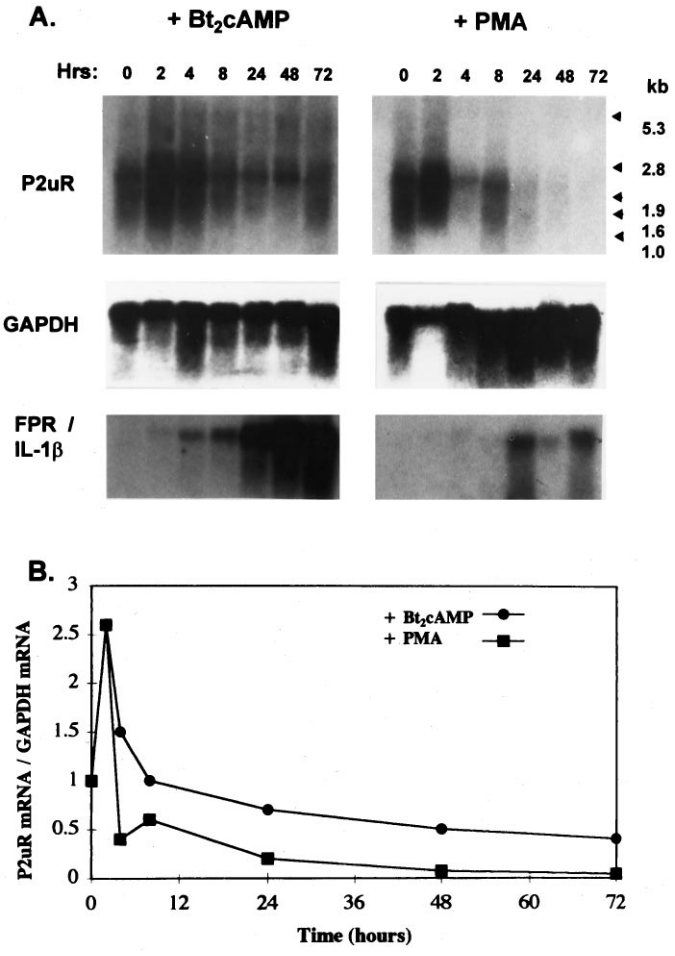


Fig. 3. Northern blot analysis of $P_{2U}R$ mRNA levels during differentiation of HL-60 cells by Bt_2cAMP or PMA. A, The levels of $P_{2U}R$ mRNA were assayed in HL-60 cells differentiated with 500 μM Bt_2cAMP or 100 nM PMA over a 72-hr time course. Poly(A)$^+$ RNA (3.0 μg/lane) was subjected to Northern blot analysis and sequentially probed with ^{32}P-labeled cDNAs corresponding to the human $P_{2U}R$ (*top left* and *right*), GAPDH (*middle left* and *right*), FPR (*bottom left*), or IL-1β (*bottom right*). B, Hybridization signals from the above Northern blots were quantified using a PhosphorImager. The $P_{2U}R$ signals were then normalized to the corresponding GAPDH signal at each time point. Finally, the normalized $P_{2U}R$ signal at each time point was expressed as a ratio relative to the normalized $P_{2U}R$ signal at time zero.

observed that FPR mRNA was strongly up-regulated during Bt_2cAMP-induced differentiation (Fig. 3A, *bottom left*) but not during PMA induction (data not shown).

A semiquantitative RT-PCR assay was used to further characterize the regulation of $P_{2U}R$ mRNA levels and other gene products during granulocytic or monocytic differentiation of HL-60 cells. This method also indicated that Bt_2cAMP induced a transient increase in the $P_{2U}R$ mRNA at 2 hr. This was followed by a gradual reduction over the next 70 hr to levels 0.5–2-fold lower than the control level (Fig. 4). GAPDH levels were constant during the initial 48 hr of differentiation and then slightly declined. The PCR product corresponding to the FPR mRNA (as a granulocytic marker gene product) was up-regulated 5–30-fold in different cell preparations after 48–72-hr treatments with Bt_2cAMP (Table 2).

RT-PCR analysis of PMA-induced HL-60 cells indicated that the $P_{2U}R$ mRNA was transiently increased by 1.8-fold

TABLE 2

Down-regulation of $P_{2U}R$ mRNA by PMA in terminally differentiated HL-60 granulocytes

HL-60 cells were treated with 100 nM PMA for 24 hr, with 500 μM Bt_2cAMP for 48 hr, or with 500 μM Bt_2cAMP for 48 hr followed by a 24-hr exposure to 100 nM PMA. Control cells were cultured in the absence of any differentiating agent. RNA, isolated from each sample of cells, was subjected to semiquantitative RT-PCR analysis using primers for the human $P_{2U}R$ cDNA, the human FPR cDNA, and GAPDH cDNA. Each PCR was supplemented with [^{32}P]dCTP to label the amplified DNA products. After electrophoresis, each PCR band was excised, melted, and analyzed by liquid scintillation counting. The total number of cpm of ^{32}P that were associated with each RT-PCR product is listed, together with normalized values that were calculated as the ratio: ^{32}P in PCR product from differentiated cells/^{32}P in PCR product from control cells.

