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Expression of purinergic receptors (ionotropic P2X1–7 and metabotropic P2Y1–11) during myeloid differentiation of HL60 cells

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Abstract

The expression of human purinergic P2 receptors (P2X1–7 and P2Y1–11) as well as the ecto-enzymes apyrase (CD39) and 5′-nucleotidase (CD73) was investigated on the nucleic acid level during granulocytic and monocytic differentiation of HL60 cells and on peripheral human blood leukocytes. RT-PCR and dot-blot hybridization assays indicated that mRNA transcripts of all analyzed P2 receptors apart from the P2X3 receptor were expressed during myeloid development of HL60 cells, showing a distinct regulation during the course of differentiation. In blood leukocytes, transcripts of P2X5, P2X7 and all P2Y receptors, except for P2Y6, receptor were found. CD39 and CD73 showed a marked upregulation during myeloid maturation. Functional analysis of P2 receptor-mediated intracellular Ca^{2+}-increase after stimulation with ATP revealed no change during granulocytic differentiation, but showed a strong attenuation in both potency and efficacy during monocytic development of HL60 cells. © 2000 Published by Elsevier Science B.V.

Keywords: RT-PCR; Dot-blot hybridization; ATP; Ecto-nucleotidase; Intracellular Ca^{2+}

1. Introduction

Extracellular ATP and other extracellular nucleotides have been identified as important signaling molecules that mediate diverse biological effects via cell surface receptors termed purinergic receptors or P2 receptors. Physiological responses to ATP include contraction of smooth muscles as well as rapid depolarization of neurons or stimulation of the inflammatory system. Purinergic receptors have been found in virtually every cell and tissue studied [1,2].

P2 receptors are divided into two main classes, P2X and P2Y receptors [3]. P2X receptors are ATP-gated ion channels which mediate rapid and selective permeability to cations, such as Na^+, K^+ and Ca^{2+} [4]. In contrast, P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins. Activation results in mobilization of inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores, activation of plasma membrane influx pathways and stimulation of diacylglyceride-dependent protein kinase C enzymes [5].

Recently, several P2X and P2Y receptor subtypes have been cloned and characterized. Seven mammalian P2X receptor proteins (P2X1–7) are currently known, five of which have been cloned from human tissues (P2X1, X3, X4, X5, X7) [1]. The P2X7 receptor is functionally unique among the P2X receptor family as it acts as a non-selective membrane pore [6].

Eleven P2Y receptors have been identified to date (P2Y1–11) [1]. The chicken P2Y3 receptor was recently shown to be the species homologue of the human P2Y6 receptor [7]. The P2Y7 receptor had originally been suggested to be a nucleotide receptor [8], but recent data identified it as a leukotriene B4 receptor [9,10]. It is still a matter of discussion whether the P2Y5 receptor is a member of the P2Y receptor family or not [11,12]. The P2Y8 receptor was identified as a nucleotide receptor in *Xenopus* neural plate [13]. The human P2Y8, P2Y9 and P2Y10 receptors have been submitted to GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/entrez/) and classified as members of the nucleotide receptor family on the basis of amino acid sequence homologies.

Magnitude and duration of purinergic signaling is also influenced by the activity of nucleotide-metabolizing ecto-enzymes, such as the apyrase CD39 [14] and the 5′-nucleotidase CD73 [15].

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The expression of purinergic receptors has been described for several types of blood cells. Previous studies of P2X1, P2Y2 and P2Y11 receptors indicated that the expression levels of purinergic receptors underlie a significant change of expression levels during hematopoietic differentiation [16–18].

The aim of our present study was to analyze the expression of the whole pattern of P2 purinergic receptor mRNA as well as of the ecto-nucleotidases CD39 and CD73 mRNA during myeloid differentiation. As a hematopoietic differentiation model, we chose the HL60 cell line, a human cell line derived from peripheral blood leukocytes of a patient with acute promyelocytic leukemia [19]. Addition of dimethylsulfoxide to the growth medium induces differentiation of these progenitors to granulocytic cells [20], whereas treatment with phorbol esters leads to a monocytic/macrophage phenotype [21].

This study describes dramatic up- and downregulation of most P2 purinergic receptor subtypes. The changes in expression of the ecto-nucleotidases mRNA confirmed previously published data.

2. Materials and methods

2.1. Cell culture

All Chemicals were obtained from Sigma (Deisenhofen, Germany) except where otherwise stated. HL60 promyelocytes were obtained from the American Type Culture Center (Rockville, USA) and were grown in RPMI 1640 containing 10% FCS (Boehringer, Mannheim, Germany), 2 mM glutamine and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin). Cultures were kept at 37°C in humidified atmosphere with 5% CO_2, and the cells were diluted every 4–5 days to maintain densities between 5×10^4 and 1×10^6 cells/ml. Growth was determined from triplicate counts of the cell number after exclusion of trypan blue-stained cells.

Differentiation of HL60 cells into granulocyte-type cells was achieved by culturing in medium containing 1.25% DMSO for 5 days [20]. Differentiation into monocyte-type cells was induced by treatment for 12, 18, 24, 36 and 48 h with 20 nM phorbol-12-myristate-13-acetate (PMA) [21].

Undifferentiated as well as DMSO-treated HL60 cells grow as single-cell suspension cultures, whereas PMA-treated cells become adherent. For experimental purposes, these cells were removed from the tissue culture dish by gentle scraping.

2.2. Measurement of intracellular calcium

Free intracellular calcium was measured using the fluorescence indicator Fura-2/AM as described by Treves et al. [22]. In brief, 10^7 cells were harvested, centrifuged and resuspended in 1 ml RPMI 1640 supplemented with 10% FCS, 50 mM HEPES and 2.5 mM Probenecid, pH 7.4. Cells were incubated with 4 μM Fura-2/AM for 15 min at 37°C, diluted 1:5, centrifuged at $300\times g$ for 10 min and resuspended in 6 ml medium for measurement (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mM NaH_2PO_4 and 5.5 mM glucose, pH 7.4).

Fura-2/AM loaded cells were assayed at 8.3×10^5 cells/ml in a SFM25 fluorometer (Kontron Analytical, Zurich, Switzerland) equipped with a chart recorder. Fluorescence was measured using 340 nm excitation and 510 nm emission. Maximal fluorescence was determined by addition of 0.1% Triton X-100, minimal fluorescence by addition of 40 μl EGTA/Tris (1.2 M EGTA/70 mM Tris pH 8.4). All measurements were carried out at room temperature.

2.3. Isolation of peripheral blood leukocytes

A 30-ml amount of whole blood in acid citrate dextrose was drawn from healthy volunteers. All subsequent steps were performed at 4°C: The citrated blood was centrifuged at $400\times g$ for 15 min. The supernatant, consisting of plasma and buffy coat containing the leukocytes, was pipetted into a separate tube and again centrifuged at $400\times g$ for 15 min. The erythrocytes contaminating the pellet were lyzed in 10 ml 155 mM ammonium chloride and 10 mM potassium bicarbonate (pH 7.4). The leukocytes were then washed twice in Hank's balanced salt solution containing 20 mM HEPES without Ca^{2+} and Mg^{2+}. The cell count for each sample was adjusted to 1×10^7 cells/ml.

