Escherichia coli Hemolysin Is a Potent Inductor of Phosphoinositide Hydrolysis and Related Metabolic Responses in Human Neutrophils

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Abstract

***Escherichia coli* hemolysin (Hly) is a proteinaceous pore-forming exotoxin that probably represents a significant virulence factor in *E. coli* infections. We investigated its influence on human polymorphonuclear neutrophils (PMN), previously identified as highly susceptible targets. Hly provoked rapid secretion of elastase and myeloperoxidase, generation of superoxide, and synthesis of platelet-activating factor (PAF) and lyso-PAF. Concomitantly, marked phosphatidylinositol (PtdIns) hydrolysis with sequential appearance of the inositolphosphates, inositol triphosphate, diphosphate, and monophosphate, respectively, and formation of diacylglycerol, occurred. The metabolic responses displayed distinct bell-shaped dose dependencies, with maximum events noted at low toxin concentrations of 0.1–0.5 hemolytic units per milliliter. PtdIns hydrolysis and metabolic responses elicited by Hly exceeded those evoked by optimal concentrations of formylmethionyl-leucyl phenylalanine, PAF, leukotriene B_4, A23187, or staphylococcal α-toxin. The toxin-induced effects were sensitive toward modulators of PMN stimulus transmission pathways (pertussis toxin, the kinase C inhibitor H7, and phorbol myristate acetate "priming"). We conclude that the marked capacity of low doses of Hly to elicit degranulation, respiratory burst, and lipid mediator generation in human PMN probably envolves signal transduction via PtdIns hydrolysis. (*J. Clin. Invest.* 1991. 88:1531–1539.) Key words: *Escherichia coli* hemolysin • neutrophil activation • phosphoinositide**

Introduction

Certain strains of *Escherichia coli* produce a proteinaceous exotoxin, designated hemolysin (Hly),[1] which was originally characterized by its lytic effect on erythrocytes (1–4). Secretion of active Hly requires the expression of four genes (hlyA, hlyB, hlyC, hlyD), constituting the hly operon (5, 6). The hlyA gene encodes the 110-kD hemolysin protein, which is activated by the hlyC gene product. Secretion of HlyA occurs via a transport mechanism involving the HlyB and HlyD proteins. Approximately 50% of *E. coli* isolates causing extraintestinal infections in humans produce Hly; this agent represents one of the most prevalent bacterial exotoxins encountered in humans (1, 3, 4, 7–9). Its pathogenetic relevance has been established in animal models with the use of genetically engineered bacterial strains (1, 7, 10). An analogous role in human infections has been inferred from the high association of hemolysin production with disease, including pyelonephritis and septicemia (8, 9).

The primary structure of Hly has been deduced from the nucleotide sequence (11). Several cytotoxins elaborated by other gram-negative rods including *Morganella morganii, Proteus spp.*, and *Pasteurella haemolytica* exhibit structural and functional similarities to Hly, indicating that these proteins constitute a new family of pore-forming toxins (12–16). The membrane permeabilizing capacities of Hly have been demonstrated in erythrocytes, neutrophils (PMN), monocytes, endothelial cells, and artificial lipid bilayers (2, 17–22). A high-affinity calcium binding domain, required for toxin membrane insertion, has been disclosed (22–24).

Although basically endowed with the capacity to permeabilize a large variety of target cells, Hly exerts a particularly potent action on granulocytes (20, 25, 26). Release of elastase and other granule constituents and loss of phagocytic killing capacity were observed upon exposure of PMN to Hly concentrations far below those causing erythrocyte lysis. In a recent study, we showed that subhemolytic doses of the toxin induce marked leukotriene (LT) generation in human neutrophils (27). In the present investigation, we sought to determine whether low-dose effects of Hly on neutrophils might be related to phosphatidylinositol (PtdIns) hydrolysis, which serves as major signal transduction pathway in PMN challenged with ligands such as lipid (leukotriene B_4 [LTB_4] and platelet-activating factor [PAF]) and peptide chemoattractants (formylmethionyl-leucyl-phenylalanine [FMLP]) (28–32). Inositol phosphate and diacylglycerol (DAG) formation was found to parallel degranulation, respiratory burst, and lipid mediator generation in Hly-treated PMN. Indeed, when compared to FMLP, PAF, LTB_4, and A23187, Hly emerged as the most potent inductor of PtdIns hydrolysis hitherto described.

Methods

Materials. FMLP, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), superoxide dismutase, cytochrome *c* type IV, diethylenetriaminepentaacetic acid, Hepes, phorbol myristate acetate (PMA), and phospholipids were obtained from Sigma Chemical Co. (Deisenhofen, FRG); octyl-β-D-glucoside from Behring Diagnostics (Frankfurt, FRG). LT B_4 was purchased from Paesel AG (Frankfurt, FRG), purified by high-pressure liquid chromatography (HPLC), and quantified before use as described (33, 34). The PAF receptor antagonist

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1. *Abbreviations used in this paper:* DAG, diacylglycerol; GPC, glycerophosphorylcholine; Hly, *Escherichia coli* hemolysin; HU, hemolytic unit(s); IP_1, IP_2, and IP_3, inositol monophosphate, diphosphate, and triphosphate, respectively; LDH, lactate dehydrogenase; PAF, platelet-activating factor; PT, pertussis toxin; PtdIns, phosphatidylinositols.