RT-PCR product	Differentiation conditions			
	Control	+PMA (24 hr)	+Bt_2cAMP (48 hr)	+Bt_2cAMP (48 hr) > +PMA (24 hr)
$P_{2U}R$				
cpm of ^{32}P	5000	800	1200	200
Normalized	1.00	0.16	0.24	0.04
FPR				
cpm of ^{32}P	200	400	5800	1000
Normalized	1.00	2.00	29.0	5.00
GAPDH				
cpm of ^{32}P	4900	4000	5900	5100
Normalized	1.00	0.81	1.20	1.04

during the initial 2 hr of induction (Fig. 4B) and then fell sharply during the next 20 hr to levels 10-fold lower than those observed in undifferentiated cells. This down-regulation was first apparent at 8 hr and near-maximal at 24 hr. Maximal 15-fold increases in IL-1β mRNA were observed during this PMA-induced differentiation (Fig. 4B). The time course that characterized this up-regulation of IL-1β expression was well correlated with the down-regulation of $P_{2U}R$ expression. Fig. 5 illustrates the concentration-response relationships that characterize the effects of PMA induction (during a constant 48-hr incubation) on $P_{2U}R$ down-regulation and IL-1β up-regulation. PMA (100 nM) was sufficient for maximal down-regulation of $P_{2U}R$ mRNA levels, whereas the EC_{50} value was ~20 nM. The slightly reduced efficacy of higher PMA concentrations ($\geq$500 nM) in reducing $P_{2U}R$ mRNA may reflect down-regulation of PKC expression. The likely involvement of PKC in mediating these effects of PMA was supported by the observation that down-regulation of $P_{2U}R$ mRNA was completely inhibited when the cells were coincubated with 3 μM bisindolylmaleimide (19), a reasonably selective inhibitor of most PKC isoforms (data not shown).

Stability of $P_{2U}R$ mRNA in HL-60 cells. The modulation of $P_{2U}R$ mRNA levels during myeloid differentiation may reflect changes in mRNA stability and/or changes in steady state transcription. To further characterize the regulation of the $P_{2U}R$ mRNA levels, the stability of $P_{2U}R$ mRNA and GAPDH mRNA transcripts was assayed by treating HL-60 cells with actinomycin D for 1–4 hr before isolation of total RNA or poly(A)$^+$ RNA. Both Northern blot (Fig. 6) and RT-PCR (Fig. 7) analyses indicated that the $P_{2U}R$ mRNA levels rapidly decreased after the addition of actinomycin. In contrast, no decrease in GAPDH mRNA was observed during the initial 4 hr of actinomycin D treatment. Given the relative stability of the GAPDH mRNA during these actinomycin treatments, $P_{2U}R$ mRNA levels at each time point were normalized relative to the corresponding GAPDH mRNA levels.