2.4. Reverse transcription-coupled PCR (RT-PCR)

Total RNA was isolated from blood leukocytes or culture cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity of RNA was determined photometrically at 260 nm. Five micrograms of RNA were reverse transcribed to cDNA in a 20 μl reaction volume containing 300 ng random hexamers, 0.5 mM dNTPs, 10 mM DTT, 40 U RNasin, and 400 U Superscript II reverse transcriptase dissolved in the supplied buffer (all reagents from Gibco-BRL Life Technologies, Karlsruhe, Germany). The reactions were allowed to proceed for 2 h at 42°C, stopped by heating at 70°C for 15 min, and were finally diluted to 100 μl.

The PCR reactions were performed in 50 μl final volumes containing 2.0 μl cDNA diluted 1:5, 7.5 pmol of each primer (Table 1), 250 μM dNTPs, 2.0 U of Taq DNA polymerase and 2.0 mM $MgCl_2$ dissolved in the supplied buffer (all reagents from Boehringer, Mannheim, Germany). After initial denaturation for 2 min at 94°C, amplifications were carried out for 35 cycles (GAPDH for 30 cycles) as follows: denaturation at 94°C for 45 s, annealing at primer-specific temperature (see below) for 45 s and extension at 72°C for 90 s. After the final PCR cycle,

Table 1
Receptor specific primers used

Primer	Strand[a]	Sequence	Corresponds to nt	GenBank accession no.
GAPDH	S	5′-cac cac cat gga gaa ggc tgg-3′	366–386	
	AS	5′-gaa gtc aga gga gac cac cag-3′	918–898	M33197
P2X1	S	5′-ctg tga aga cgt gtg aga tct ttg g-3′	660–684	
	AS	5′-ttg aag agg tga cgg tag ttg gtc-3′	1122–1099	U45448
P2X3	S	5′-atc aac cga gta gtt cag c-3′	241–259	
	AS	5′-gat gca ctg gtc cca gg-3′	936–919	Y07683
P2X4	S	5′-gag att cca gat gcg acc-3′	661–678	
	AS	5′-gac ttg agg taa gta gtg g-3′	956–938	U83993
P2X5	S	5′-tcg act aca aga ccg aga ag-3′	77–96	
	AS	5′-ctt gac gtc cat cac att g-3′	672–654	AF016709
P2X7	S	5′-aac atc act tgt acc ttc c-3′	663–681	
	AS	5′-tgt gaa gtc cat cgc agg-3′	1337–1320	Y09561
P2Y1	S	5′-cgg tcc ggg ttc gtc c-3′	194–209	
	AS	5′-cgg acc ccg gta cct-3′	721–707	U42030
P2Y2	S	5′-ctc tac ttt gtc acc acc agc g-3′	750–777	
	AS	5′-ttc tgc tcc tac agc cga atg tcc-3′	1387–1364	S74902
P2Y4	S	5′-cca cct ggc att gtc aga cac c-3′	405–426	
	AS	5′-gag tga cca ggc agg gca cgc-3′	829–809	X91852
P2Y5	S	5′-tgg tta act gtg atc gga gg-3′	465–484	
	AS	5′-agt cac ttc tcc tga cag acc-3′	984–964	AF000546
P2Y6	S	5′-cgc ttc ctc ttc tat gcc aac c-3′	510–531	
	AS	5′-cca tcc tgg cgg cac agg cgg c-3′	874–853	U52464
P2Y8	S	5′-cat cct gtt acc tgg aga cc-3′	229–248	
	AS	5′-cag cat cag gtc cgt gac g-3′	431–413	AA804531
P2Y9	S	5′-ttt ggt gac acc ctc tgc-3′	383–399	
	AS	5′-gag ttg tag ggt aca aag c-3′	866–848	U90322
P2Y10	S	5′-cat cag tct tca aag gtg c-3′	378–396	
	AS	5′-gga caa ctg cta atg atg g-3′	833–815	AF000545
P2Y11	S	5′-cag cgt cat ctt cat cac c-3′	336–354	
	AS	5′-gct ata cgc tct gta ggc-3′	608–593	AF030335
CD39	S	5′-gac cca gaa caa agc att gcc-3′	178–199	
	AS	5′-tgt agt cct tgc cag aga ggc g-3′	806–785	AJ133134
CD73	S	5′-cac caa ggt tca gca gat ccg c-3′	244–265	
	AS	5′-gtt cat caa tgg gcg acc gg-3′	1249–1230	NM002526

[a]S: sense oligonucleotide; AS: antisense oligonucleotide

extension was allowed to proceed for 15 min at 72°C. The annealing temperatures were 64°C for P2X1, 62°C for P2X3, 54°C for P2X4, 57°C for P2X5, 54°C for P2X7, 56.5°C for P2Y1, 67°C for P2Y2, 56.5°C for P2Y4, 54°C for P2Y5, 56.5°C for P2Y6, 59°C for P2Y8, 55°C for P2Y9, 52°C for P2Y10, 61°C for P2Y11, 62°C for CD39, 64°C for CD73, and 60°C for GAPDH. Oligonucleotides were obtained from Amersham Pharmacia Biotech (Braunschweig, Germany).

Representative for all analyzed P2 receptors and ectoenzymes, the linear amplification range of the PCR products of GAPDH and CD39 was determined from several test reactions with varying numbers of amplification cycles (Fig. 1).

As a control for contamination of the RNA preparation with genomic DNA, the crude product of the RNA extraction procedure without any reverse transcription reaction was used as template for a PCR reaction. No signal was detected in these RNA samples without cDNA synthesis indicating that the samples were free of genomic DNA contamination.

For quantification, ethidium bromide-stained gels of the PCR products were analyzed by densitometric scanning with a FluorImager (Molecular Dynamics, Sunnyvale, USA), using ImageQuant software (Molecular Dynamics).

Sequence analysis of both strands of the PCR products was performed on an ABI377 Automatic Sequencer (Perkin Elmer, Weiterstadt, Germany).

2.5. Dot-blot analysis

For determination of the relative amounts of specific mRNA in the total RNA extracts, a modified dot-blot hybridization technique was performed according to Wassarman and DePamphilis [23] and Ausubel et al. [24].

Two micrograms of total RNA were blotted onto a Qiagen Nylon Plus Membrane (Qiagen) using the S and S Minifold I Dot-Blot System (Schleicher and Schüll, Dassel, Germany).