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WEB 2086 was generously supplied by Boehringer (Ingelheim, FRG), and the 5-lipoxygenase inhibitor L-663-536 was a kind gift from Dr. C. Rouzer; Merck Frosst (Canada). Highly purified *Bordetella* pertussis toxin (PT) as well as its inactive B oligomer and the kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Diglyceride kinase was obtained from Lipidex, Inc. (Middleton, WI). [γ-^{32}P]ATP (sp act 4,500 Ci/mmol) was from ICN Radiochemicals (Irvine CA), and S-2484, a chromogenic substrate for granulocyte elastase, from Kabi-Vitrum (Stockholm, Sweden). RPMI 1640 medium, medium 199, Hanks' balanced salt solution, and fetal calf serum were from Gibco Laboratories (Grand Island, NY), and Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). Tritiated inositol phosphates, [^{3}H]PAF, [^{3}H]lyso-PAF, [^{3}H]phosphatidylcholine, [^{14}C]phosphatidic acid, and [^{3}H]serotonin were obtained from Amersham Corp. (Dreieich, FRG). Myo-[2-^{3}H]inositol was purchased from New England Nuclear (Boston, MA). Chromatographic supplies included silica gel 5-μm column packing (Machery Nagel, Düren, FRG), HPLC-grade solvents, distilled in glass (Fluka KG, Heidelberg, FRG), and silica gel 60 F_{254} plates (Merck & Co., Darmstadt, FRG). All other biochemicals were obtained from Merck & Co., (Munich, FRG).

Preparation of human granulocytes. Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient (35) to yield a PMN fraction of ~ 97% purity. Before experiments with measurement of granule secretion, superoxide production, PAF, or DAG synthesis, PMN were kept in RPMI 1640 medium with 10% calf serum for 2 h. For analysis of phosphoinositide metabolism, preincubation with myo-[2-^{3}H]inositol was carried out in medium 199 (Gibco Laboratories) with 2% calf serum for 2 h. After cell washing, PT (1.5 μg/ml), its B oligomer (1.3 μg/ml), or a corresponding buffer volume were added, followed by another 2-h incubation period. Immediately before stimulus application, the cells were washed twice and suspended in Hanks' Hepes buffer. Cell viability, as assessed by trypan blue exclusion, ranged above 96% and lactate dehydrogenase (LDH) release was consistently below 3% at this time.

Preparation of E. coli hemolysin. The hemolysin was prepared as described (2, 36). The endotoxin content of the preparation was reduced to ~ 3 ng of lipopolysaccharide (LPS)/μg protein. The hemolytic titer was assessed directly before use and expressed in hemolytic units (HU)/ml; 1 HU/ml corresponded to $\approx$ 100 ng protein/ml. At the optimum Hly concentration used (0.1 HU/ml), endotoxin levels ranged between 25 and 35 pg/ml (assessed with *Limulus* lysate assay using chromogenic substrate, according to Friberger [37]).

Superoxide anion generation. PMN O_2^- generation was measured as superoxide dismutase inhibitable cytochrome *c* reduction as described (38). Duplicate reaction mixtures containing 5×10^6/ml PMN and 75 μM ferricytochrome *c* were incubated for the indicated time intervals at 37°C in the presence or absence of 10 μg/ml superoxide dismutase. Incubations were terminated by centrifugation at 4°C for 5 min at 1,200 *g*. O_2^- production is expressed as nanomoles of cytochrome reduced, using an extinction coefficient of 21 mM^{-1} cm^{-1} at 549 nm.

Release of granule constituents and LDH. Elastase, myeloperoxidase, and LDH release were taken as markers for PMN degranulation and cell lysis, respectively, and were measured according to standard procedures (39, 40).

Measurement of PAF precursor, PAF, and lyso-PAF in prelabeled cells. Metabolism of PAF was determined by stimulating neutrophils containing 1-*O*-[^{3}H]alkyl-2-acyl-glycerophosphorylcholine (GPC), obtained by prelabeling the cells with 1-*O*-[^{3}H]alkyl-2-lyso-GPC (56 Ci/mmol), using a modification of the method described by Chilton et al. (41). Briefly, PMN (1×10^8 cells/ml) were incubated for 2 h with 100 μCi/ml [^{3}H]lyso-PAF in RPMI (10% calf serum) in a shaking water bath (37°C). During this period, 95% of the radiolabel became cell associated with 85% of the label extractable as 1-*O*-[^{3}H]alkyl-2-acyl-GPC. Before stimulation, cells were washed twice and resuspended in Hanks' balanced salt solution (1×10^7 cells/ml). Reactions were stopped by addition of three volumes of chloroform/methanol (2:1, vol/vol) and extracted according to Bligh and Dyer (42). The entire lipid extract was evaporated to dryness, redissolved in 60 μl of the mobile phase, and subjected to straight-phase HPLC separation. The column (25 × 0.46 cm) was packed with silica gel 5-μm particles and eluted isocratically with acetonitrile/methanol/phosphoric acid (95.5:3.5:1) at a flow rate of 1.8 ml/min. Eluate fractions corresponding to appropriate standard retention times were collected and assayed for radioactivity by liquid scintillation counting. In selected experiments, the elution of radiolabel was monitored using a radiochromatogram imaging system (5LS Raytest). In additional studies, PAF synthesis was quantified by incorporation of labeled acetate. 10^7 PMN were stimulated in the presence of 50 μCi [^{3}H]acetate (7.75 Ci/mmol) in a total volume of 1 ml according to Tessner et al. (43). Extraction and HPLC processing were performed as indicated above. Up to 0.6% of the exogenously offered [^{3}H]acetate was incorporated into total lipid fraction during this procedure.