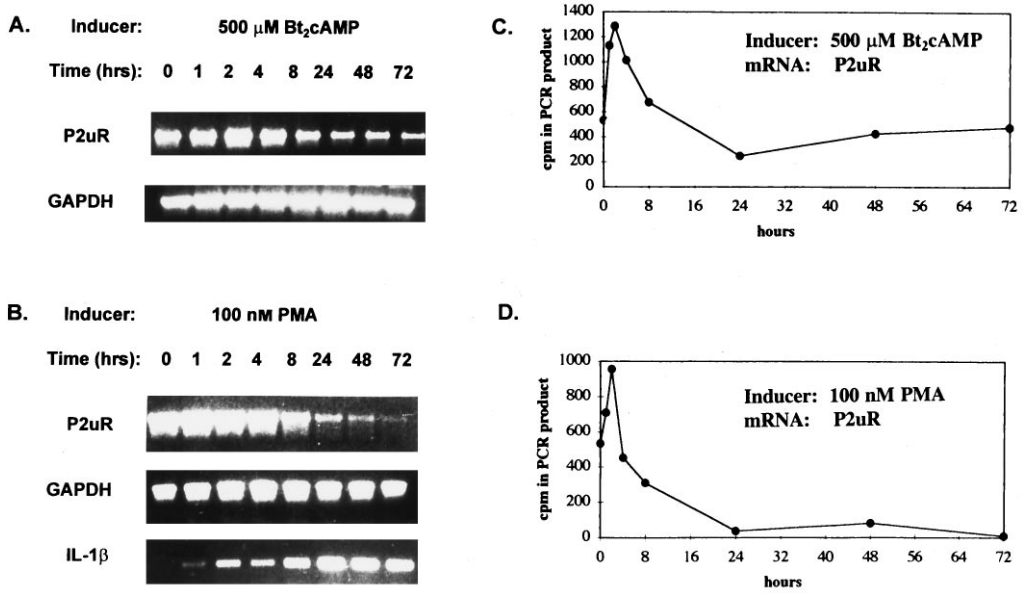


Fig. 4. RT-PCR analysis of $P_{2U}R$ mRNA levels during differentiation of HL-60 cells by Bt_2cAMP or PMA. A, $P_{2U}R$ and GAPDH mRNA levels in HL-60 cells differentiated with 500 μM Bt_2cAMP over a 72-hr time course. B, $P_{2U}R$, IL-1β, and GAPDH mRNA levels in HL-60 cells differentiated with 100 nM PMA over a 72-hr time course. C and D, Quantification of the amplified $P_{2U}R$ RT-PCR products from the experiments illustrated in A and B, respectively. Each ^{32}P-labeled PCR product band was excised, melted, and analyzed by scintillation counting. Data represent cpm of ^{32}P for each band after subtraction of background radioactivity and are representative of results from three similar experiments.

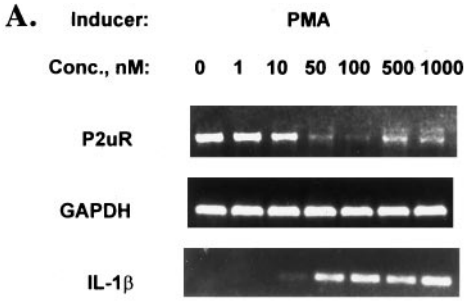
These measurements indicated that the $P_{2U}R$ mRNA half-life was ~60 min (range, 30–75 min in five experiments) in undifferentiated HL-60 cells. This relatively short half-life of the $P_{2U}R$ mRNA suggests that expression of functional $P_{2U}R$ can be rapidly altered by changes in transcription or mRNA stability. Other experiments tested whether the stability of $P_{2U}R$ mRNA was acutely altered by Bt_2cAMP or PMA, the agents used to induce *in vitro* differentiation along the granulocytic or monocytic pathways (Fig. 7). RT-PCR analysis indicated that the ~60-min half-life of the $P_{2U}R$ mRNA transcripts was not significantly affected by either agent during the 4-hr treatment with actinomycin D.

Effects of PMA on $P_{2U}R$ mRNA levels in other myeloid and nonmyeloid cell types. $P_{2U}R$ expression was assayed during the PMA-induced differentiation of THP-1 monocytes, another human myeloid leukocyte line. Unlike the pluripotent HL-60 line, THP-1 cells are irreversibly committed to the monocyte/macrophage lineage (20). $P_{2U}R$ mRNA levels were reduced by 10-fold in THP-1 cells treated with 100 nM PMA for 1 or 2 days (Fig. 8A). Like in HL-60 cells, these changes in $P_{2U}R$ transcript levels were inversely correlated with the up-regulation of IL-1β mRNA. After 3 days of induction with PMA, the level of $P_{2U}R$ mRNA increased slightly, whereas the amount of IL-1β mRNA was markedly reduced.

We also tested whether PMA might similarly down-regulate $P_{2U}R$ expression in A431 ovarian epithelial carcinoma cells and primary keratinocytes, two nonmyeloid human cell types that also express this particular ATP receptor. PMA treatment had no major effect on $P_{2U}R$ mRNA levels in either cell type over a time course of 48 hr (Fig. 8B). Consistent with these results, chronic PMA treatment of these cells did not change the potency or efficacy of UTP as a Ca^{2+}-mobilizing agonist (data not shown). RT-PCR measurements revealed that the half-life of $P_{2U}R$ mRNA in actinomycin D-treated keratinocytes was ~90 min (data not shown). This indicates that a relatively short half-life is an intrinsic characteristic of the $P_{2U}R$ mRNA and is not a unique feature of $P_{2U}R$ regulation in myeloid leukocytes.

Myeloid progenitor cells, such as HL-60 promyelocytes and THP-1 promonocytes, are actively proliferating cells, and most differentiating agents induce these cells to withdraw from the cell cycle (14, 20, 21). Thus, the ability of PMA to markedly down-regulate $P_{2U}R$ expression in these myeloid cell types might be secondary to effects of PMA as a cytostatic agent. We tested whether PMA could further down-regulate $P_{2U}R$ expression in HL-60 cells that had been terminally differentiated into granulocytes by an initial 48-hr induction with Bt_2cAMP (Table 2). In this experiment, treatment with Bt_2cAMP alone decreased $P_{2U}R$ mRNA levels by 4-fold relative to those assayed in untreated cells. However, the additional presence of PMA further down-regulated the $P_{2U}R$ mRNA to <5% of the level observed in untreated cells. The level of FPR mRNA was greatly up-regulated (29-fold in the experiment described in Table 2) during the 48-hr induction with Bt_2cAMP alone but then was substantially reduced after the additional 24 hr of PMA exposure. These data suggest that down-regulation of $P_{2U}R$ mRNA levels by PMA is characteristic of both progenitor and differentiated myeloid cells and that mechanisms other than cell cycle withdrawal contribute to this down-regulation.

