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Nucleotide-, Chemotactic Peptide- and Phorbol Ester-Induced Exocytosis in HL-60 Leukemic Cells

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Abstract

Undifferentiated and differentiated HL-60 leukemic cells possess nucleotide receptors which functionally couple to phospholipase C via pertussis toxin-sensitive guanine nucleotide-binding proteins (G-proteins). We investigated the role of extracellular nucleotides in the regulation of β-glucuronidase release in HL-60 cells. In dibutyryl cyclic AMP (Bt_2cAMP)-differentiated HL-60 cells, the chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), the phosphorothioate analogue of ATP, adenosine 5′-O-[3-thio]triphosphate (ATP[γS]), and UTP increased cytosolic Ca^{2+} from 100 nM up to 1.2 μM with EC_{50} values of 4 nM, 1 μM and 100 nM, respectively. In these cells, ATP[γS] induced exocytosis with an EC_{50} of 4 μM and an effectiveness amounting to 50–70 % of that of fMet-Leu-Phe. ATP, ITP, UTP, CTP, and uridine 5′-O-[2-thio]diphosphate activated exocytosis as well. Phorbol myristate acetate (PMA) induced exocytosis with an EC_{50} of 115 ng/ml and an effectiveness similar to that of ATP[γS]. Cytochalasin B (CB) differently potentiated exocytosis induced by ATP[γS], fMet-Leu-Phe and PMA. Treatment of Bt_2cAMP-differentiated HL-60 cells with pertussis toxin (500 ng/ml) for 24 h resulted in ADP-ribosylation of more than 97.5 % of the G-proteins. Under these conditions, pertussis toxin almost completely inhibited the increase in cytosolic Ca^{2+} and β-glucuronidase release induced by fMet-Leu-Phe but only partially inhibited the effects of ATP[γS] and UTP. fMet-Leu-Phe at a non-stimulatory concentration (1 nM) potentiated ATP[γS]-induced β-glucuronidase release in the presence but not in the absence of CB. In contrast, ATP[γS] and fMet-Leu-Phe synergistically activated superoxide formation in the absence of CB. PMA potentiated superoxide formation induced by ATP[γS] or fMet-Leu-Phe and did not affect exocytosis induced by ATP[γS] or fMet-Leu-Phe. In undifferentiated HL-60 cells, fMet-Leu-Phe, ATP[γS], UTP and PMA did not induce β-glucuronidase release. fMet-Leu-Phe did not increase cytosolic Ca^{2+} in undifferentiated HL-60 cells, whereas ATP[γS] and UTP were similarly potent and effective as in Bt_2cAMP-differentiated cells. In differentiated HL-60 cells, fMet-Leu-Phe induced aggregation, and ATP[γS] induced a transient shape change. Our results show (I) that exocytosis in HL-60 cells does not obligatorily depend on CB. (II) Purine and pyrimidine nucleotides activate exocytosis via pertussis toxin-sensitive and -insensitive signal transduction pathways.

Abbreviations: ADP[βS] = adenosine 5′-O-[2-thio]diphosphate; *ATP[γS]* = adenosine 5′-O-[3-thio]triphosphate; *Bt_2cAMP* = dibutyryl cyclic AMP; *Fura-2/AM* = Fura-2 acetoxymethylester; *GDP[βS]* = guanosine 5′-O-[2-thio]diphosphate; *GTP[γS]* = guanosine 5′-O-[3-thio]triphosphate; *[β,γ-NH]GTP* = guanosine 5′-[β,γ-imido]triphosphate; *CB* = cytochalasin B; *fMet-Leu-Phe* = N-formyl-L-methionyl-L-leucyl-L-phenylalanine; *G-proteins* = guanine nucleotide-binding proteins; *O_2^-* = superoxide; *PDD* = phorbol didecanoate; *PMA* = phorbol myristate acetate; *UDP[βS]* = uridine 5′-O-[2-thio]diphosphate

(III) Specific signal transduction components for exocytosis are expressed during myeloid differentiation which are apparently different from G-proteins, phospholipase C, protein kinase C, and the Ca^{2+}-mobilizing system. (IV) Exocytosis and superoxide formation are independently regulated.

Introduction

The chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), binds to formyl peptide receptors in human neutrophils and differentiated HL-60 leukemic cells and activates phospholipase C via pertussis toxin-sensitive guanine nucleotide-binding proteins (G-proteins) (1, 2). Phospholipase C catalyzes degradation of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol triphosphate, leading to activation of protein kinase C and Ca^{2+} mobilization (1, 2). In addition, fMet-Leu-Phe activates NADPH oxidase-catalyzed superoxide (O_2^-) formation, β-glucuronidase release from azurophilic granules and aggregation of neutrophils (3–6). fMet-Leu-Phe may activate NADPH oxidase through Ca^{2+}/protein kinase C-dependent and -independent pathways (1, 7, 8). Cytochalasin B (CB) potentiates agonist-induced O_2^- formation, presumably by inhibiting actin polymerization and sequestration of formyl peptide receptors (9).

Induction of β-glucuronidase release by fMet-Leu-Phe in human neutrophils depends absolutely on the presence of CB (3, 4). Ca^{2+}- and protein kinase C-dependent mechanisms and arachidonic acid have been suggested to be involved in the activation of azurophilic granule release (10–13). The role of protein kinase C in this process, however, is more complex. Phorbol myristate acetate (PMA) and cell-permeable diacylglycerols are only poor inducers of β-glucuronidase release in neutrophils (3, 4, 10, 14). In addition, PMA may potentiate or inhibit fMet-Leu-Phe-induced β-glucuronidase release or is without effect (11–13, 15, 16). The mechanism by which PMA inhibits exocytosis may involve inhibition of phospholipase C and Ca^{2+} mobilization (11, 16–18). Finally, a putative G-protein, G_E, has been claimed to be involved in exocytosis (19, 20).

In addition to chemotactic peptides, extracellular nucleotides activate human neutrophils and HL-60 cells via purino- and pyrimidinoceptors, respectively (6, 21, 22). Myeloid purinoceptors have been shown to couple functionally to pertussis toxin-sensitive and -insensitive G-proteins, and pyrimidinoceptors couple functionally to pertussis toxin-sensitive G-proteins (6, 12, 13, 22, 23). In human neutrophils and dimethyl sulfoxide-differentiated HL-60 cells, nucleotides potentiate fMet-Leu-Phe-induced O_2^- formation (6, 22, 24). In dibutyryl cyclic AMP (Bt_2cAMP)-differentiated HL-60 cells, extracellular nucleotides per se activate O_2^- formation and β-glucuronidase release (12, 13, 22). Interestingly, the phosphorothioate analogue of ATP, adenosine 5′-O-[3-thio]triphosphate (ATP[γS]), activates phospholipase C in undifferentiated HL-60 cells (23).