Labeling reactions of the receptor-specific PCR products were carried out with the Prime-a-Gene Kit (Promega, Madison, USA) and [α-^{32}P]dATP (ICN Biomedicals, Irvine, USA). Blots were then hybridized with the receptor-specific ^{32}P-oligolabeled PCR products for 16 h at

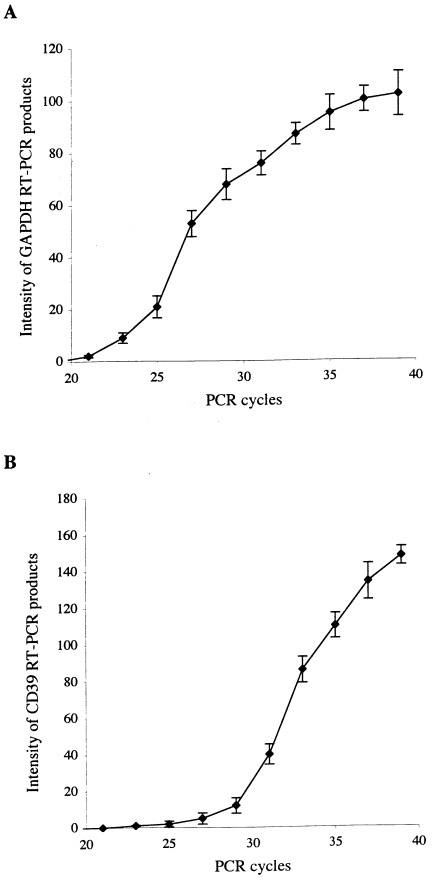

Fig. 1. Determination of the linear amplification range of the PCR reactions. Specific primers for GAPDH and CD39 were used as shown in Table 1. Complementary DNA from HL60 cells differentiated with 20 nM PMA for 48 h (where expression was maximal) was used as a template in the RT-PCR reactions. Amplification for (A) GAPDH RT-PCR products and (B) CD39 RT-PCR products was carried out with varying numbers of amplification cycles. For experimental conditions see Section 2.

42°C under constant shaking in 0.1 ml/cm^2 hybridization solution (50% formamide, 1 M NaCl, 50 mM $NaHPO_4$, pH 7.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 5×Denhardt's solution and 200 μg/ml denatured herring sperm). The membranes were washed for 5 min in 2×SSC/0.1% SDS, twice for 5 min in 0.2×SSC/0.1% SDS, and twice for 15 min in 0.2×SSC/0.1% SDS at 42°C.

A storage phosphor screen (Molecular Dynamics) was exposed to the radioactive membranes for 15 h and then scanned on a PhosphorImager (Molecular Dynamics). Image analysis was performed to quantitate the relative amount of radiolabel hybridized to each sample using ImageQuant software (Molecular Dynamics).

3. Results

Expression levels of 14 cloned human P2X and P2Y receptors, and the ecto-nucleotidases CD39 and CD73 were analyzed in HL60 cells and in peripheral blood leukocytes by a semiquantitative RT-PCR assay using specific primers. Undifferentiated HL60 promyelocytes were used as control cells. To determine the effects of granulocytic differentiation on the mRNA expression, HL60 cells were treated with 1.25% DMSO for 5 days. Stimulation with 20 nM PMA over a time course of 48 h induced differentiation to a monocytic/macrophage phenotype. Analysis of mRNA levels was performed after 12, 18, 24, 36 and 48 h of incubation. To characterize the expression pattern of the P2 receptors and ecto-nucleotidases in non-malignant mature cells, whole blood leukocytes were assayed.

RT-PCR products were eluted from the gels and analyzed by sequencing The relative amounts of the amplified RT-PCR products were quantified by FluorImager analysis and normalized to levels of GAPDH RT-PCR products in the same cells. All quantification experiments were repeated three times, using three independent batches of cell preparation. Triplicate determinations were performed for each set of measurements.

3.1. RT-PCR analysis of the P2 receptors and ecto-nucleotidases

We observed a widespread distribution pattern for the expression of P2X and P2Y receptor and ecto-nucleotidase mRNA in undifferentiated, DMSO- and PMA-treated HL60 cells as well as in human blood leukocytes. Most of the analyzed receptors and enzymes showed a distinct regulation which often fluctuated during myeloid differentiation.

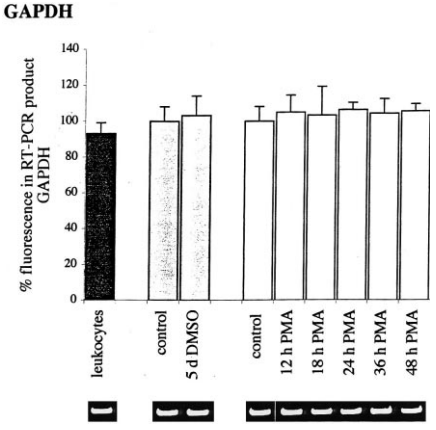

Fig. 2. RT-PCR analysis of GAPDH mRNA in blood leukocytes, and during the differentiation of HL60 cells. HL60 cells were differentiated by 1.25% DMSO for 5 days, or 20 nM PMA for 12, 18, 24, 36 and 48 h as described in Section 2. The gel prints are representative of results from three independent experiments. The bar graphs show mean and standard deviation from triplicate determinations (control level was set at 100%). The relative amounts of the RT-PCR products were quantified by fluorography. For other experimental conditions see Section 2.