Down-regulation of $P_{2U}R$ mRNA expression during inflammatory activation of myeloid leukocytes. In addition to inducing HL-60 promyelocytes and THP-1 monocytes to differentiate along the monocyte/macrophage pathway, PMA modulates the expression of many genes associated with inflammatory activation of mature monocyte/macrophages (13, 20, 21). Several observations suggested that the marked down-regulation of $P_{2U}R$ expression in PMA-treated HL-60 cells may be indicative of inflammatory activation rather than simple commitment to monocyte/macrophage differentiation. First, freshly isolated blood monocytes exhibit robust Ca^{2+}-mobilizing responses to micromolar ATP and UTP (9). Second, the PMA-induced down-regulation of $P_{2U}R$ mRNA in both HL-60 cells and THP-1 cells correlates with the up-regulation of proinflammatory cytokines, such as IL-1β (Figs. 3–5 and 8) and TNF-α (data not shown). To further address this possibility, we tested whether physiological inflammatory agents, such as IFN-γ and bacterial endotoxin/LPS, might also induce down-regulation of $P_{2U}R$ ex-



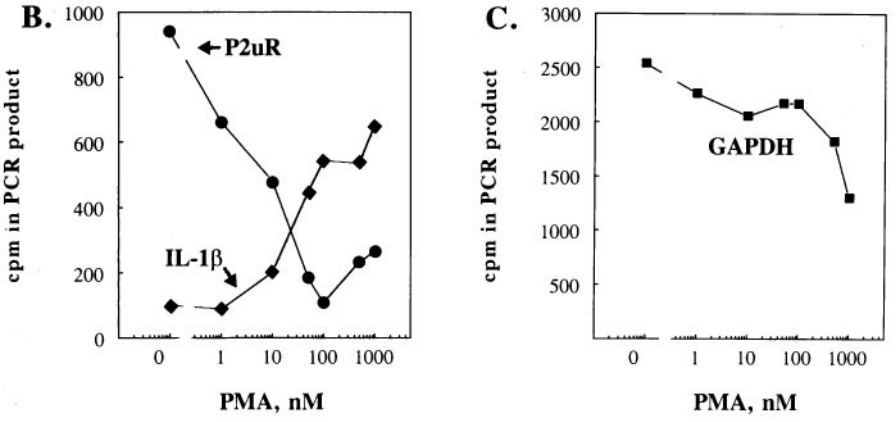


Fig. 5. Concentration-response relationship characterizing the down-regulation of $P_{2U}R$ mRNA by PMA. A, HL-60 cells were treated with the indicated concentrations of PMA for 48 hr, and total RNA was prepared and subjected to semiquantitative RT-PCR analysis using primers for the human $P_{2U}R$, IL-1β, and GAPDH cDNAs. B and C, Quantification of the PCR products was performed as described in the legend to Fig. 4. Data represent cpm of ^{32}P in each PCR product band after correction for background radioactivity.

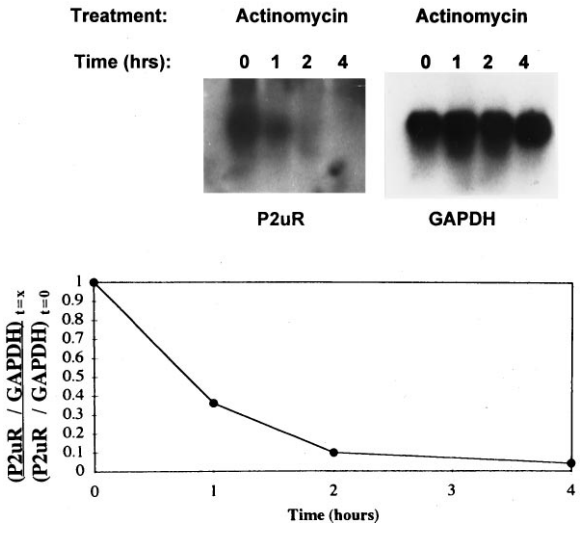


Fig. 6. Half-life of $P_{2U}R$ mRNA in undifferentiated HL-60 cells. HL-60 cells were treated with 5 μg/ml actinomycin D for the indicated times. Poly(A)$^+$ RNA was subjected to Northern blot analysis, which was performed and quantified as described in the legend to Fig. 3. Data represent the changes in $P_{2U}R$ mRNA level relative to time zero after normalization to the corresponding GAPDH mRNA level at each time point and are representative of duplicate experiments.