These findings prompted us to study β-glucuronidase release, O_2^- formation and aggregation in HL-60 cells. We report that fMet-Leu-Phe, purine, and pyrimidine nucleotides induce exocytosis in Bt_2cAMP-differentiated HL-60 cells. The effects of fMet-Leu-Phe show a greater pertussis toxin-sensitivity than those of nucleotides. Undifferentiated HL-60 cells do not release β-glucuronidase. We characterize the interaction of fMet-Leu-Phe, ATP[γS], PMA, and CB on exocytosis and O_2^- formation and show that both cell functions are independently regulated.

Materials and Methods

Materials

Uridine 5'-O-[2-thio]diphosphate (UDP[βS]) was kindly provided by Dr. F. ECKSTEIN (Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, FRG). Pertussis toxin was a gift of Dr. M. YAJIMA (Kyoto, Japan). Phorbol didecanoate (PDD) was purchased from Sigma Chemie (Taufkirchen, FRG). A stock solution of PDD (1 mg/ml) was prepared in dimethyl sulfoxide and was stored at −20 °C. Fura-2 acetoxy-methylester (Fura-2/AM) was purchased from Boehringer Mannheim (Mannheim, FRG). A stock solution of Fura-2/AM (2 mM) was prepared in dimethyl sulfoxide and was stored at −20 °C. All cell culture media were obtained from Biochrom (Berlin, FRG). Sources of other materials have been described elsewhere (6, 7, 22, 25, 26).

Cell culture

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10 % (v/v) horse serum, 1 % (v/v) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere with 7 % CO_2 at 37 °C. To induce differentiation, HL-60 cells were seeded at 10^6 cells/ml and were cultured for 48 h with 0.2 mM Bt_2cAMP (27, 28). Pappenheim-stained smears showed that Bt_2cAMP-differentiated HL-60 cells consisted of more than 98 % myelocytes or cells beyond this stage. Undifferentiated HL-60 cells consisted of more than 95 % promyelocytes (data not shown).

Pertussis toxin treatment of Bt_2cAMP-differentiated HL-60 cells

Differentiation of HL-60 cells was induced as described above. After 24 h of incubation with Bt_2cAMP, pertussis toxin (500 ng/ml) or its vehicle (control) were added to the cell cultures. Cells were incubated for an additional period of 24 h. Thereafter, functional studies with HL-60 cells or preparation of HL-60 membranes were performed. In agreement with a recent report (29), we found that long-term incubation of HL-60 cells with pertussis toxin did neither affect cell viability nor Bt_2cAMP-induced differentiation (data not shown).

Measurement of cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} was determined with the dye, Fura-2/AM (30), according to the protocol described in Reference 23 with modifications. Briefly, HL-60 cells were suspended at 10^7 cells/ml in a buffer consisting of (mM) 138 NaCl, 6 KCl, 1 $MgSO_4$, 1.1 $CaCl_2$, 0.1 EGTA, 1 Na_2HPO_4, 5 $NaHCO_3$, 5.5 glucose, and 20 Hepes/NaOH, pH 7.4, supplemented with 0.1 % bovine serum albumin (w/v). Fura-2/AM was added at a concentration of 4 μM and cells were incubated for 10 min at 37 °C. Thereafter, cells were diluted with the above buffer to a concentration of 5×10^6 cells/ml and were incubated for a period of 50 min at 37 °C. Subsequently, cells were diluted with the above buffer to a final concentration of 0.5×10^6 cells/ml and were centrifuged at 250 × g for 10 min at 20 °C. Cells were suspended at 5×10^6

cells/ml in the above buffer and were kept at 20 °C until measurement of cytosolic Ca^{2+}. HL-60 cells (2.5×10^6 cells) were suspended in 2 ml of the above buffer using acryl fluorescence cuvettes (Sarstedt, Nümbrecht, FRG). Fluorescence was determined at 37 °C under constant stirring of the cells at 10^3 rpm, using a Ratio II™ spectrofluorometer (Aminco, Silver Spring, Maryland, USA). Cells were incubated for 3 min prior to the addition of stimuli. The excitation wavelength was 340 nm, the emission wavelength was 500 nm. Fluorescence signals were calibrated after lysis of the cells with 0.1 % Triton X-100 (w/v) (maximal fluorescence) and subsequent addition of 20 mM EGTA (minimal fluorescence). Peak cytosolic Ca^{2+} was calculated according to equation 6 given in Reference 30. Autofluorescence of HL-60 cells was determined by loading the cells with dimethyl sulfoxide instead of Fura-2/AM.

Assay for β-glucuronidase release

HL-60 cells ($0.5–1.0 \times 10^7$ Bt_2cAMP-differentiated cells or $1.0–1.5 \times 10^7$ undifferentiated cells) were suspended in 500 µl of a buffer consisting of (mM) 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose, and 20 Hepes/NaOH, pH 7.4, and were incubated for 5 min at 37 °C in the absence or presence of CB (5 µg/ml) prior to the addition of stimuli. Unless stated otherwise, reactions were terminated after 10 min by placing the tubes onto melting ice. Reaction mixtures were centrifuged at 250 × g for 10 min at 4 °C. The determinations of β-glucuronidase and lactate dehydrogenase activities of the supernatant fluids of the reaction mixtures were performed as described (31). The release of lactate dehydrogenase and β-glucuronidase (in % of total cellular content) was calculated. None of the agents studied caused cell damage as revealed by the release of lactate dehydrogenase and trypan blue dye exclusion. In undifferentiated and Bt_2cAMP-differentiated HL-60 cells, the release of lactate dehydrogenase generally amounted to < 5.0 % (data not shown).

Assay for superoxide formation

O_2^- formation was monitored by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase using a Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, FRG) (28, 32). Reaction mixtures (0.5 ml) contained 2.5×10^6 Bt_2cAMP-differentiated HL-60 cells, 100 µM ferricytochrome C and the buffer used for the determination of β-glucuronidase release. Reaction mixtures were incubated for 5 min at 37 °C in the absence or presence of CB (5 µg/ml) prior to the addition of stimuli. V_{max} of O_2^- formation was calculated (28, 32).