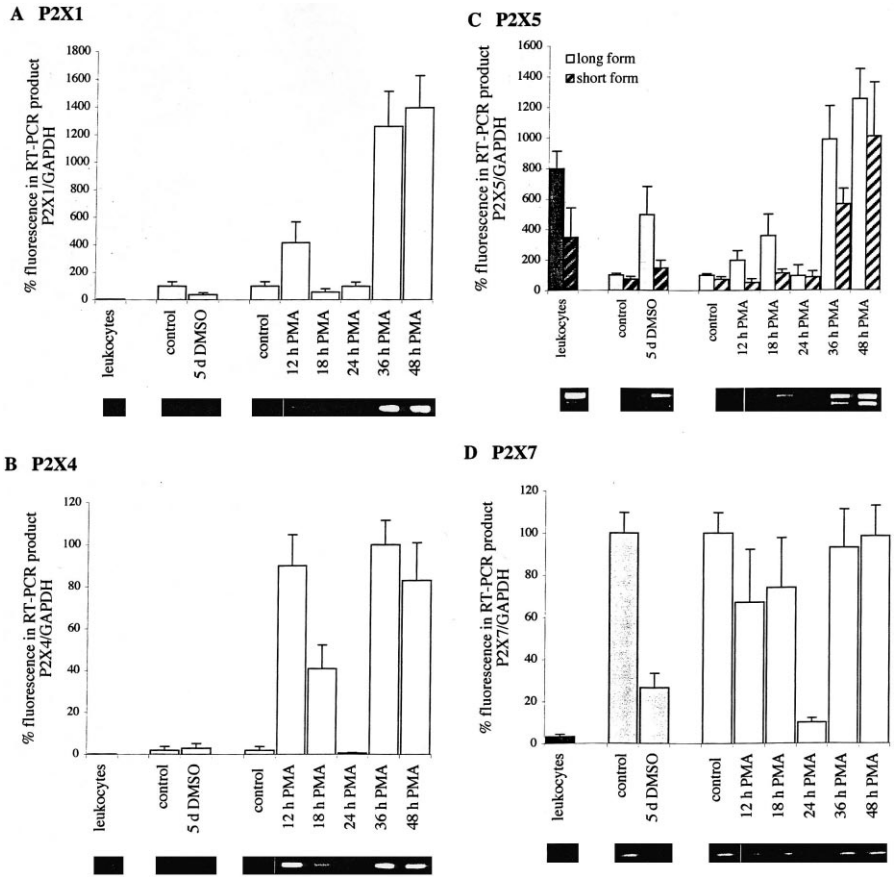

Fig. 3. RT-PCR analysis of P2X receptor mRNA. Messenger RNA expression in blood leukocytes, and during the differentiation of HL60 cells by 1.25% DMSO for 5 days, or 20 nM PMA for 12, 18, 24, 36 and 48 h of the (A) P2X1, (B) P2X4, (C) P2X5 and (D) P2X7 receptor. The gel prints are representative of results from three independent experiments. The bar graphs show mean and standard deviation from triplicate determinations (control levels were set at 100%; for the P2X4 receptor, the expression level of HL60 cells differentiated with PMA for 36 h was set at 100%). The relative amounts of the RT-PCR products were quantified by fluorography and normalized to the GAPDH RT-PCR product levels.

3.1.1. GAPDH (Fig. 2)

GAPDH mRNA was equally expressed in all samples analyzed.

3.1.2. P2X1 (Fig. 3A)

Low levels of P2X1 mRNA were found in undifferentiated HL60 cells. Granulocytic differentiation did not result in a change of the expression level. Monocytic cells showed an initial 3- to 5-fold increase in the transcript level at 12 h. The most apparent expression, up to a 10- to 14-fold upregulation compared to preinduction levels, was observed after 36 h and stayed at this level. In blood leukocytes, P2X1 mRNA could not be detected.

3.1.3. P2X3

P2X3 mRNA could not be detected in any of the analyzed cell types (data not shown).

3.1.4. P2X4 (Fig. 3B)

P2X4 mRNA was nearly undetectable in HL60 promyelocytes and granulocytes. During monocytic differentiation the transcript was highly expressed showing a biphasic peak in early and later stages of differentiation, while being nearly undetectable at 24 h. Blood leukocytes did not express P2X4 mRNA.

3.1.5. P2X5 (Fig. 3C)

Two splice variant forms of P2X5 mRNA differing by 72 bp were found in HL60 cells and in blood leukocytes. Both alternatively spliced forms appeared to be regulated parallel with the full length form being expressed at somewhat higher levels. Both splice variants were weakly expressed in undifferentiated HL60 cells. Upon granulocytic differentiation, transcript levels increased 2- to 5-fold during monocytic differentiation, P2X5 mRNA was upregulated up to 10- to 14-fold with a transient decrease to nearly preinduction levels at 24 h. Both P2X5 transcripts were strongly expressed in blood leukocytes.

3.1.6. P2X7 (Fig. 3D)

P2X7 mRNA was moderately expressed in undifferentiated HL60 cells. Compared to control cells, transcript levels declined by $>70\%$ in granulocytic cells. Monocytic differentiation resulted in moderate transcript levels with a 10-fold downregulation at 24 h. Negligible amounts of P2X7 receptor mRNA were observed in blood leukocytes.

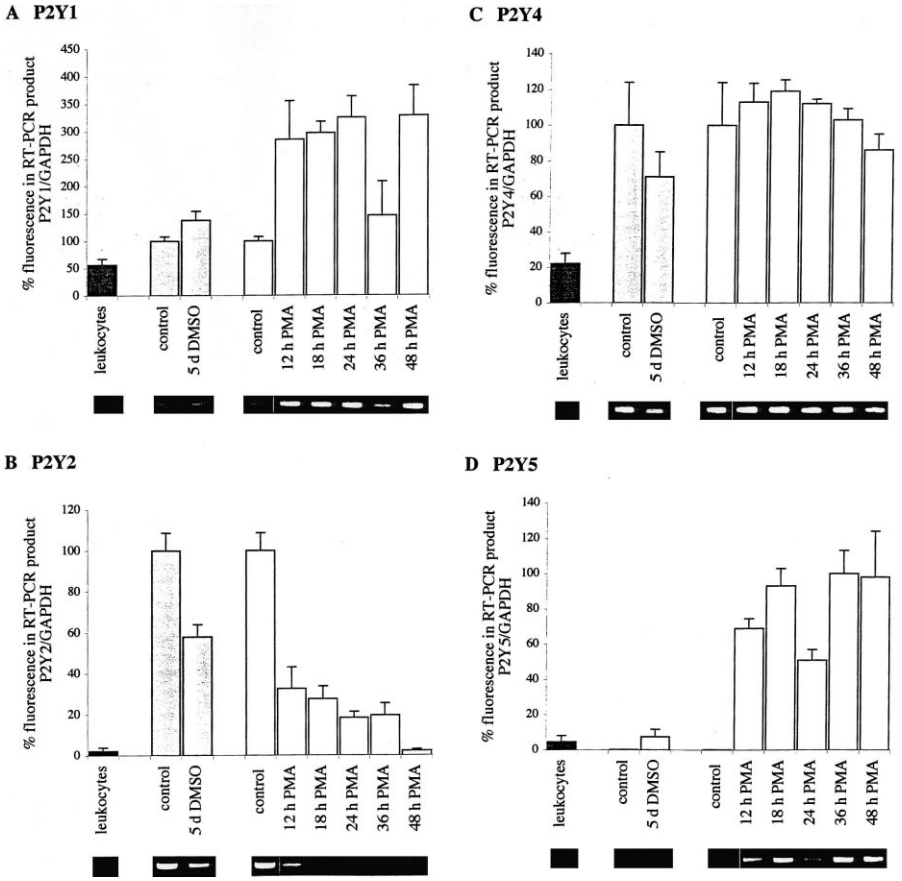

Fig. 4. RT-PCR analysis of P2Y receptor mRNA. Messenger RNA expression in blood leukocytes, and during the differentiation of HL60 cells by 1.25% DMSO for 5 days, or 20 nM PMA for 12, 18, 24, 36 and 48 h of the (A) P2Y1, (B) P2Y2, (C) P2Y4, (D) P2Y5, (E) P2Y6, (F) P2Y8, (G) P2Y9, (H) P2Y10 and (I) P2Y11 receptor. The gel prints are representative of results from three independent experiments. The bar graphs show mean and standard deviation from triplicate determinations (control levels were set at 100%; for the P2Y5 and P2Y10 receptor, the expression levels of HL60 cells differentiated with PMA for 36 h, respectively, 24 h were set at 100%). The relative amounts of the RT-PCR products were quantified by fluorography and normalized to the GAPDH RT-PCR product levels.