pression in THP-1 cells that are already committed to the monocyte/macrophage lineage. It should be noted that IFN-γ induces some but not all phenotypic changes that characterize inflammatory activation of mononuclear phagocytes (13, 22). Likewise, although LPS alone can induce most phenotypic changes that characterize inflammatory activation of macrophages, the rate of such induction by LPS can be greatly potentiated when monocyte/macrophages are simultaneously exposed to IFN-γ (13). Fig. 9A shows that IFN-γ alone induced no changes in $P_{2U}R$ expression at 24 hr and only a minor decrease at 48 hr. Consistent with its being a priming agent rather than a full inflammatory activator, IFN-γ induced a delayed up-regulation of TNF-α (particularly evident at 48 hr) but no increase in IL-1β mRNA. In contrast, cotreatment of THP-1 cells with both IFN-γ and LPS greatly up-regulated the expression of TNF-α and IL-1β within 24 hr. This up-regulation of inflammatory cytokines was correlated with a ~5-fold decrease in $P_{2U}R$ mRNA levels. UTP-induced Ca^{2+} mobilization was also significantly decreased in THP-1 cells treated with both LPS and IFN-γ (data not shown). The ability of IFN-γ to prime THP-1 cells for the down-regulation of $P_{2U}R$ mRNA by either LPS or PMA was further characterized by pretreating these cells with interferon for 48 hr before relatively short (8-hr) exposures to either PMA or LPS (Fig. 9B). We also tested the potential priming effects of 1,25-dihydroxy-vitamin D_3, which can induce monocytic differentiation but not inflammatory activation of human myeloid cell lines (20). Treatment of nonprimed THP-1 cells with LPS or PMA for 8 hr did not change the intensities of the $P_{2U}R$ RT-PCR signals (as visually indicated by ethidium fluorescence). In cells primed with 1,25-dihydroxy-vitamin D_3, an 8-hr exposure to PMA but not LPS reduced the intensity of the $P_{2U}R$ RT-PCR signal relative to that observed in the untreated cells. In contrast, the short term exposure to either LPS and PMA induced a more significant down-regulation of $P_{2U}R$ mRNA in THP-1 cells primed with IFN-γ for 48 hr.

Discussion

Neutrophils and monocytes express P_{2U}-purinergic receptors that mobilize intracellular Ca^{2+} in response to extracel-

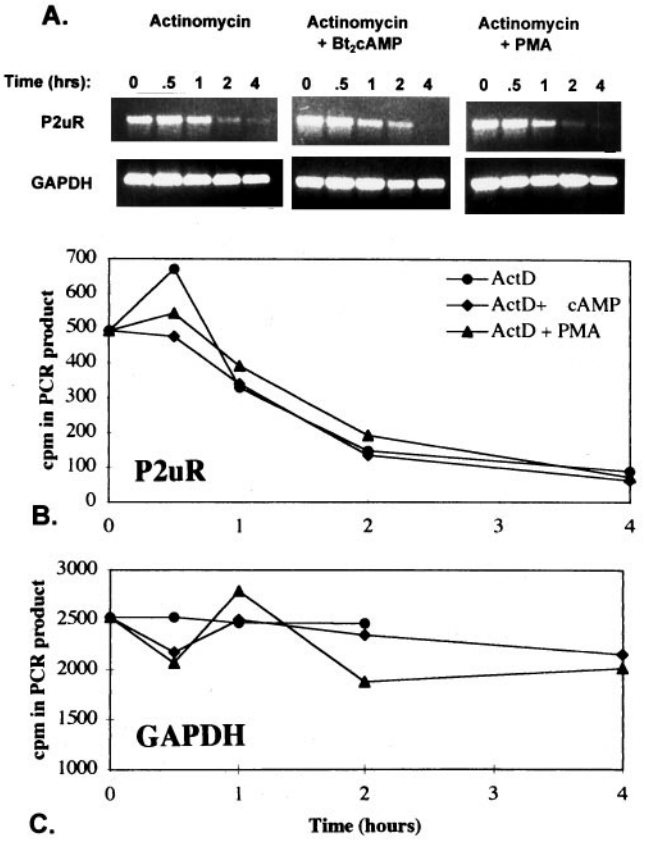


Fig. 7. RT-PCR analysis of $P_{2U}R$ mRNA half-life in control HL-60 cells and HL-60 cells acutely treated with Bt_2cAMP or PMA. A, HL-60 cells were treated with 5 μg/ml actinomycin D (*ActD*) alone (*left*) or in the additional presence of 500 μM Bt_2AMP (*middle*) or 100 nM PMA (*right*) for the indicated times. Total RNA was prepared and subjected to semiquantitative RT-PCR analysis using primers to the human $P_{2U}R$ and GAPDH as described in the legend to Fig. 4. B and C, Quantification of the $P_{2U}R$ and GAPDH PCR products was performed as described in the legend to Fig. 4. These values are representative of results from two experiments.

lular ATP or UTP (9, 10). Although cDNAs encoding this G protein-coupled receptor have been isolated from several species and tissue sources (2), the promoter sequence of the human $P_{2U}R$ gene has not been characterized, and little is known regarding the factors that regulate expression of this receptor. Because traditional methods for studying receptor number at the cell surface, such as ligand or antibody binding, are unavailable for most ATP receptors, we studied the regulation of $P_{2U}R$ expression at the mRNA level. We demonstrate that the expression of the $P_{2U}R$ mRNA in human myeloid leukocytes is regulated by both agents that induce differentiation of myeloid progenitor cells and agents that trigger inflammatory activation of these leukocytes. This is the first evidence for plasticity in the expression of $P_{2U}R$ during defined programs of cellular differentiation.