Aggregation of HL-60 cells

Aggregation was measured by turbidometry (6, 33). Bt_2cAMP-differentiated HL-60 cells (1×10^7 cells) were suspended in 900 µl of the buffer used for the determination of β-glucuronidase release. Cells were incubated for 5 min at 37 °C in the presence of CB (5 µg/ml) prior to the addition of stimuli. Aggregation experiments were carried out under constant stirring of cells at 10^3 rpm, using a Uvikon 810 dual-beam spectrophotometer.

Preparation of HL-60 membranes and determination of protein

Membranes from Bt_2cAMP-differentiated HL-60 cells treated with pertussis toxin or its vehicle were prepared as described (34). Protein determination was performed according to Lowry et al. (35).

[^{32}P]ADP-ribosylation of HL-60 membranes and gel electrophoresis

Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of HL-60 membranes was performed as described (25, 26, 36). Briefly, preactivated pertussis toxin was diluted with dilution buffer to a final concentration of 1.7 µg/ml. Control samples received dilution buffer without toxin. The final NAD concentration was 1 µM with 75 kBq [^{32}P]NAD/assay tube (60 µl). Incubations were conducted for 30 min at 30 °C. Prior to electrophoresis, proteins were precipitated by

acetone. The precipitates were washed with trichloroacetic acid and subsequently with methanol/chloroform (1:2, v/v).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to LAEMMLI (37). Fifty μg of membrane protein were loaded/lane. Autoradiography of dried polyacrylamide gels was performed as described (25). Gels were cut on the basis of autoradiographs, and radioactivity incorporated into gel bands corresponding to 40–41 kDa proteins, presumably representing the α-subunits of G_{i2} and G_{i3} (38), was determined by liquid scintillation counting.

Results

Cytosolic Ca^{2+} in undifferentiated and Bt_2cAMP-differentiated HL-60 cells was determined (Fig. 1). Basal cytosolic Ca^{2+} in undifferentiated and Bt_2cAMP-differentiated HL-60 cells amounted to about 100 nM. In undifferentiated HL-60 cells, fMet-Leu-Phe did not increase cytosolic Ca^{2+}. In contrast, ATP[γS] and UTP increased cytosolic Ca^{2+} up to 1 μM in undifferentiated HL-60 cells. The effects of ATP[γS] and UTP were half-maximal at 1 μM and 100 nM, respectively, and reached a maximum at 10 μM. In Bt_2cAMP-differentiated HL-60 cells, ATP[γS] and UTP increased cytosolic Ca^{2+} up to 1.2 μM and the potencies of the nucleotides were similar to the ones in undifferentiated HL-60 cells. In Bt_2cAMP-

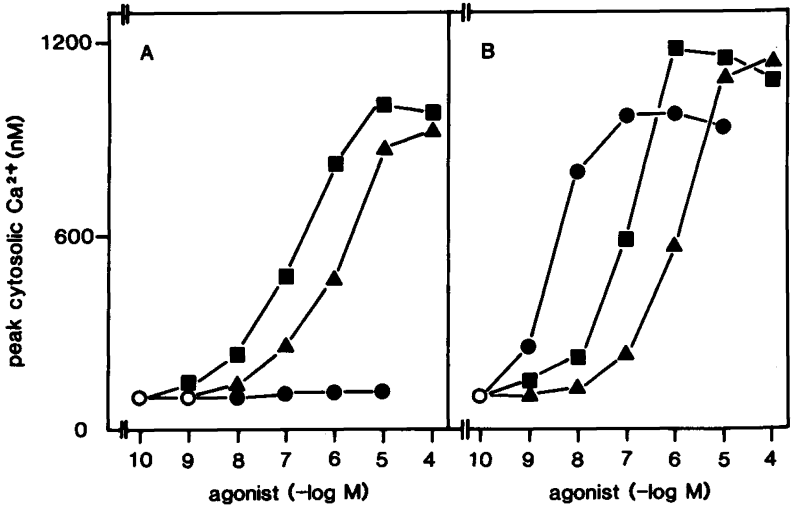

Figure 1. Ca^{2+} mobilization by fMet-Leu-Phe, ATP[γS] and UTP in undifferentiated and Bt_2cAMP-differentiated HL-60 cells. HL-60 cells were loaded with Fura-2/AM and preincubated for 3 min prior to addition of stimuli. fMet-Leu-Phe, (●); ATP[γS], (▲); UTP, (■). Open circles indicate that agonists at the indicated concentrations were without effect. A: concentration response curves to agonists in undifferentiated HL-60 cells. B: concentration response curves to agonists in Bt_2cAMP-differentiated HL-60 cells. Data shown are the means of assay duplicates; these varied by less than 10%. Similar results were obtained in three experiments performed with different preparations of HL-60 cells.

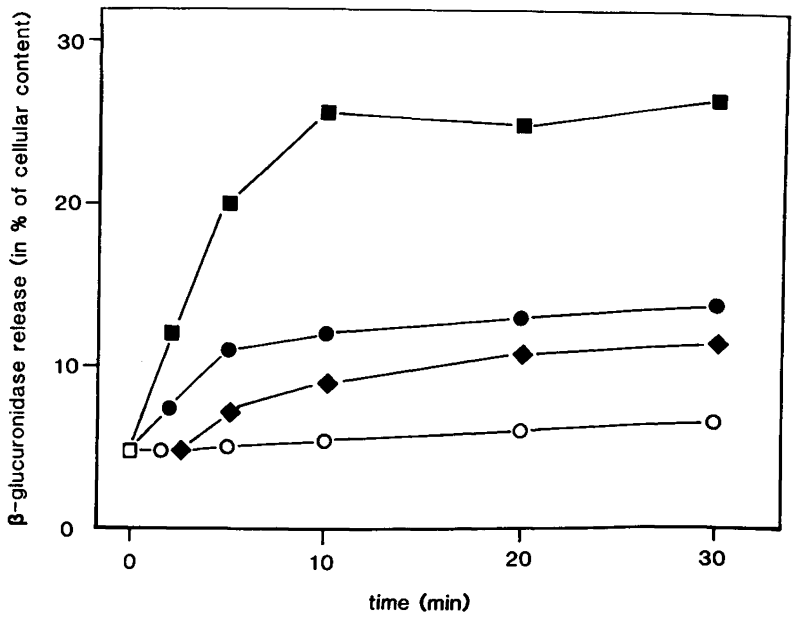

Figure 2. Time course of β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells. HL-60 cells were treated with CB (5 μg/ml) for 5 min prior to the addition of stimuli or solvent (control). The release of β-glucuronidase was measured for the indicated periods of time. fMet-Leu-Phe (1 μM), (■); ATP[γS] (100 μM), (●); PMA (100 ng/ml), (◆); control, (○). The open square indicates that the substances were without stimulatory effect. Data shown are the means of assay duplicates which varied by less than 5 %. Similar results were obtained in three experiments performed with different preparations of HL-60 cells.

differentiated HL-60 cells, fMet-Leu-Phe increased cytosolic Ca^{2+} with an EC_{50} of 4 nM and a maximum at 100 nM. fMet-Leu-Phe was slightly less effective than nucleotides to increase cytosolic Ca^{2+} in Bt_2cAMP-differentiated HL-60 cells and cytosolic Ca^{2+} returned more slowly to basal values in ATP[γS]- and UTP-stimulated cells than in chemotactic peptide-stimulated cells (data not shown).