3.1.7. P2Y1 (Fig. 4A)

P2Y1 transcript levels were moderately expressed in undifferentiated HL60 cells. Granulocytic differentiation did not change this expression level. Monocytic differentiation resulted in a transient, circa 3-fold upregulation of mRNA which returned to nearly preinduction levels after 36 h. Low levels of P2Y1 mRNA were also detectable in blood leukocytes.

3.1.8. P2Y2 (Fig. 4B)

P2Y2 mRNA was strongly expressed in HL60 promyelocytes. The relatively high levels in undifferentiated HL60 declined during granulocytic differentiation. P2Y2 mRNA levels in PMA-induced cells decreased to a third of the control levels at 12 h, and were almost undetectable after 48 h, indicating a continuous downregulation during monocytic differentiation. Minimal amounts were observed in blood leukocytes.

3.1.9. P2Y4 (Fig. 4C)

P2Y4 mRNA was highly expressed in HL60 promyelocytes. During granulocytic differentiation, amounts of transcript decreased slightly. Compared with control cells, stimulation with PMA did not change the mRNA levels in early stages of monocytic differentiation, but resulted in a gradual reduction during further differentiation. Low levels of P2Y4 mRNA were identified in blood leukocytes.

3.1.10. P2Y5 (Fig. 4D)

P2Y5 mRNA expression could not be detected in undifferentiated HL60 cells. Only low levels were visible after DMSO treatment. Differentiation to monocytic cells resulted in a marked upregulation, with a transient decrease in expression at 24 h. Blood leukocytes expressed only low levels of P2Y5 transcript.

3.1.11. P2Y6 (Fig. 4E)

Undifferentiated HL60 cells showed high levels of the P2Y6 receptor transcript, which was unaffected during granulocytic differentiation. During monocytic differentiation the expression fluctuated around preinductional mRNA levels. Blood leukocytes did not express P2Y6 mRNA.

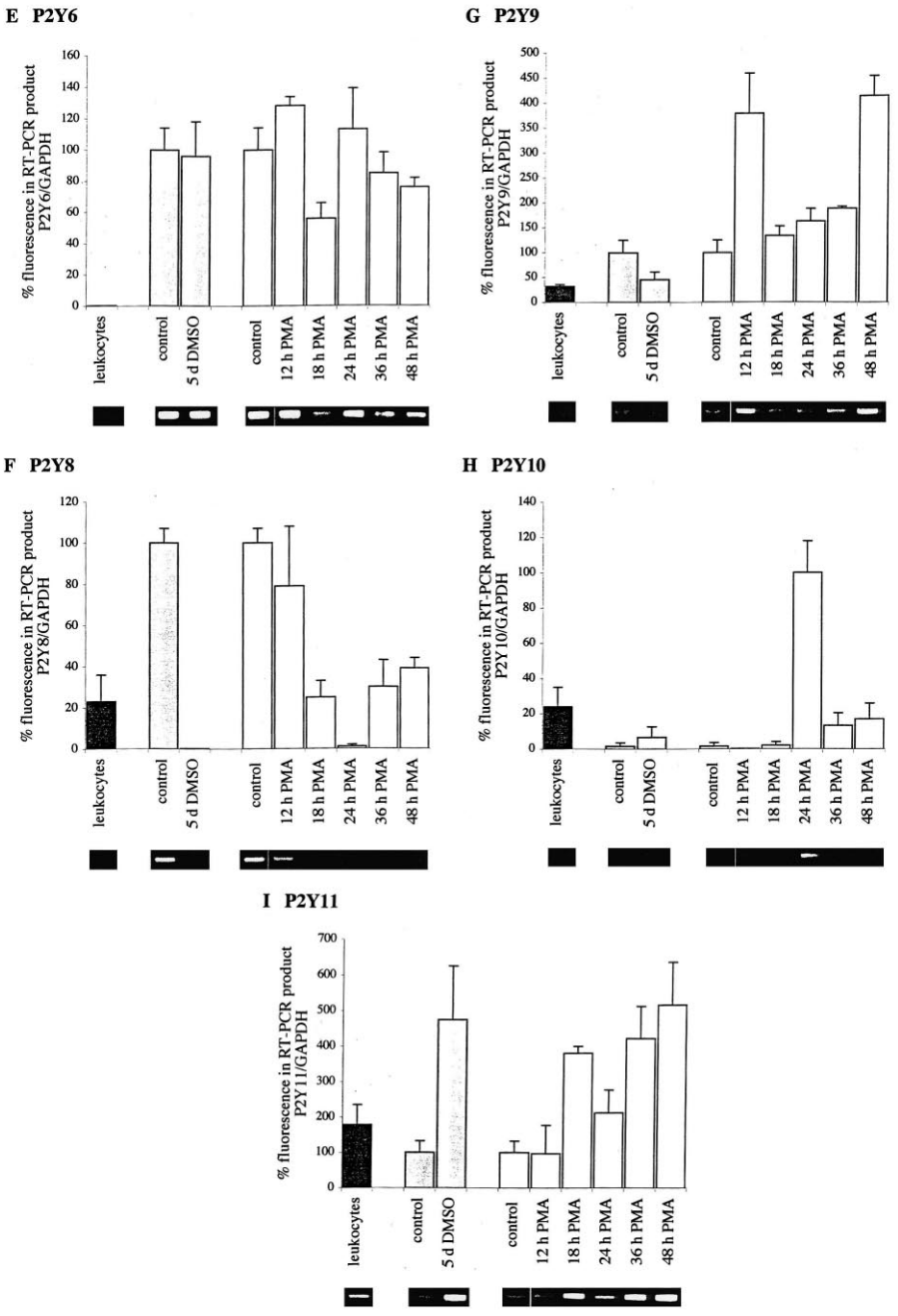

Fig. 4 (*continued*).

3.1.12. P2Y8 (Fig. 4F)

Moderate levels of P2Y8 mRNA were detected in undifferentiated HL60 cells. During granulocytic differentiation, this receptor was completely downregulated. Monocytic cells showed a reduction of P2Y8 mRNA up to 24 h of differentiation, at later times an upregulation was observed. P2Y8 receptor mRNA was barely detectable in blood leukocytes.

3.1.13. P2Y9 (Fig. 4G)

P2Y9 mRNA was moderately expressed in undifferentiated cells. In granulocytic cells, the expression level decreased to 50%. During monocytic differentiation P2Y9 mRNA was increased up to 4-fold showing distinct maximum at 12 and 48 h of incubation time. Low levels of P2Y9 mRNA were found in blood leukocytes.