Bt_2cAMP induces HL-60 cells to acquire morphological characteristics of granulocytes/neutrophils (14, 17, 23). We have previously reported that the potency and efficacy of ATP as a Ca^{2+}-mobilizing agonist were virtually identical in undifferentiated HL-60 promyelocytes and in HL-60 granulocytes (4). Although $P_{2U}R$ mRNA levels always decreased during prolonged treatment of HL-60 cells with Bt_2cAMP, the steady state levels after 2 days of induction were usually only 2–3-fold lower than that in uninduced cells (Figs. 3 and 4). $P_{2U}R$ mRNA was also expressed at approximately similar levels in HL-60 granulocytes and freshly isolated human neutrophils (data not shown). The relatively modest effect of a granulocytic-inducing agent, such as Bt_2cAMP, on the level of $P_{2U}R$ mRNA in HL-60 cells is consistent with the maintained expression of functional $P_{2U}R$ in both HL-60 granulocytes and human blood neutrophils. The reduction in $P_{2U}R$ mRNA was preceded by a transient increase in $P_{2U}R$ transcript levels, which peaked within 2 hr (Figs. 3 and 4). This transient increase was also observed in PMA-treated cells and presumably involved enhanced transcription because no increase was observed in the presence of actinomycin D (Fig. 8). The physiological significance of this transient increase is unclear, and it remains to be determined whether these effects can be mimicked by receptor agonists that elevate cAMP. However, Collins *et al*. (24) observed a similar, rapid increase in β_2-adrenergic receptor mRNA, followed by a modest decrease in DDT1-MF2 smooth cells treated with either Bt_2cAMP or epinephrine, a physiological agonist for β_2-adrenergic receptor. A cAMP-response element present in the promoter of β_2-adrenergic receptor gene was implicated in the up-regulation of that receptor mRNA. The very similar, triphasic changes in $P_{2U}R$ or β_2-adrenergic receptor mRNA induced by Bt_2cAMP suggests that a cAMP-response element may be present in the human $P_{2U}R$ gene promoter.

HL-60 cells treated with PMA acquire many of the morphological and functional characteristics of monocytes and macrophages (14, 21). These PMA-differentiated cells were largely unresponsive to even maximally activating concentrations of UTP in both Ca^{2+} mobilization and $InsP_3$ production assays (Figs. 1 and 2). This lack of $P_{2U}R$ functional activity correlated with a significant down-regulation of $P_{2U}R$ mRNA to values ~10-fold lower than that measured in undifferentiated cells (Figs. 3–5). The correlation between the loss of $P_{2U}R$ function in cell membranes (Table 1) and the reduction in $P_{2U}R$ mRNA levels suggests that PMA-induced down-regulation of these receptors primarily reflects decreased expression of functional receptor protein at the cell surface. Although $P_{2U}R$ mRNA levels were down-regulated in both Bt_2cAMP- and PMA-differentiated HL-60 cells, a significant attenuation of $P_{2U}R$ functional activity (as indicated by the agonistic potencies and efficacies of UTP or ATP) was observed only in the PMA-treated HL-60 cells. It is possible that chronic PMA treatment also increases the internalization and degradation of $P_{2U}R$. A PMA-induced increase in $P_{2U}R$ internalization/degradation in combination with the large reduction in steady state $P_{2U}R$ mRNA levels may lead to the dramatic loss of $P_{2U}R$ function that was observed in PMA-differentiated HL-60 cells but not in the Bt_2cAMP-induced cells (4). It should also be noted that phorbol esters have been shown to induce a profound down-regulation of other G protein-coupled receptors at both mRNA and protein levels; these include the β_3-adrenergic receptor in adipocytes (25), the M2-muscarinic receptor in lung cells (26), and the thrombin receptor in mesangial cells (27).