The time course of β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells in the presence of CB is shown in Figure 2. Similar to human neutrophils, the basal release of β-glucuronidase in HL-60 cells slightly increased during the entire incubation period (3, 5). ATP[γS] and fMet-Leu-Phe rapidly induced β-glucuronidase release. Exocytosis induced by these stimuli was half-maximal after 3 min. In contrast, the PMA-induced β-glucuronidase release was delayed in onset, and a stimulatory effect of PMA was not evident until 5 min. PMA was similarly effective as ATP[γS].

Concentration response functions for ATP[γS] and fMet-Leu-Phe on β-glucuronidase release in Bt_2cAMP-differentiated and undifferentiated HL-60 cells are shown in Figure 3. In the absence of CB, fMet-Leu-Phe induced β-glucuronidase release with an EC_{50} of 25 nM and a maximum at 0.3 μM.

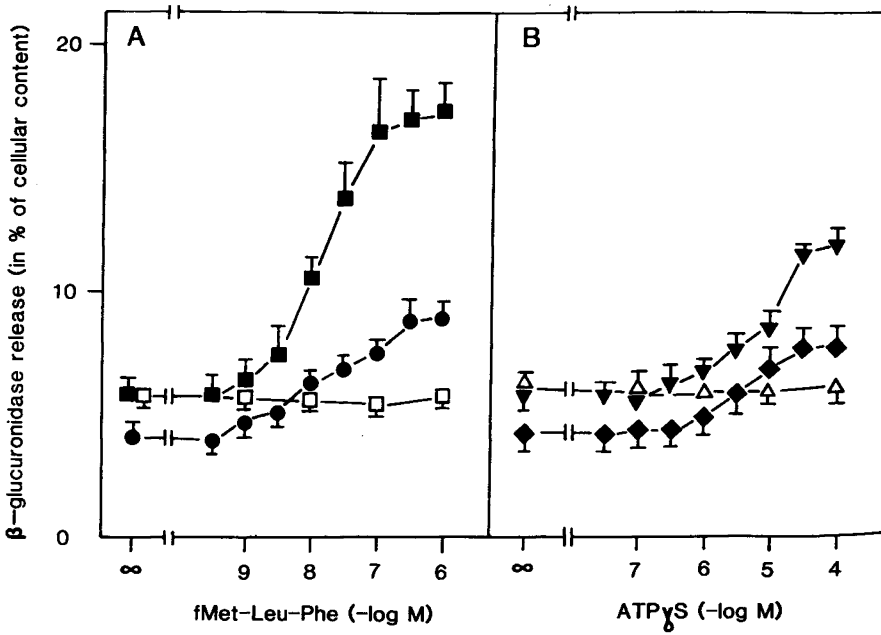

Figure 3. Activation of β-glucuronidase release by fMet-Leu-Phe and ATP[γS] in Bt_2cAMP-differentiated and undifferentiated HL-60 cells. HL-60 cells were treated with CB (5 μg/ml) or solvent (control) for 5 min prior to the addition of stimuli. A: concentration response curves to fMet-Leu-Phe: fMet-Leu-Phe, (●); fMet-Leu-Phe plus CB, (■, □). B: concentration response curves to ATP[γS]: ATP[γS], (♦); ATP[γS] plus CB, (▼, △). Closed symbols denote the experiments carried out with Bt_2cAMP-differentiated HL-60 cells, open symbols denote the experiments carried out with undifferentiated HL-60 cells. Data shown represent the means ± SEM of seven to eight experiments carried out with different preparations of HL-60 cells.

CB (5 μg/ml) per se significantly stimulated β-glucuronidase release ($p < 0.02$, $n = 30$). CB potentiated β-glucuronidase release induced by fMet-Leu-Phe at concentrations > 3 nM and decreased the EC_{50} for fMet-Leu-Phe to 13 nM. In the absence of CB, ATP[γS] induced β-glucuronidase release with an EC_{50} of 4 μM and a maximum at 30 μM. In contrast to fMet-Leu-Phe, CB potentiated ATP[γS]-induced exocytosis only in the presence of the agonist at maximal stimulatory concentrations. The effectiveness of ATP[γS] to induce β-glucuronidase release in the absence and presence of CB amounted to about 50–70 % of that of fMet-Leu-Phe.

In contrast to Bt_2cAMP-differentiated HL-60 cells, undifferentiated HL-60 cells did not release β-glucuronidase upon stimulation with fMet-Leu-Phe or ATP[γS]. ATP and UTP also did not induce exocytosis in undifferentiated HL-60 cells (data not shown). The cellular content of β-glucuronidase in undifferentiated HL-60 cells amounted to 1.01 ± 0.13 nmoles/10^6 cells/min ($n = 17$) and increased to 1.27 ± 0.07 nmoles/10^6 cells/min ($n = 30$) (not significant) in Bt_2cAMP-differentiated cells.

The effects of various nucleotides on β-glucuronidase release are summarized in Table 1. Among the purine nucleotides, ATP[γS], ATP, and ITP significantly stimulated exocytosis. The pyrimidines, UTP, CTP, and UDP[βS], significantly induced β-glucuronidase release as well. ADP, adenosine 5′-O-[2-thio]diphosphate (ADP[βS]), AMP, adenosine, GTP, guanosine 5′-O-[3-thio]triphosphate (GTP[γS]), guanosine 5′-O-[2-thio]diphosphate (GDP[βS]), guanosine 5′-[β, γ-imido]triphosphate ([β, γ-NH]GTP) and TTP did not significantly stimulate exocytosis.