3.1.14. P2Y10 (Fig. 4H)

P2Y10 mRNA was nearly undetectable in undifferentiated HL60 cells. Granulocytic differentiation did not result in a change of the expression level. During monocytic differentiation, P2Y10 mRNA was first apparent at 18 h and markedly increased at 24 h followed by a low expres-

sion level at 36 and 48 h. P2Y10 mRNA was weakly expressed in blood leukocytes.

3.1.15. P2Y11 (Fig. 4I)

P2Y11 mRNA could be detected in undifferentiated HL60 cells. The relatively low levels of transcript in the control cells were upregulated 3- to 6-fold in granulocytic cells. Monocytic differentiation resulted in 2- to 5-fold upregulation after 12 h. Moderate amounts of P2Y11 mRNA were observed in blood leukocytes.

3.1.16. Ecto-apyrase CD39 (Fig. 5A)

CD39 mRNA was nearly undetectable in undifferentiated HL60 cells. Differentiation to granulocytic and monocytic cells caused a strong increase in the amount of the transcript. CD39 mRNA was moderately expressed in blood leukocytes.

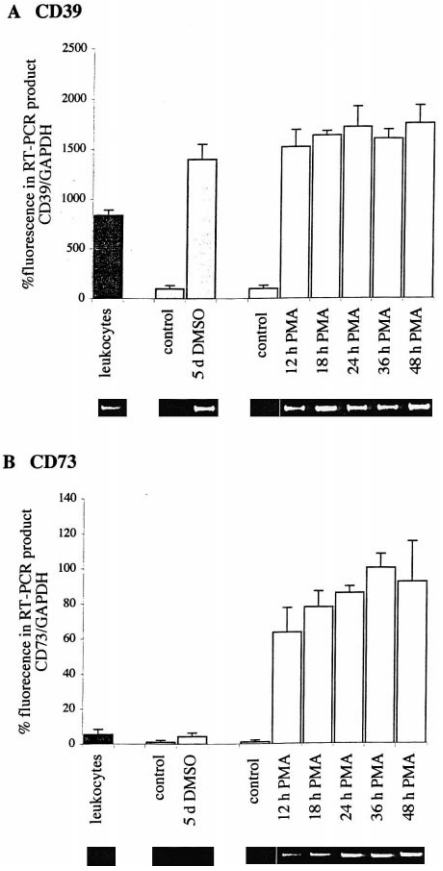

Fig. 5. RT-PCR analysis of CD39 (ecto-apyrase) and CD73 (ecto-5′-nucleotidase) mRNA. Messenger RNA expression in blood leukocytes, and during the differentiation of HL60 cells by 1.25% DMSO for 5 days, or 20 nM PMA for 12 h, 18 h, 24 h, 36 h and 48 h of (A) CD39 and (B) CD73. The gel prints are representative of results from three independent experiments. The bar graphs show mean and standard deviation from triplicate determinations (for CD39, control level was set at 100%; for CD73, expression level of HL60 cells differentiated with PMA for 36 h was set at 100%). The relative amounts of the RT-PCR products were quantified by fluorography and normalized to the GAPDH RT-PCR product levels.

3.1.17. Ecto-5′-nucleotidase CD73 (Fig. 5B)

CD73 mRNA was hardly expressed in undifferentiated HL60 cells. Granulocytic differentiation did not change the expression of the transcript. Monocytic differentiation resulted in a marked upregulation of CD73 mRNA. Low levels were observed in blood leukocytes.

3.2. Determination of the mRNA levels by dot-blot analysis

Representative of all analyzed P2 receptors, the specific mRNA levels for GAPDH, P2Y1 and the long splice variant of P2X5 were quantified by dot-blot assays. Total RNA extracts of HL60 promyelocytes and the differentiated HL60 cells (as described above) were dot-blotted and probed with receptor-specific ^{32}P-oligolabeled PCR products. For analysis of the GAPDH mRNA levels, the probes were stripped from the membranes and rehybridized with the labeled GAPDH probe. The relative changes in mRNA levels, as quantified by PhosphorImager analysis and normalized to GAPDH-mRNA levels are illustrated in Fig. 6.

GAPDH transcripts were nearly constant in all analyzed development stages of the HL60 cells.

Moderate levels of the long splicing variant form of P2X5 mRNA were observed in undifferentiated HL60 cells. The transcript levels increased in granulocytic cells. During monocytic differentiation, the expression levels were also significantly upregulated with a transient reduction in expression at 24 h.

P2Y1 mRNA was weakly expressed in undifferentiated HL60 cells. The mRNA levels were slightly upregulated in granulocytic cells. Monocytic differentiation resulted in a clear rise in mRNA levels, showing a temporary downregulation at 36 h.

These observations indicate, by two independent quantification techniques, that the relative amounts of mRNA, as determined by the semi-quantitative RT-PCR method agree with the analysis using the more precise dot-blot assay. While an absolute determination of transcript levels is not possible using RT-PCR method, relative changes (i.e. up- and downregulation during differentiation) can be measured with sufficient accuracy.

3.3. Ca^{2+} response to stimulation with ATP

As activation of most P2Y receptors results in an increase in intracellular Ca^{2+} [1], the measurement of intracellular Ca^{2+} was used as an indicator for P2Y-receptor function. Cells were loaded with the Ca^{2+}-sensitive fluorescence dye Fura-2 as described in Section 2. Stimulation with ATP led to an increase of fluorescence intensity reflecting the rise in intracellular Ca^{2+}.

We observed that the functional responses to ATP were similar in both undifferentiated and granulocytic HL60 cells. In contrast, during monocytic differentiation, a distinct attenuation of ATP-induced Ca^{2+}-liberation was observed.

Fig. 7 shows a comparison of the concentration response plots of ATP as a Ca^{2+}-mobilizing agonist in undifferentiated versus DMSO- and PMA- differentiated HL60 cells. Concentrations of >0.01 μM ATP were sufficient to increase intracellular Ca^{2+} in HL60 promyelocytes and granulocytes. In cells treated with PMA for 12 h, Ca^{2+}-mobilization was only detectable after stimulation with ATP concentrations of >1 μM. Incubation with PMA for 48 h induced a more than 4-log unit decrease in ATP potency. ATP at concentrations of 1–2 μM effected supramaximal Ca^{2+}-responses in undifferentiated and DMSO differentiated HL60 cells, but hardly increased the intracellular Ca^{2+}-level in later stages of monocytic differentiated HL60 cells. Total Ca^{2+}-response during monocytic differentiation was reduced more than 10-fold.