Inflammatory activation constitutes a second stage of phagocyte development, distal to the primary commitment to monocytic or granulocytic differentiation. PMA is a potent inducer of the inflammatory phenotype (13, 20). For example,

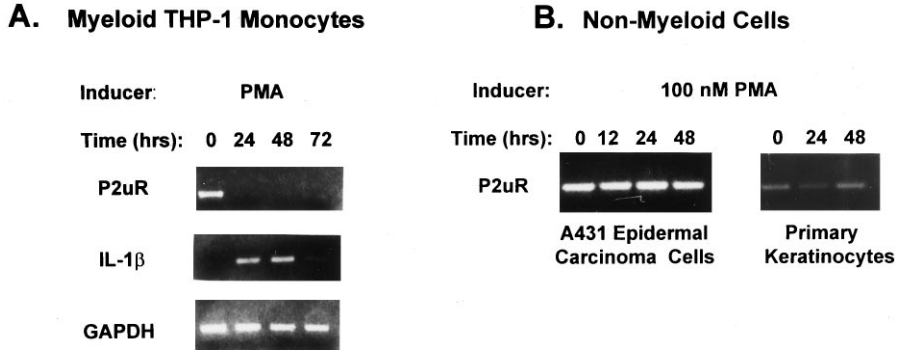


Fig. 8. Differential effects of chronic PMA treatment on the down-regulation of $P_{2U}R$ mRNA in myeloid and nonmyeloid cells. A, THP-1 promonocytes were treated with 100 nM PMA for 0, 24, or 48 hr. RNA, isolated from each cell sample, was analyzed by RT-PCR using primers for the human $P_{2U}R$, IL-1β, and GAPDH cDNAs. B, A431 human epidermal cells were treated with 100 nM PMA for 0, 12, 24, or 48 hr; human keratinocytes were treated with 100 nM PMA 0, 24, or 48 hr. RNA, isolated from each sample, was analyzed by RT-PCR using primers to the human $P_{2U}R$ and GAPDH cDNAs. Only the $P_{2U}R$ products are shown.

the mRNA for IL-1β, a major proinflammatory cytokine, was strongly induced during PMA treatment of HL-60 promyelocytes (Figs. 3 and 4) and THP-1 monocytes (Fig. 8A). This suggested that PMA-induced down-regulation of $P_{2U}R$ expression might be mimicked by physiological activators of inflammation. Consistent with this possibility, we observed that cotreatment of THP-1 monocytes with IFN-γ and LPS strongly down-regulated $P_{2U}R$ mRNA levels (Fig. 9). Therefore, the profound down-regulation of $P_{2U}R$ mRNA and functional $P_{2U}R$ observed in PMA-treated myeloid leukocyte may be associated with the second, inflammatory stage of differentiation rather than the primary differentiation to the monocytic phenotype. It remains to determined whether down-regulation of the $P_{2U}R$ mRNA by chronic PMA treatment is an exclusively myeloid-specific phenomenon. The absence of significant $P_{2U}R$ mRNA down-regulation by PMA in A431 epithelial carcinoma cells and human keratinocytes (Fig. 8B) indicates that down-regulation of $P_{2U}R$ mRNA is not a generalized response to PKC activation. The strong temporal correlation between down-regulation of $P_{2U}R$ expression and the up-regulation of IL-1β expression in PMA-induced HL-60 cells raises the possibility that $P_{2U}R$ down-regulation also involves autocrine input from proinflammatory cytokines, such as IL-1β and TNF-α.

Another significant finding was that the $P_{2U}R$ transcript is relatively short lived ($t_{1/2}$ = 60–90 min) in both myeloid and nonmyeloid cells. A short half-life may facilitate rapid modulation of $P_{2U}R$ expression in different functional or developmental states of both myeloid and nonmyeloid tissues. The mechanism underlying this rapid turnover of the $P_{2U}R$ mRNA remains to be determined. The half-life of the $P_{2U}R$ mRNA was unchanged during 4-hr treatments of HL-60 cells with Bt_2AMP or PMA (Fig. 7). This indicates that down-regulation of $P_{2U}R$ mRNA levels cannot be due simply to acute changes in mRNA stability triggered by PKC- or PKA-dependent phosphorylation of pre-existing RNA stability factors. However, this does not rule out a delayed effect on $P_{2U}R$ mRNA stability in cells treated for prolonged times (>8 hr) with PMA or Bt_2cAMP. A delayed effect could indicate a requirement for *de novo* synthesis of a factor that further decreases the stability of $P_{2U}R$ mRNA.

The ability of phorbol esters to down-regulate $P_{2U}R$ expression in myeloid leukocytes raises the question of whether a similar down-regulation might be elicited by physiological agents that activate diglyceride accumulation and PKC-based signaling. Other than $P_{2U}R$, undifferentiated HL-60 cells do not express most of the PI-PLC-coupled receptor types present in mature myeloid leukocytes (e.g., the receptors for formyl peptides, platelet activating factor, or leukotriene B; for a review, see Ref. 27). We previously reported that chronic treatment (5 days) of HL-60 cells with adenosine-5′-*O*-(3-thio)triphosphate (added every 12 hr to offset breakdown) induces a partial down-regulation of $P_{2U}R$, as assayed by reduced Ca^{2+} mobilization in response to ATP (28). Preliminary RT-PCR analyses also suggest that $P_{2U}R$ mRNA levels are reduced by ~50% in HL-60 cells treated with 100 μM ATP every 6–8 hr for 2 days. Further experiments are required to verify this autocrine down-regulation. However, such results may be similar to the findings of Chau *et al.* (29), who reported an autocrine down-regulation of platelet activating factor receptor mRNA in U937 human promonocytes (a related human myeloid line) chronically stimulated with a poorly metabolizable platelet-activating factor analog.