In order to elucidate the role of G-proteins in activation of HL-60 cells by extracellular nucleotides, Bt_2cAMP-differentiated HL-60 cells were treated with pertussis toxin at 500 ng/ml for 24 h. Under these conditions, pertussis toxin catalyzed the ADP-ribosylation of more than 97.5 % of the G-proteins in HL-60 cells as assessed by subsequent [^{32}P]ADP-ribosylation of 40–41 kDa proteins by preactivated pertussis toxin in membranes obtained from the toxin- or toxin carrier-treated cells (Table 2).

The effect of pertussis toxin on Ca^{2+} mobilization induced by fMet-Leu-Phe, ATP[γS], and UTP at similarly effective concentrations was evaluated (Table 3). Pertussis toxin was without effect on basal cytosolic Ca^{2+} in HL-60 cells and inhibited the increase in cytosolic Ca^{2+} by fMet-Leu-Phe by about 85 %. In contrast, the stimulatory effects of ATP[γS] and UTP on

Table 1. Nucleotide specificity for stimulation of β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells

Nucleotide		β-glucuronidase release (in % of cellular content)	
Control		5.2 ± 0.4	(7)
Purines	ATP[γS]	10.1 ± 0.7[a]	(7)
	ATP	8.0 ± 0.7[b]	(4)
	ADP	5.6 ± 0.7[c]	(4)
	ADP [βS]	5.2 ± 0.1[c]	(6)
	AMP	5.3 ± 0.1[c]	(4)
	adenosine	4.8 ± 0.5[c]	(4)
	ITP	8.6 ± 1.0[b]	(4)
	GTP	5.3 ± 0.4[c]	(4)
	GTP[γS]	5.4 ± 0.2[c]	(4)
	GDP[βS]	5.3 ± 0.4[c]	(5)
	[β, γ-NH]GTP	5.2 ± 0.3[c]	(4)
Pyrimidines	UTP	10.1 ± 0.4[a]	(6)
	CTP	8.2 ± 0.5[b]	(4)
	TTP	5.3 ± 0.9[c]	(4)
	UDP[βS]	7.5 ± 0.3[b]	(6)

The effect of various nucleotides on β-glucuronidase release was measured. Cells were incubated with CB (5 μg/ml) for 5 min prior to the addition of nucleotides (100 μM each) or solvent (control). Data shown represent the means ± SEM of four to seven experiments carried out with different preparations of HL-60 cells. Figures in parentheses indicate the number of experiments performed. Statistical significance of the effectiveness of nucleotides was assessed using the Wilcoxon test. [a] $p < 0.01$, [b] $p < 0.05$, [c] not significant.

Table 2. Effectiveness of pertussis toxin to ADP-ribosylate G-proteins in intact Bt_2cAMP-differentiated HL-60 cells

Source of membranes	[^{32}P]ADP-ribosylation of 40–41 kDa proteins in HL-60 membranes	
	– pertussis toxin	+ pertussis toxin
	incorporated radioactivity (cpm)	
control cells	17	1305
pertussis toxin-treated cells	20	49

Bt_2cAMP-differentiated HL-60 cells were treated with pertussis toxin (500 ng/ml) or its vehicle (control) for 24 h. Thereafter, membranes from these cells were prepared and pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of 40–41 kDa proteins in HL-60 membranes was assessed using [^{32}P]NAD as substrate. Equal amounts of protein loaded on the gel are the basis for comparison. Data shown are the means of assay duplicates; these varied by less than 15%. Similar results were obtained in two experiments.

cytosolic Ca^{2+} were inhibited only by about 30–35 % and unlike O_2^- formation (22) there was no differential pertussis toxin-sensitivity between purine and pyrimidine nucleotides. A qualitatively similar pattern of pertussis toxin-sensitivity on cytosolic Ca^{2+} as with fMet-Leu-Phe and nucleotides at submaximally effective concentrations was also obtained with agonists at higher concentrations. However, the inhibitory effects of pertussis toxin were smaller (data not shown). Similar observations were made for ATP-induced Ca^{2+} mobilization in undifferentiated and Bt_2cAMP-differentiated HL-60 cells (23).

With regard to exocytosis, pertussis toxin almost completely abolished the stimulatory effect of fMet-Leu-Phe and reduced the ones of ATP[γS] and UTP by about 40 % and 60 %, respectively (Table 4). When Bt_2cAMP-

Table 3. Effect of pertussis toxin to cytosolic Ca^{2+} in Bt_2cAMP-differentiated HL-60 cells

Stimulus	treatment	
	none	pertussis toxin
	peak cytosolic Ca^{2+} (nM)	
none	109 ± 10	112 ± 5[b]
fMet-Leu-Phe (1 nM)	265 ± 9	133 ± 5[a]
ATP[γS] (100 nM)	236 ± 6	198 ± 4[a]
UTP (10 nM)	229 ± 10	186 ± 7[a]

Bt_2cAMP-differentiated HL-60 cells were treated with pertussis toxin (500 ng/ml) or its vehicle (control) for 24 h. Thereafter, cells were harvested, loaded with Fura-2/AM and cytosolic Ca^{2+} was measured. Cells were incubated for 3 min prior to the addition stimuli or solvent (control). Data shown are the means ± SEM of assay quadruplicates. Similar results were obtained with two different preparations of HL-60 cells. The effect of pertussis toxin on cytosolic Ca^{2+} was assessed statistically using the Wilcoxon test. [a] $p < 0.05$, [b] not significant.

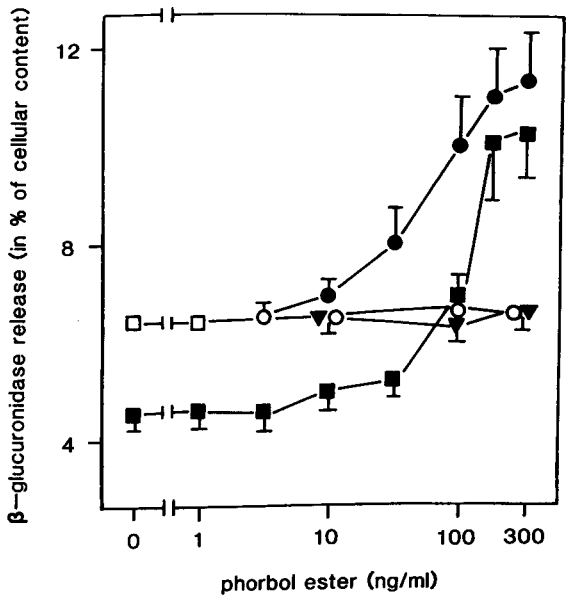

Figure 4. Activation of β-glucuronidase release by phorbol esters in Bt_2cAMP-differentiated and undifferentiated HL-60 cells. The effect of various concentrations of PMA and PDD on the release of β-glucuronidase was studied. HL-60 cells were treated with CB (5 μg/ml) or solvent (control) for 5 min prior to the addition of stimuli. PMA, (■); PMA plus CB, (●, ○); PDD plus CB (▼). Closed symbols denote the experiments carried out with Bt_2cAMP-differentiated HL-60 cells, open circles denote the experiments carried out with undifferentiated HL-60 cells. The open squares indicate that phorbol esters at the indicated concentrations did not activate exocytosis. Data shown represent the means ± SEM of seven to eight experiments carried out with different preparations of HL-60 cells.