Post-HPLC PAF bioassay. PAF production in nonlabeled PMN was quantified by induction of [^{3}H]serotonin release from prelabeled rabbit platelets. After termination of PMN incubation, the total cellular and extracellular PAF content was lipid-extracted and subjected to HPLC separation as indicated above. Eluate fractions were collected at the appropriate PAF retention time, again lipid-extracted for removal of phosphoric acid present in the mobile phase, evaporated to dryness, and redissolved in 50 μl of assay buffer for induction of platelet serotonin release. Preparation of platelets and the protocol of the bioassay were essentially as published by Pinkard et al. (44). [^{3}H]serotonin-labeled platelets (250,000 cells/μl in a total volume of 0.5 ml) were incubated for 60 s. A 200-μl aliquot then was rapidly removed, added to a chilled tube containing 20 μl of 1.5 mM formaldehyde, and centrifuged at 12,000 *g* for 2 min. The serotonin secretion into the platelet supernatant was determined by liquid scintillation counting and related to that released from the same volume of platelet suspension after cell lysis with Triton X-100 (final concentration 0.83% wt/vol). Known quantities of PAF were used to establish a calibration curve for the bioassay. Aliquots of each sample were used to ascertain the specificity of platelet secretion by the inhibitory effect of the PAF receptor antagonist WEB 2086 (1 μM).

Phosphoinositide metabolism. The PtdIns turnover of stimulated neutrophils was investigated by measuring the accumulation of inositol phosphates according to Berridge et al. (45). For prelabeling of cellular phospholipid pools, PMN were resuspended to 1×10^8 cells/ml with medium 199 containing 2% fetal calf serum plus 40 mM Hepes buffer, pH 7.4. Myo-[^{3}H]inositol (50 μCi/ml) was added, and cells were incubated at 37°C for 2 h on a shaking water bath. Before experimental use, cells were washed twice and resuspended in Hanks' balanced salt solution containing 20 mM Hepes and 10 mM LiCl (1×10^7 PMN/ml). At different times after stimulus application, samples were quenched with trichloroacetic acid (final concentration 7.5%), kept on ice for 15 min, and extracted four times with diethylether. The aqueous phase was neutralized with sodium tetraborate to pH 8.0, and processed to separate inositol phosphates on Dowex anion exchange columns as described by Berridge et al. (45). The column was eluted sequentially with water (for free [^{3}H]inositol); 5 mM Na-tetraborate/60 mM Na-formate (for glycerophospho-[^{3}H]inositol); 0.1 M formic acid/0.2 M ammonium formate (for [^{3}H]inositol monophosphate [IP_1]; 0.1 M formic acid/0.5 M ammonium formate (for [^{3}H]inositol diphosphate [IP_2]); 0.1 M formic acid/1.0 M ammonium formate (for [^{3}H]inositol triphosphate [IP_3]; and samples were processed for liquid scintillation counting.

DAG assay. 0.8-ml samples of PMN suspension (1×10^7 cells/ml) were mixed with 3 ml of chloroform/methanol with subsequent splitting of the monophase by addition of chloroform and 1 M NaCl (1 ml each) according to Bligh and Dyer (42). The chloroform phase was removed, kept at −20°C to minimize acyl group migration and DAG was quantified within 24 h by enzymatic conversion to [^{32}P]phosphatidic as described (46). Briefly, an aliquot of the chloroform phase was evaporated under nitrogen, and the lipid film solubilized in 20 μl of 7.5% octyl-β-D-glucoside, 5 mM cardiolipin, 1 mM diethylenetriaminepentaacetic acid by water bath sonication. The resulting mi-

celles were then reacted with *E. coli sn*-1,2-DAG kinase in the presence of 5 mM [γ-^{32}P]ATP. After subsequent neutral lipid extraction, an aliquot of the lipid phase was subjected to thin-layer chromatography on a Silica gel 60 F_{254} plate and developed with chloroform/methanol/acetic acid (65:15:5, vol/vol/vol). Identification of DAG and its separation from labeled ceramide phosphate was ascertained by autoradiography before liquid scintillation counting of the DAG spot. Samples of sn-1,2-diolein were carried through the same procedure and spotted onto each plate as controls. Thereby, DAG recovery and conversion were ascertained to range consistently above 85%. The amounts of *sn*-1,2-DAG present in the original samples were calculated from the respective phosphatidic acid counts and the specific activity of the adenosine triphosphate (ATP) batch employed.