The PI-PLC signaling pathway in mature phagocytic leukocytes is rapidly activated by many inflammatory agonists and is involved in the regulation of chemotaxis, secretion, phagocytosis, and superoxide release. The activation of $P_{2U}R$ in mature human neutrophils and monocytes primes these cells for enhanced superoxide release in response to other inflammatory agonists, such as formyl peptides (10, 11). ATP or UTP stimulation of the $P_{2U}R$ also increases neutrophil adherence to endothelial cells (30, 31) and the activation of CD11b/CD18 integrins in neutrophils (32). Recent studies have verified that ATP and UTP are very effective chemotactic stimuli for HL-60 granulocytes and human neutrophils (33). Our data suggest that $P_{2U}R$ are primarily expressed in the marrow-restricted myeloid progenitor cells and blood-borne neutrophils and monocytes. Like other G protein-coupled chemoattractant receptors (34), $P_{2U}R$ may play a role in the recruitment of blood neutrophils and monocytes to sites of tissue inflammation. Once neutrophils or monocytes have entered these sites, the continued expression of primarily chemotactic receptors may be counterproductive and subject to down-regulation by inflammatory cytokines. Lloyd *et al.* (35) reported that expression of IL-8 receptor mRNA and protein is markedly reduced in neutrophils treated with LPS or TNF-α. This is similar to the down-regulation of $P_{2U}R$ mRNA observed in THP-1 monocytes treated with LPS and IFN-γ. It will be interesting to determine whether $P_{2U}R$ are down-regulated in other myeloid cell types, such as neutrophils, and in nonmyeloid cells, such as endothelial cells,

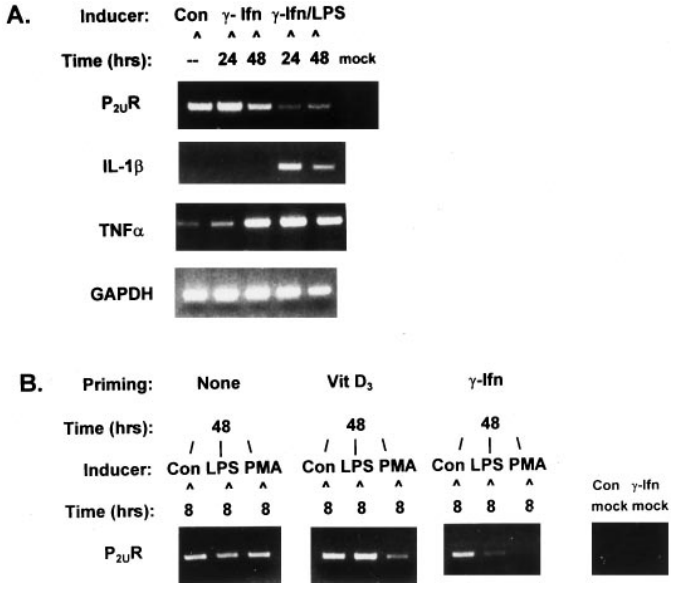


Fig. 9. Down-regulation of $P_{2U}R$ mRNA during inflammatory activation of THP-1 monocytes with IFN-γ and endotoxin/LPS. A, THP-1 monocytes were treated for 24 or 48 hr with 10^3 units/ml IFN-γ alone (*Ifn*) or with 10^3 units/ml IFN-γ plus 1 μg/ml endotoxin/LPS (*Ifn/LPS*). RNA from these treated cells and from control, untreated THP-1 monocytes (*Con*) was analyzed by RT-PCR using primers for $P_{2U}R$, IL-1β, TNF-α, and GAPDH. A parallel sample of RNA from the control THP-1 cells was subjected to the same RT-PCR protocol in the absence of RT enzyme (*mock*). B, THP-1 monocytes were cultured for 48 hr (priming incubation) with no priming agents (*None*), with 10 ng/ml 1,25-dihydroxyvitamin D_3 (*Vit D_3*), or with 1000 μ/ml IFN-γ (γ-*Ifn*). Each suspension of primed cells was divided into three aliquots that were cultured for an additional 8 hr with no inducing agent (*Con*), with 1 μg/ml LPS, or with 100 nM PMA. RNA, isolated from each group of primed/induced cells, was analyzed by RT-PCR using primers for human $P_{2U}R$ cDNA and GAPDH cDNA. Only the $P_{2U}R$ PCR products are shown. Parallel samples of RNA from the control and γ-IFN-treated cells were subjected to mock RT-PCR analysis (*mock*).

which exhibit rapid changes in gene expression in response to LPS, TNF-α, and IL-1.

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