differentiated HL-60 cells were treated with pertussis toxin (100 ng/ml) for 4 h, i.e. under less effective conditions, the effects of fMet-Leu-Phe and UTP on β-glucuronidase release were reduced by about 80 % and 50 %, respectively, whereas exocytosis induced by ATP[γS] was not inhibited by the toxin treatment (data not shown). Finally, pertussis toxin did not affect basal and PMA-induced β-glucuronidase release (see Table 4).

Concentration response functions for exocytosis by PMA and the inactive phorbol ester, PDD (16), in HL-60 cells are shown in Figure 4. In the absence of CB, PMA induced β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells with an EC_{50} of 115 ng/ml and a maximum at 300 ng/ml. CB decreased the EC_{50} for PMA to 65 ng/ml but did not significantly enhance β-glucuronidase release by PMA at a maximal stimulatory concentration. PDD did not stimulate exocytosis in Bt_2cAMP-differentiated HL-60 cells, and PMA did not induce β-glucuronidase release in undifferentiated HL-60 cells.

The interaction of ATP[γS], fMet-Leu-Phe, and PMA on exocytosis and O_2^- formation in Bt_2cAMP-differentiated HL-60 cells was studied (Table

Table 4. Effect of pertussis toxin on β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells

Stimulus	treatment	
	none	pertussis toxin
	β-glucuronidase release (in % of cellular content)	
none	4.8 ± 0.2	4.5 ± 0.2[b]
fMet-Leu-Phe (1 μM)	22.5 ± 0.6	5.2 ± 0.4[a]
ATP[γS] (100 μM)	12.1 ± 0.3	8.9 ± 0.4[a]
UTP (100 μM)	11.9 ± 0.3	7.6 ± 0.3[a]
PMA (300 ng/ml)	10.1 ± 0.2	10.3 ± 0.2[b]

Bt_2cAMP-differentiated HL-60 cells were treated with pertussis toxin (500 ng/ml) or its vehicle (control) for 24 h. Thereafter, cells were harvested and assayed for β-glucuronidase release. Cells were incubated for 5 min in the presence of CB (5 μg/ml) prior to the addition stimuli or solvent (control). Data shown are the means ± SEM of assay quadruplicates. Similar results were obtained with two different preparations of HL-60 cells. The effect of pertussis toxin on β-glucuronidase release was assessed statistically using the Wilcoxon test. [a] $p < 0.05$, [b] not significant.

5). In the absence of CB, ATP[γS] plus fMet-Leu-Phe did not synergistically stimulate exocytosis. In the presence of CB, fMet-Leu-Phe at a non-stimulatory concentration (1 nM) enhanced ATP[γS]-induced exocytosis up to 14.8 ± 0.7 % ($p <= 0.02$, $n = 7$), but in the presence of fMet-Leu-Phe at 1 μM no synergism between ATP[γS] and the chemotactic peptide was apparent. PMA did not significantly affect ATP[γS]- or fMet-Leu-Phe-induced β-glucuronidase release in the absence or presence of CB.

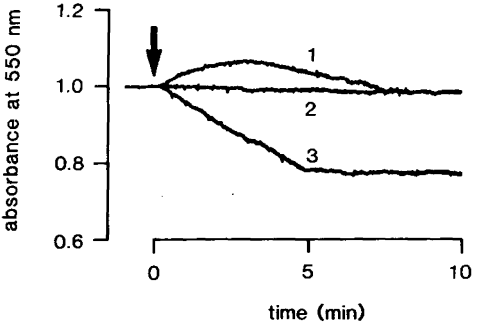

Figure 5. Induction of aggregation of Bt_2cAMP-differentiated HL-60 cells by ATP[γS] and fMet-Leu-Phe. Cells were incubated with CB (5 μg/ml) for 5 min prior to the addition of stimuli. The arrow indicates the addition of stimuli to stirred suspensions of HL-60 cells. Superimposed original registrations from one representative experiment performed in duplicate are shown. The variation of responses within one experiment was less than 10 %. Similar results were obtained in four experiments carried out with different preparations of HL-60 cells. Trace 1, ATP[γS] (100 μM); trace 2, solvent (control); trace 3, fMet-Leu-Phe (1 μM).

Table 5. Interactions of PMA, ATP[γS] and fMet-Leu-Phe on β-glucuronidase release and O_2^--formation in Bt_2cAMP-differentiated HL-60 cells

Stimulus	β-glucuronidase release (in % of cellular content)		O_2^--formation (nmol/10^7 cells/min)	
	− CB	+ CB	− CB	+ CB
none	4.1 ± 0.3	5.2 ± 0.3	0	0.9 ± 0.4
PMA	9.3 ± 0.7	9.9 ± 0.7	3.9 ± 0.4	4.1 ± 0.8
ATP[γS]	7.3 ± 0.6	10.8 ± 1.3	1.6 ± 0.3	4.7 ± 1.2
fMet-Leu-Phe	10.0 ± 0.7	20.2 ± 1.5	13.0 ± 2.6	52.4 ± 10.7
PMA + ATP[γS]	9.3 ± 1.3[b]	12.2 ± 1.1[b]	11.5 ± 1.9[a]	11.6 ± 2.4[a]
PMA + fMet-Leu-Phe	9.5 ± 0.9[b]	18.7 ± 1.3[b]	24.5 ± 2.2[a]	46.6 ± 5.6[b]
ATP[γS] + fMet-Leu-Phe	9.3 ± 1.0[b]	20.4 ± 1.4[b]	25.3 ± 1.2[a]	48.5 ± 4.5[b]

The effects of PMA, ATP[γS] and fMet-Leu-Phe or combinations of these stimuli on β-glucuronidase release and O_2^- formation were studied in the absence or presence of CB. Cells were incubated in the absence or presence of CB (5 μg/ml) for 5 min prior to the addition of stimuli. The concentrations of stimuli were as follows. PMA, 300 ng/ml; ATP[γS], 100 μM; fMet-Leu-Phe, 1 μM. Data shown are the means ± SEM of seven experiments carried out with different preparations of HL-60 cells. The effectiveness of combinations of stimuli on β-glucuronidase release and O_2^- formation was assessed statistically using the Wilcoxon test. [a] $p < 0.01$, [b] not significant.