Results

Incubation of human neutrophils with Hly caused a rapid, dose-dependent secretion of elastase (Fig. 1). This response commenced at toxin doses below 0.1 HU/ml and reached its maximum at 0.5–2.5 HU/ml. At these concentrations, elastase liberation surpassed that elicited by optimum concentrations of established receptor-operated stimuli, the Ca^{++} ionophore

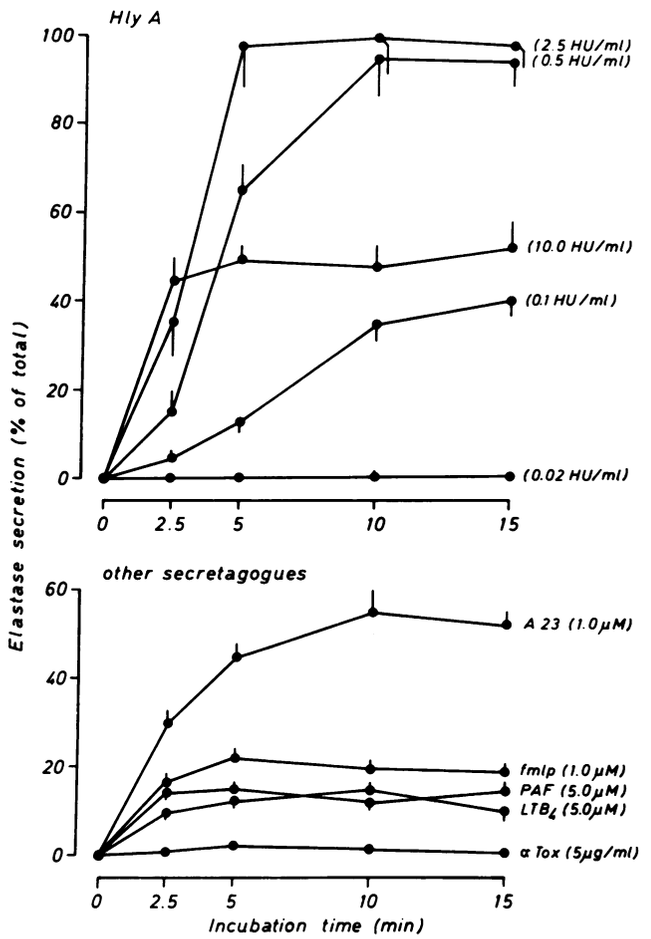

Figure 1. Time course of neutrophil elastase secretion evoked by Hly in comparison with different other secretagogues. 10^7 PMN were incubated with different concentrations of Hly (*top*) or different other secretagogues at optimum concentrations (*bottom*) for various time periods. Elastase release is expressed as percentage of total cellular amount. Means±SEM of five (*top*) and four (*bottom*) independent experiments are given.

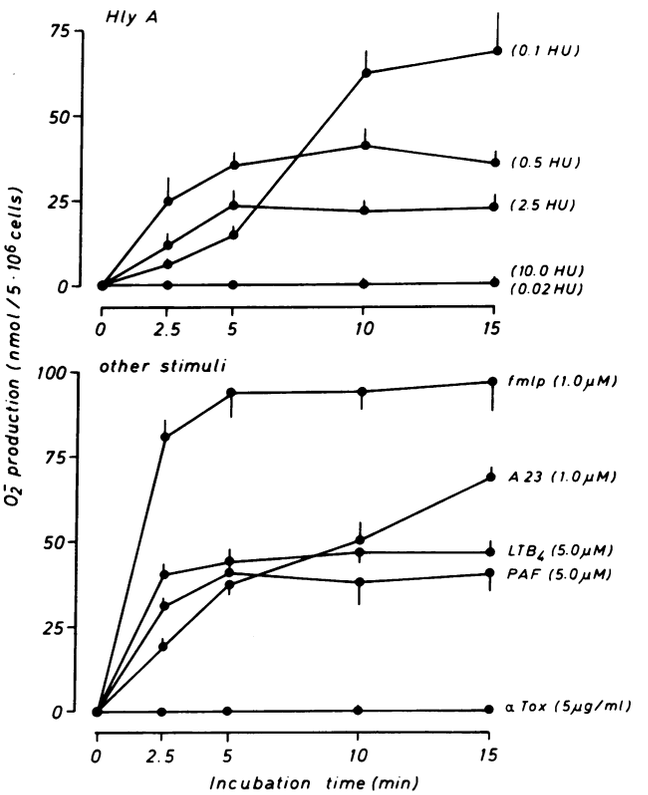

Figure 2. Time course of neutrophil superoxide production evoked by Hly in comparison with different other stimuli. 10^7 PMN were incubated with different concentrations of Hly (*top*) or different other stimuli at optimum concentrations (*bottom*) for various time periods. Means±SEM of five (*top*) and four (*bottom*) independent experiments are given.

A23187 and the pore-forming staphylococcal α-toxin by 2- to > 10-fold. At higher Hly doses (10 HU/ml, Fig. 1), there was a rapid onset but somewhat reduced extent of elastase secretion. In addition, toxin-treated neutrophils released myeloperoxidase with similar dose and time dependencies (data not shown).

Respiratory burst, measured as O_2^- release, was also noted in Hly-treated PMN. This response was maximal at very low Hly doses ($\approx$ 0.1 HU/ml; Fig. 2); at 10 HU/ml, no O_2^- release was detected. The magnitude of the toxin-evoked O_2^- liberation was in the range of that elicited by optimum concentrations of PAF, LTB_4, A23187, and FMLP.