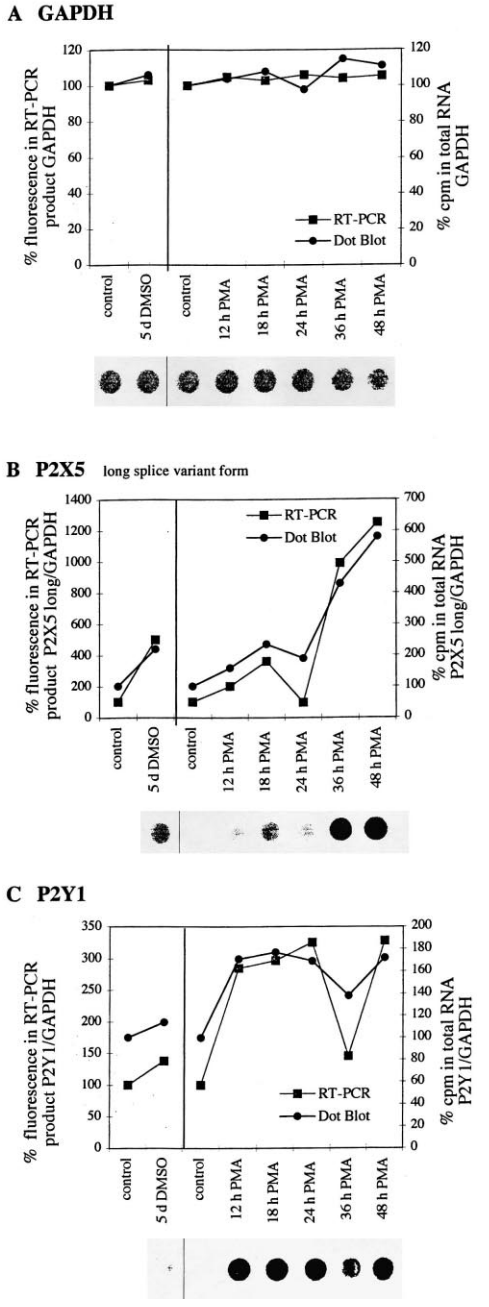

Fig. 6. Dot-blot analysis of GAPDH, P2X5 (long splicing variant) and P2Y1 mRNA levels during the differentiation of HL60 cells by DMSO, and PMA, respectively. For comparison, RT-PCR-data are also shown (see Fig. 2 for the GAPDH, Fig. 3C for the P2X5 and Fig. 4A for the P2Y1 receptor). The levels of (A) GAPDH mRNA, (B) P2X5 mRNA (long splicing variant) and (C) P2Y1 mRNA were assayed in HL60 cells differentiated with 1.25% DMSO for 5 days, or 20 nM PMA for 12, 18, 24, 36 and 48 h. Total RNA (2 μg/dot) was dot blotted and probed with receptor specific ^{32}P-oligolabeled PCR products. The relative amounts of the mRNA were quantified using a PhosphorImager. The P2X5 and the P2Y1 receptor signals were normalized to the corresponding GAPDH signals (control levels were set at 100%).

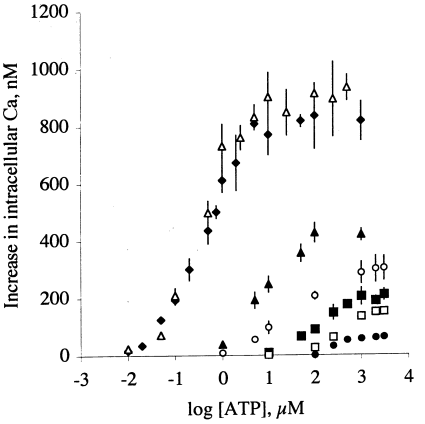

Fig. 7. ATP-induced Ca^{2+}-mobilization in undiffentiated, DMSO- or PMA-differentiated HL60 cells. HL60 cells were loaded with Fura-2/AM as described in Section 2, and assayed in a fluorimeter for Ca^{2+}-mobilization in response to indicated concentrations of ATP. Fig. 7 shows concentration–response relationships describing ATP-induced Ca^{2+}-mobilization in control HL60 cells (♦), in cells treated with 1.25% DMSO (△) for 5 days, or in cells treated with 20 nM PMA for 12 h (▲), 18 h (○), 24 h (■), 36 h (□) and 48 h (●). Data represent the mean of three independent experiments.

4. Discussion

In this study, we determined and compared the mRNA expression levels of all cloned P2X and P2Y receptor subtypes and the ecto-enzymes CD39 and CD73 during granulocytic and monocytic differentiation of HL60 cells and in peripheral blood leukocytes. Since the usual methods for determining the number of receptors on a cell surface, like binding of specific ligands or antibodies, are unavailable for most purinergic receptors, their expression levels were studied using RT-PCR and dot-blot techniques. The direct quantification of specific mRNA by dot-blot hybridization showed that the indirect RT-PCR method can be used with sufficient accuracy to determine relative changes of transcript levels. The analyzed cells showed a great variability in the expression of P2 receptors. Most receptors revealed fluctuating changes in transcript levels, i.e. a decrease in transcript amounts at the 24 h time point. This effect was found most pronounced for P2X7 and P2Y8 receptors. These fluctuations were consistently observed in three independent sets of experiments using three

unique batches of cell preparations. Although highly reproducible, there is currently no obvious physiological explanation for these changes in transcript abundancy.

The phenomenon of fluctuating expression levels during differentiation has already been described for other receptors. For example, the β_3-adrenergic receptor in 3T3-F442A adipocytes showed significant up- and downregulations during PMA-incubation [25]. Concerning P2 receptors, a similar feature has been reported for the P2Y2 receptor mRNA in the early PMA differentiation stages of activated thymocytes [26].

Our study is the first synopsis of the mRNA expression of all known P2 receptors during granulocytic and monocytic differentiation of HL60 cells. Our data confirm previous studies of the expression of single P2 receptor subtypes and the ecto-nucleotidases CD39 and CD73.

We detected P2X1 receptor expression in all development stages of HL60 cells with a strong upregulation during monocytic differentiation. This observation is in agreement with reported P2X1 receptor expression in undifferentiated HL60 cells and in PMA stimulated HL60 monocytes [27–29]. Furthermore, Buell et al. [16] showed a 20-fold increase of P2X1 mRNA levels in 72-h PMA-treated cells. In agreement with findings by Clifford et al. [30] and MacKenzie et al. [31], we could not detect P2X1 mRNA in blood leukocytes.

We did not observe P2X3 receptor expression in any of the analyzed blood cells. This is consistent with the restricted tissue distribution of P2X3 receptor mRNA to the spinal cord and heart as shown by Garcia-Guzman et al. [32].

Consistent with observations on rat leukocytes [33], we did not find P2X4 mRNA in peripheral human blood leukocytes.

We detected two splicing variant forms of P2X5 mRNA in HL60 cells and in blood leukocytes. Our findings agree with recent data of the existence of the two alternative splice variants, both being highly expressed in human lymphocytes and leukocytes [34].

We found expression of P2X7 mRNA in all analyzed stages of HL60 myeloid differentiation as well as in blood leukocytes. Corresponding with our data the P2X7 receptor has been identified on human macrophages [35–37] and in peripheral blood leukocytes [38].