ATP[γS] was a less effective activator of O_2^- formation than fMet-Leu-Phe, and the effects of both agonists but not the one of PMA were potentiated by CB. In contrast to exocytosis, fMet-Leu-Phe and ATP[γS] synergistically activated O_2^- formation in the absence of CB, but in the presence of CB no synergism was evident. Similar to exocytosis, CB per se weakly activated O_2^- formation. In the absence of CB, PMA synergistically enhanced O_2^- formation induced by ATP[γS] or fMet-Leu-Phe. In the presence of CB, ATP[γS] and PMA also synergistically activated O_2^- formation, but the extent of synergism was not greater than in the absence of CB. PMA and fMet-Leu-Phe did not synergistically activate O_2^- formation in the presence of CB.

Finally, we studied the effects of ATP[γS] and fMet-Leu-Phe on aggregation of Bt_2cAMP-differentiated HL-60 cells. Representative aggregation traces of HL-60 cells are shown in Figure 5. In the presence of CB, fMet-Leu-Phe induced monophasic and irreversible aggregation of HL-60 cells, and ATP[γS] caused a transient increase in absorbance, a phenomenon which correlates to a shape change (39). However, ATP[γS] did not induce aggregation of HL-60 cells.

Discussion

We studied the effects of extracellular nucleotides on Ca^{2+} mobilization and exocytosis in HL-60 cells. The kinetics of exocytosis induced by fMet-Leu-Phe and extracellular nucleotides are similar, and CB preferentially potentiates exocytosis induced by fMet-Leu-Phe and nucleotides at high concentrations (see Fig. 2 and 3). These data indicate that nucleotides and fMet-Leu-Phe activate exocytosis through similar mechanisms. In addition, the stimulatory effects of nucleotides on exocytosis show a structure/activity relationship which is similar to the one for activation of O_2^- formation in HL-60 cells (see Table 1) (22). Interestingly, the stimulatory effects of ATP[γS] and UTP on cytosolic Ca^{2+} and exocytosis are less sensitive to inhibition by pertussis toxin than those of fMet-Leu-Phe (see Table 3 and 4). Partial or complete insensitivity to inhibition by pertussis toxin of adenine nucleotide-induced activation of phospholipase C, arachidonic acid release, exocytosis and O_2^- formation in human myeloid cells has been repeatedly observed (12, 13, 22–24), but partial pertussis toxin-insensitivity of the effects of pyrimidine nucleotides on cytosolic Ca^{2+} and exocytosis has not yet been reported (see Table 3 and 4). Possibly, ADP-ribosylation of G-proteins by pertussis toxin in human myeloid cells impairs the interaction of nucleotide receptors with the known pertussis toxin-sensitive G-proteins to a lesser degree than the one of formyl peptide receptors with G-proteins. Alternatively, the small percentage of the G-proteins not ADP-ribosylated by pertussis toxin (less than 2.5 %, see Table 2) may be sufficient to effectively transduce signals from nucleotide recep-

tors to intracellular effector systems as G-proteins are highly abundant in HL-60 cells (38). Moreover, pertussis toxin-insensitive signal transduction pathways involving low molecular mass GTP-binding proteins (40) or ecto-protein kinases (41) may play a role in the activation of HL-60 cells by extracellular nucleoside triphosphates. Considering these possibilities it is not surprising that the effects of ATP[γS] which is more resistant to cleavage by phosphatases than ATP on β-glucuronidase release are less sensitive to inhibition by pertussis toxin than those of ATP (12). Interestingly, DUBYAK and coworkers (23) reported on partial pertussis toxin-sensitivity of the effects of ATP on cytosolic Ca^{2+} and inositol polyphosphate accumulation in HL-60 cells. These findings are in agreement with our finding that pertussis toxin only partially inhibits ATP[γS]-induced increase in cytosolic Ca^{2+} (see Table 3). Other differences between the results obtained by various laboratories are discussed below.

Recently, we suggested that purine and pyrimidine nucleotides activate neutrophils and HL-60 cells through different receptors (6, 21, 22). Our present finding that UDP[βS] but not the corresponding adenine nucleotide, ADP[βS], induces β-glucuronidase release in HL-60 cells supports this concept (see Table 1). The same holds true for the differential effects of pertussis toxin on purine- and pyrimidine nucleotide-induced exocytosis (see Table 4 and text in Results). Interestingly, UTP is about one order of magnitude more potent than ATP[γS] to increase cytosolic Ca^{2+} in undifferentiated and Bt_2cAMP-differentiated HL-60 cells (see Fig. 1 and Table 3). With respect to O_2^- formation in Bt_2cAMP-differentiated HL-60 cells, both nucleotides are similarly potent (22). Differences concerning the potency and/or effectiveness of purine and pyrimidine nucleotides to activate various cell functions were also observed in human neutrophils (6). We did not analyze in detail the structure/activity relationship of purine and pyrimidine nucleotides with respect to Ca^{2+} mobilization, but this may be an interesting task for future studies. Pyrimidine nucleotide-induced O_2^- formation is more sensitive to inhibition by pertussis toxin than the one induced by purine nucleotides (22). With respect to aggregation of human neutrophils, no dissociation between the effects of purine and pyrimidine nucleotides is apparent (6). The fact that the effects of purine and pyrimidine nucleotides on cytosolic Ca^{2+} show no substantial differences in their pertussis toxin-sensitivity (see Table 3) does not necessarily argue against the existence of distinct purino- and pyrimidinoceptors. It is well-known that the effects of a given agonist on different cell functions may show differential pertussis toxin-sensitivity (42, 43).