Analysis of liberated and cell-bound PAF by post-HPLC bioassay and [^{3}H]acetate and [^{3}H]lyso-PAF bioincorporation revealed marked formation of this lipid mediator in response to Hly challenge (Figs. 3 and 4). Maximum amounts of PAF were elicited by 0.1–0.5 HU/ml of the toxin. PAF liberation was paralleled by the appearance of lyso-PAF in similar amounts in all experiments.

Neutrophil activation with Hly caused rapid-onset, sustained PtdIns hydrolysis with sequential appearance of the inositol phosphates IP_3 and IP_2 and accumulation of IP_1 (Figs. 5 and 6). Maximum responses were again elicited by 0.1–0.5 HU/ml. Total amounts of liberated inositol phosphates surpassed those evoked by optimum concentrations of the receptor-operated stimuli PAF, FMLP, and LTB_4 and of A23187 and α-toxin by greater than twofold. Correspondingly, marked

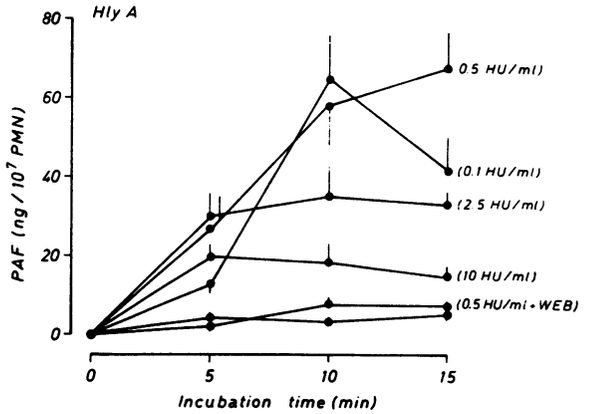

Figure 3. Time course of neutrophil PAF generation in response to Hly challenge. 10^7 PMN were incubated with different concentrations of Hly for various time periods. Secreted and cell-bound PAF was lipid-extracted, purified by HPLC, and quantified by induction of [^{3}H]serotonin release from prelabeled platelets. Specificity of the platelet release reaction was ascertained by use of the PAF antagonist WEB 2086 (indicated for 0.5 HU/ml in the figure). Means±SEM of five (*top*) and four (*bottom*) independent experiments are given.

generation of DAG was detectable in Hly-exposed PMN, exceeding the amount of this second messenger in FMLP-treated cells by about threefold (Fig. 7).

In the concentration range used, Hly incubation evoked only moderate, protracted LDH release as a marker of overt cell lysis (Fig. 8). At ≤ 0.5 HU/ml, only ≈ 3–5% of total PMN LDH was liberated within 15 min of toxin challenge. LDH release amounted to > 15% within the same incubation time in the presence of 10 HU/ml.

In PMN pretreated with PT, Hly-evoked PtdIns hydrolysis and elastase secretion were significantly inhibited (Table I). The PT sensitivity was inversely related to the Hly dose. The extent of suppression effected by PT was slightly lower as compared to FMLP-, PAF-, and LTB_4-stimulated neutrophils. In control experiments, equal amounts of the inactive B oligomer of PT did not inhibit Hly-, FMLP-, or lipid mediator-evoked cellular events. As anticipated, PMN activation elicited by the ionophore was not significantly inhibited by PT.

In the presence of the protein kinase C inhibitor H7, the Hly-evoked oxygen burst was reduced to < 50%, whereas elastase liberation and inositol phosphate accumulation were not significantly affected (Table II). The PAF antagonist