According to the observations of other authors, our studies revealed P2Y1 mRNA in undifferentiated HL60 cells and in blood leukocytes [39,40]. In contrast to Léon et al. [40] and us, Janssens et al. [41], as well as Berchthold et al. [42] found no evidence for P2Y1 mRNA in blood leukocytes and undifferentiated HL60 cells, respectively.

We observed expression of P2Y2 mRNA in both HL60 promyelocytes and granulocytes as well as in blood leucocytes. During stimulation with PMA, transcript levels were dramatically downregulated. A similar expression pattern of the P2Y2 receptor has been reported for blood leukocytes and HL60 cells [39,43]. Martin et al. [17] have also shown the changes of P2Y2 mRNA levels during myeloid differentiation of HL60 cells. Their study demonstrated that P2Y2 mRNA levels were only modestly attenuated during granulocytic differentiation by DMSO. In contrast, PMA treatment induced, after an early transient upregulation of P2Y2 mRNA, a rapidly following decrease of P2Y2 mRNA to a level 5–10-fold lower than in undifferentiated HL60 cells [17].

We identified P2Y4 transcripts in all analyzed development stages of HL60 cells and in blood leukocytes. In contrast with our findings, P2Y4 mRNA was not observed in undifferentiated HL60 [39,44]. Our results agree with findings by Jin et al. [39], who also demonstrated the expression of P2Y4 in blood leukocytes.

To our knowledge, the distribution patterns of the human P2Y5, P2Y8 and P2Y10 receptors have not yet been analyzed. We found mRNA of these receptors in most development stages of the HL60 cells and also in blood leukocytes. Previously, these receptors, or their species analogues, were detected only in animals: the species homologue of P2Y5 in chicken showed a restricted expression to activated T-cells [45]. Bogdanov et al. [13] demonstrated that P2Y8 mRNA is expressed transiently in the neural plate and tailbud during *Xenopus* development.

We found P2Y6 transcripts in the HL60 cells, but not in blood leukocytes. In agreement with our data, P2Y6 mRNA was detected in undifferentiated HL60 cells [39]. A low expression of P2Y6 mRNA in blood leukocytes has been described [39,46]. It should be noted that differences in the preparation methods may underlie these variations. For example, an apparently higher receptor expression was described using heparinized versus citrated blood for leukocyte preparations [16].

We detected P2Y9 mRNA in blood leukocytes, while others were not able to observe a positive signal by Northern blot hybridization [47], which might be less sensitive than the RT-PCR assay we used [48,49].

Our study revealed P2Y11 mRNA in all analyzed samples. In granulocytic as well as in the later stages of monocytic differentiation, this receptor was strongly upregulated. In agreement with our results, others have reported that low levels of P2Y11 mRNA were expressed in HL60 promyelocytes and in HL60 cells stimulated with PMA for 8 h, whereas differentiation to granulocytes with DMSO led to increased mRNA signals [18].

It is interesting that with the only exception of P2X3 receptor, expression of all analyzed P2 receptors occurred during myeloid differentiation of HL60 cells. Each receptor showed a distinct regulation sometimes with unexpected fluctuations during the course of differentiation. In whole blood leukocytes, transcripts of P2X5, P2X7 and all P2Y receptors except for P2Y6 receptor were found.

The data presented here show that P2X receptors are essentially absent on undifferentiated HL60 promyelocytes and on HL60 granulocytes, however they are strongly ex-

pressed in macrophage/monocyte-differentiated HL60 cells. Therefore it appears likely that P2X receptor signalling is important only in macrophages [16], and seems not to be significant on undifferentiated promyelocytes and granulocytes.

A study carried out in collaboration with our group found expression of P2X1, P2X4, P2X5, P2X7 and P2Y1, P2Y2, P2Y4, P2Y5, P2Y6, P2Y10, P2Y11 receptors on adherent macrophages [42]. In our experiments, these receptors were also found on PMA-differentiated HL60 cells, but showed a markedly different expression pattern on the undifferentiated promyelocytes. Thus, the observed regulation of purinergic receptor expression on HL60 cells may indeed be indicative of the regulatory processes in macrophages.

The physiological activity of P2Y receptors was measured by the analysis of ATP-induced intracellular Ca^{2+}-increase. We demonstrated that the Ca^{2+}-response was unaltered during granulocytic differentiation of HL60 cells. In contrast, both potency and efficacy of ATP were significantly attenuated during PMA differentiation.

A similar Ca^{2+}-response of HL60 promyelocytes and granulocytes to ATP-stimulation has been described by others [50]. Consistent with our observations with ATP, Martin et al. [17] showed an attenuation of the Ca^{2+}-response during PMA-differentiation with the pyrimidine UTP as a Ca^{2+}-mobilizing agonist. They explained this effect with the strong PMA-induced downregulation of P2Y2 mRNA. With the background of our RT-PCR data, we tend to interpret the change of potency and efficacy of ATP-mediated Ca^{2+}-response during HL60-monocytic differentiation as a result of the regulation of more than only one P2 receptor type.

It is of particular interest to note that at ATP levels of around 100 μM, Ca^{2+}-liberation in undifferentiated HL60 cells is at maximum, while HL60 monocytes have essentially zero Ca^{2+}-response. Thus, ATP increases intracellular Ca^{2+} only in undifferentiated HL60 cells and in HL60 granulocytes, but do not induce metabolic responses via Ca^{2+}-release after the monocytic stage has been reached. This observation is consistent with previous findings that suggest a role for ATP as a differentiation factor in HL60 cells [51].

A further important point in the characterization of the potency of an agonist to purinergic receptors is the activity of agonist-metabolizing enzymes. Metabolization of extracellular nucleotides is effected by ecto-enzymes, such as CD39 (ecto-apyrase) and CD73 (ecto-5′-nucleotidase). We demonstrated a distinct upregulation of CD39 and CD73 during myeloid maturation of HL60 cells. Others have also shown that these enzymes underlie an upregulation during differentiation of HL60 cells resulting in reduction of magnitude and duration of agonist potency [43]. Whether the reduced Ca^{2+}-response is due to receptor regulation, or to increased activity of agonist-metabolizing enzymes, cannot be deduced from these experiments. It seems likely, however, that Ca^{2+}-liberation is affected by both processes.

Therefore, it is not only the receptor-mediated response to purinergic ligands which needs to be considered in blood cell differentiation. The signal itself is also modified through extracellular enzymes, as shown here for CD39 and CD73. The presence of agonist-modifying enzymes and the resulting complexity of P2 receptor function must therefore always be seen in context, when aspects of purinergic signalling and its regulation are investigated.

Our data indicate the important role of the purinergic system during hematopoietic differentiation. As long as the physiological role of each receptor subtype has not been elucidated, the biological meaning of this fine regulation will remain unclear.

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