The failure of fMet-Leu-Phe to increase cytosolic Ca^{2+} and to induce β-glucuronidase release in undifferentiated HL-60 cells (see Fig. 1 and 3) is readily explained by the fact that HL-60 cells express formyl peptide receptors only in the differentiated state (27). However, undifferentiated and Bt_2cAMP-differentiated HL-60 cells contain similar amounts of β-glucuronidase (see text in Results) and undifferentiated HL-60 cells

express nucleotide receptors which functionally couple to phospholipase C via G-proteins resulting in an increase of cytosolic Ca^{2+} (see Fig. 1) (23). In addition, these cells possess substantial amounts of protein kinase C (44). Nonetheless, neither extracellular nucleotides nor PMA induce exocytosis in undifferentiated HL-60 cells (see Fig. 3 and 4). These data suggest that components distinct from nucleotide receptors, G-proteins, phospholipase C, protein kinase C, the Ca^{2+}-mobilizing system and β-glucuronidase are missing in undifferentiated HL-60 cells. Such a component may be the fusion protein, lipocortin III, which was recently purified from human neutrophil cytosol (45, 46). These components appear to be involved in activation of exocytosis by receptor agonists and phorbol esters and are expressed during myeloid differentiation. Interestingly, myeloid differentiation of HL-60 cells is associated with the expression of specific cytosolic activation factors for NADPH oxidase which are different from the above-mentioned signal transduction components (47, 48). It should also be noted that purine and pyrimidine nucleotides are more effective than fMet-Leu-Phe to increase cytosolic Ca^{2+} in Bt_2cAMP-differentiated HL-60 cells (see Fig. 1 and text in Results). In contrast, extracellular nucleotides are substantially less effective than fMet-Leu-Phe to induce exocytosis (see Fig. 2 and 3 and Tables 4 and 5), O_2^- formation (see Table 5) and aggregation (see Fig. 5). These data suggest that an increase in cytosolic Ca^{2+} per se is not a sufficient signal to activate the above cell functions. Dissociations between Ca^{2+} mobilization and activation of O_2^- formation have been repeatedly observed (1).

There are not only differences between the results of our present study and that of the one by COCKCROFT and STUTCHFIELD (12) concerning the effects of pertussis toxin on exocytosis (see Table 4 and text in Results), but also with regard to other parameters. In contrast to our results, STUTCHFIELD and COCKCROFT reported that ATP and UTP are similarly effective activators of β-glucuronidase in undifferentiated and Bt_2cAMP-differentiated HL-60 cells (compare Fig. 2 of this study with Table 1 in Ref. 49). In addition, COCKCROFT and STUTCHFIELD find that unlike in this study ATP is substantially more effective than ATP[γS] to induce exocytosis (compare Table 1 of this study with Table 1 in Ref. 12 and 49). Furthermore, COCKCROFT and STUTCHFIELD (13) reported on a potentiating effect of PMA on fMet-Leu-Phe- and ATP-induced exocytosis in Bt_2cAMP-differentiated HL-60 cells, whereas we did not find any effect of the phorbol ester on chemotactic peptide- and nucleotide-induced β-glucuronidase release (see Table 5). Basically, the methods applied by us (see Materials and Methods) and by the above authors (12, 13, 49) are very similar. Thus, it is possible that differences in the lineage of HL-60 cells used in the two laboratories account, at least in part, for the differences observed as strains of HL-60 cells from different laboratories are known to be heterogenous with respect to various properties (50). Finally, there are also discrepancies concerning activation of exocytosis in human neutrophils by extracellular

nucleotides between various laboratories. COCKCROFT and STUTCHFIELD (12) reported that ATP induces azurophilic granule release from human neutrophils, whereas BALAZOVICH and BOXER (51) and we (6) found that ATP per se does not induce β-glucuronidase release. However, ATP potentiates fMet-Leu-Phe-induced β-glucuronidase release (6).

Synergistic activation of exocytosis but not of O_2^- formation by extracellular nucleotides and fMet-Leu-Phe obligatorily depends on the presence of CB (see text in Results and Table 5). In the absence of CB, PMA potentiates O_2^- formation induced by ATP[γS] or fMet-Leu-Phe, but in the presence of CB, this synergism is much less prominent or is missing. In contrast, PMA does not affect ATP[γS]- or fMet-Leu-Phe-induced exocytosis in the absence or presence of CB. These data suggest that exocytosis and O_2^- formation are activated in parallel by receptor agonists but are independently regulated, at least with respect to the role of CB, protein kinase C, and receptor synergism.

In human neutrophils but not in Bt_2cAMP-differentiated HL-60 cells, activation of β-glucuronidase release by fMet-Leu-Phe obligatorily depends on the presence of CB (see Fig. 3) (3, 5). This finding may be relevant for further studies on the regulation of azurophilic granule release, as the molecular mode of action of CB on exocytosis is only poorly understood and as CB possesses no known physiological equivalent (3, 5, 9, 52).

Unlike in human neutrophils, a significant synergism between fMet-Leu-Phe and ATP[γS] on exocytosis in HL-60 cells is evident only in the presence of the chemotactic peptide at a non-stimulatory concentration (1 nM), and fMet-Leu-Phe at a maximal stimulatory concentration activates exocytosis in HL-60 cells to an extent which cannot further be increased by ATP[γS] (see text in Results and Table 5) (6). Apparently, the pool of β-glucuronidase releasable by receptor agonists in HL-60 cells is much smaller than the one in human neutrophils, and hence, the stimulatory effects of fMet-Leu-Phe and nucleotides are correspondingly smaller (3, 5, 6, 12, 13).

With respect to aggregation, there are differences between human neutrophils and HL-60 cells, too. WHITIN and COHEN (53) reported that dimethylformamide-differentiated HL-60 cells showed a much smaller aggregation response than human neutrophils upon exposure to PMA. However, these authors did not study the effects of receptor agonists such as fMet-Leu-Phe on aggregation (53). We have recently shown that extracellular nucleotides induce irreversible and monophasic aggregation in human neutrophils in the presence of CB (6). In Bt_2cAMP-differentiated HL-60 cells, fMet-Leu-Phe induces monophasic aggregation in the presence of CB (see Fig. 5). In contrast, ATP[γS] induces only a shape change. These findings are in agreement with the fact that extracellular nucleotides are less effective activators of exocytosis and O_2^- formation than chemotactic peptides in these cells (see Figs. 2 and 3) (22). In comparison to human neutrophils, fMet-Leu-Phe induces only a modest aggregation response in

Bt_2cAMP-differentiated HL-60 cells (compare Fig. 5 of this study with Fig. 5 and 6 in Ref. 6) suggesting that HL-60 cells are devoid of certain plasma membrane components involved in phagocyte aggregation.

In conclusion, HL-60 cells may be a useful model system in addition to human neutrophils to study the effects of cytochalasins on exocytosis and the differentiation-dependency of β-glucuronidase release.

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