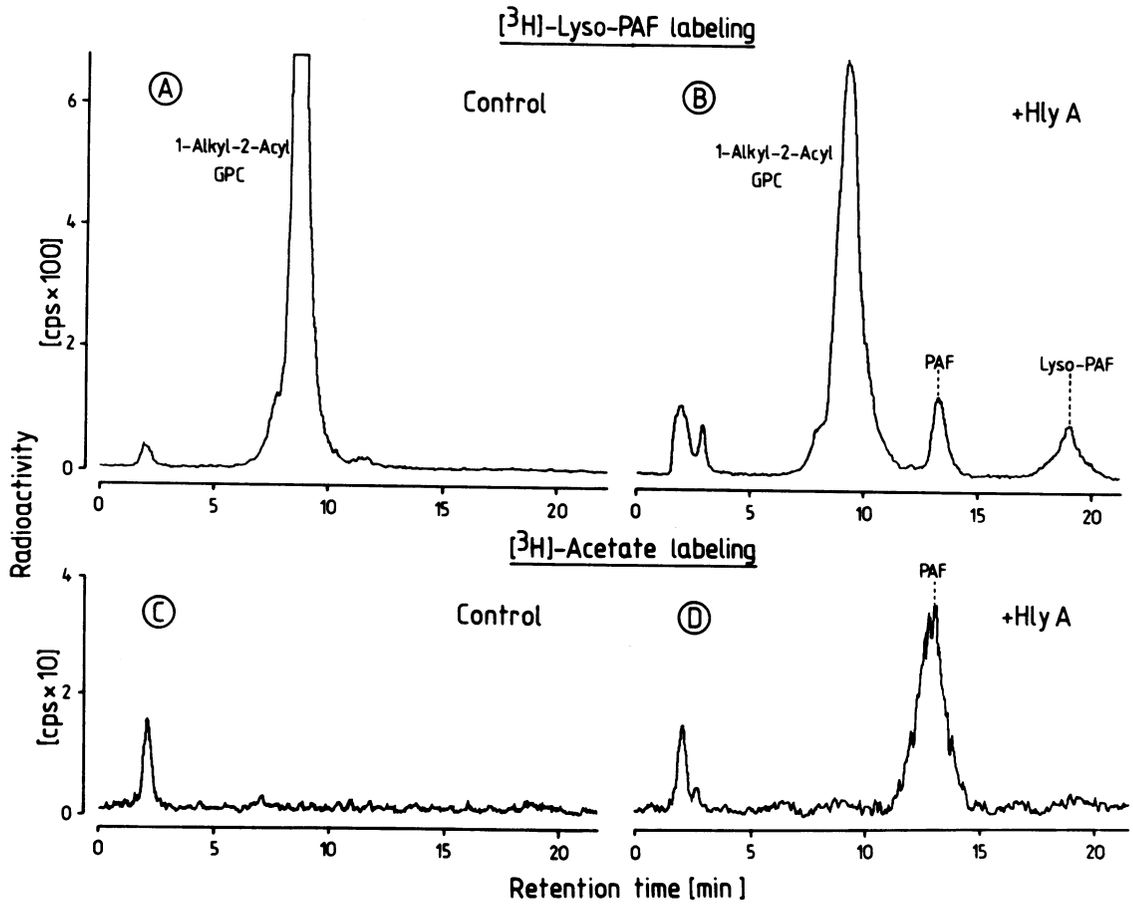

Figure 4. Analysis of PAF generation in Hly-challenged neutrophils by bioincorporation of labeled lyso-PAF and acetate. 10^7 PMN were preincubated with [^{3}H]lyso-PAF (1 h; *A* and *B*) or [^{3}H]acetate (30 s; *C* and *D*) before application of 0.1 HU/ml Hly (*B* and *D*) or sham challenge (*A* and *C*). Lipid-extracted radioactivity was separated by SP-HPLC as detailed in Methods. In quiescent PMN, only labeled phophatidylcholine (GPC) was detected upon lyso-PAF preincubation. In response to Hly challenge (10 min), the labeled phosphatidylcholine pool decreased concomitant with the appearance of PAF and lyso-PAF. Correspondingly, [^{3}H]acetate incorporation into the PAF fraction was detected in these cells. Data from one representative experiment out of five are shown.

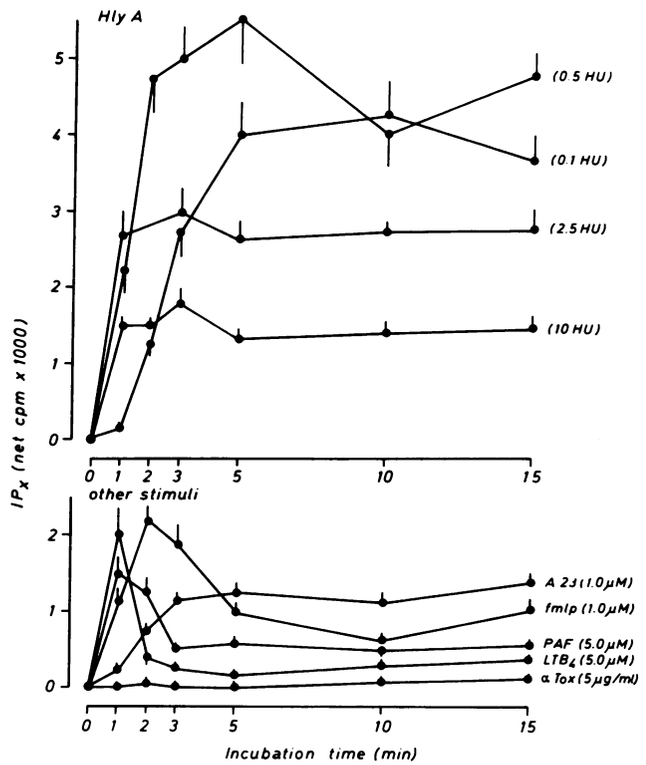

Figure 5. Time course of neutrophil inositol phosphate generation evoked by Hly in comparison with different other stimuli. 10^7 PMN, prelabeled with [^{3}H]inositol, were incubated with different concentrations of Hly (*top*) or different other stimuli at optimum concentrations (*bottom*) for various time periods. Extracted inositol phosphates were separated by anion-exchange chromatography. IP_3, IP_2, and IP_1 are composed as IP_x, corrected for baseline levels in nonchallenged PMN (net cpm; baseline range between 540 and 1,100 cpm). Means±SEM of five (*top*) and four (*bottom*) independent experiments are given.

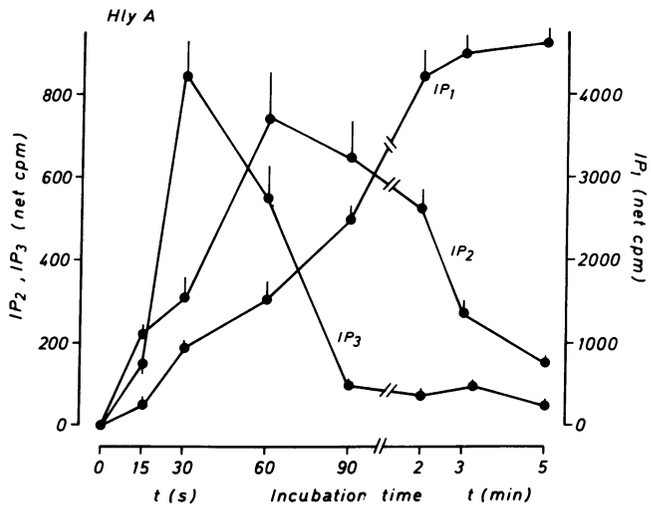

Figure 6. Sequential appearance of inositol phosphates in Hly-challenged neutrophils. 10^7 PMN, prelabeled with [^{3}H]inositol, were incubated with 0.1 HU/ml Hly for various time periods. Extracted inositol phosphates were sequentially eluted from anion-exchange columns and corrected for baseline levels in nonchallenged PMN (net cpm; baseline [^{3}H]IP_3 = 173±24 cpm, [^{3}H]IP_2 = 259±19 cpm, [^{3}H]IP_1 = 814±65 cpm). Means±SEM of five independent experiments are given.

WEB 2086 and the 5-lipoxygenase inhibitor L-663-536 suppressed neither elastase and O_2^- release nor inositol phosphate accumulation. Pretreatment ("priming") of neutrophils with a nonactivating concentration of PMA (3 nM) for 5 min augmented superoxide generation in response to a subsequent Hly challenge (0.1 HU/ml) by about twofold, but reduced toxin-dependent elastase liberation and accumulation of inositol phosphates.

As reported previously, incubation of Hly at 37°C resulted in rapid loss of its toxic properties (Fig. 9). As expected, the activity of FMLP remained unchanged under the same conditions.

In contrast to Hly, treatment of PMN with 0.1–50 μg/ml staphylococcal α-toxin was virtually ineffective with regard to PtdIns hydrolysis and secretory responses (data for 5 μg/ml given in Figs. 1, 2, and 5), despite the fact that cytolytic activity became apparent above 5–10 μg/ml.

Discussion

This study corroborates data of a previous report in showing that Hly is a potent inductor of neutrophil granule secretion (20). Virtually complete secretion of the total cellular elastase and myeloperoxidase content was evoked by toxin concentrations in the range of 0.5 to > 2.5 HU/ml. In addition to this secretory response, the present communication describes the previously undetected occurrence of marked PtdIns hydrolysis in neutrophils exposed to low Hly doses (optimum concentration range ≈ 0.1–0.5 HU/ml). This triggering of a preformed PMN stimulus transmission pathway was paralleled by metabolic responses including the formation of PAF and lyso-PAF, the generation of superoxide anion, and the synthesis and re-

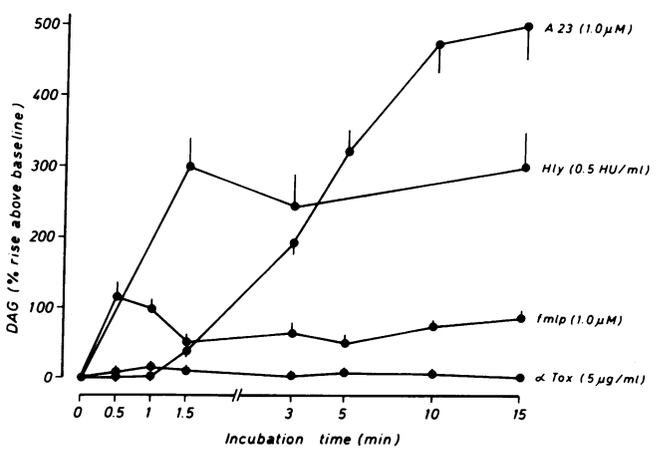

Figure 7. Time course of neutrophil DAG generation evoked by Hly in comparison with different other stimuli. 10^7 PMN were incubated with Hly or different other stimuli at optimum concentrations for various time periods. DAG formation is expressed as percent above baseline values, detected in sham-challenged PMN studied in parallel (baseline range 154.2±24 pmol/10^7 PMN). Means±SEM of four independent experiments are given.

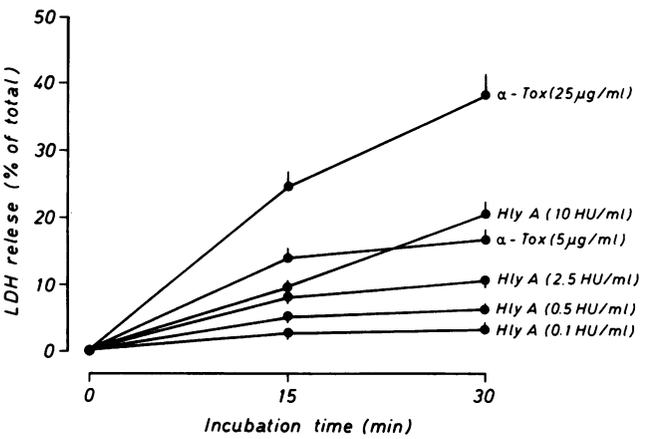

Figure 8. Time course of neutrophil LDH release evoked by Hly in comparison with staphylococcal α-toxin (*Tox*). 10^7 PMN were incubated with different concentrations of Hly or α-toxin up to 30 min. LDH release is expressed as percentage of total cellular enzyme content. Means±SEM of five independent experiments are given.

lease of leukotrienes (27). The ability of Hly to stimulate respiratory burst was previously observed in PMN (25) and renal tubular cells (47) upon use of low toxin concentrations, whereas it was missed in experiments employing overtly cytocidal toxin doses (20). This discrepancy is evidently explained by the presently noted unusual bell-shaped dose dependency of PtdIns hydrolysis and related metabolic responses. This dose–effect curve suggests predominance of signal transduction events at low Hly concentrations, in contrast to predominant cytolysis at high toxin doses. Neutrophils permeabilized owing to high toxin load (20) evidently fail to respond metabolically in terms of PtdIns hydrolysis and mediator release, most probably due to breakdown of cell homeostasis.

In Hly-treated PMN, inositol phosphate formation was detectable within 1 min, similar to the onset of signal transduction in PMN activated by ligand–receptor interaction (FMLP, PAF, LTB_4). The kinetics of sequential IP_3 and IP_2 appearance with subsequent accumulation of IP_1 and the concomitant formation of DAG were compatible with an activation of PtdIns-diphosphate-specific phospholipase C, which is operative in neutrophil stimulus response coupling (28–32). Several lines of evidence indicated that the metabolic responses observed in PMN exposed to subcytolytic Hly doses were mediated via PtdIns hydrolysis-related signal transduction pathways. First, all presently assessed PMN reactions are known to occur upon stimulation of this pathway, via DAG-mediated protein kinase C activation and/or inositol phosphate-related calcium fluxes (28–32). Second, inositol phosphate formation, respiratory burst, PAF generation, and leukotriene synthesis (27) all displayed corresponding bell-shaped dose dependencies with optimum toxin-efficacy at ≈ 0.1–0.5 HU/ml. Granule secretion (elastase, myeloperoxidase) already commenced at this low dose range, but additionally progressed at higher Hly concentrations that elicited rapid membrane permeabilization (≈ 2.5

*Table I. Pertussis Toxin Sensitivity of Hly- and Receptor-operated PMN Stimluation**

Stimulus		Elastase		IP_x	
		−PT	+PT	−PT	+PT
		% total		*net cpm*	
Hly‡ (*HU/ml*)	0.1	39.4±5.3	9.2±0.8	3,898±459	2,121±189
	0.5	66.8±7.0	28.5±3.1	5,347±668	2,879±314
	2.5	95.3±4.2	57.8±5.4	2,504±238	1,811±158
FMLP (μ*M*)	0.01	12.4±1.3	3.8±0.3	962±84	213±20
	0.1	18.5±1.4	5.8±0.4	1,837±191	424±38
	1.0	25.6±2.0	9.2±0.8	1,984±224	745±69
PAF (μ*M*)	0.1	8.9±0.4	<3	381±29	<50
	1.0	11.4±0.7	6.6±0.7	1,478±156	533±71
	5.0	18.1±1.2	16.4±1.4	1,314±104	971±64
LTB_4 (μ*M*)	0.1	6.8±0.4	<3	289±25	<50
	1.0	10.6±0.8	<3	1,977±210	549±61
	5.0	15.7±1.0	6.2±0.5	1,637±144	803±92
A 23187 (μ*M*)	1.0	56.7±4.5	50.9±5.1	1,223±95	1,092±82
	5.0	62.9±4.8	64.8±5.6	1,024±71	981±73

* 10^7 PMN, preincubated with and without 1.0 μg/ml PT for 2.0 h were used in all experiments. Elastase was measured 15 min after application of the different stimuli and is given as percentage of total cellular enzyme content. IP_3, IP_2, and IP_1 were quantified at times of maximum responses to the different stimuli and are composed as IP_x (1 min after LTB_4 and PAF application, 2 min after FMLP stimulation, and 5 min after Hly and A23187 challenge; values given as net counts per minute according to Fig. 5). Means±SEM of six independent experiments each are given.

‡ At all Hly concentrations, elastase release and IP_x generation in the presence of PT differed significantly from those in the absence of PT ($P < 0.01$; two-tailed t test for unpaired samples).

HU/ml). Third, the inositol phosphate accumulation evoked by Hly exceeded that observed in response to the chemoattractive ligands FMLP, PAF, and LTB_4 (all used at optimum concentrations) by greater than fourfold. Correspondingly, the hemolysin was markedly more efficient than these agents in eliciting degranulation, arachidonic acid 5-lipoxygenase metabolism (27), and PAF formation (data for the ligands not given in detail). Superoxide generation evoked by Hly ranged somewhat below that elicited by optimum doses of FMLP, despite the higher DAG concentrations generated in the presence of the hemolysin. This finding is reminiscent of the decrease in O_2^- generation observed in PMN in the presence of high ionomycin concentrations in spite of increased DAG levels, and might possibly be explained by a sensitivity of the DAG-activated NADPH oxidase complex to membrane perturbation and/or excessively high intracellular calcium concentrations (48). Fourth, pretreatment with PT suppressed both Hly-evoked PtdIns hydrolysis and the PMN metabolic and secretory responses. Moreover, the response to Hly after PMA pretreatment was modulated according to the previously described concept of PMA "priming" for ligand-operated stimulation in neutrophils (49, 50). Finally, H7 selectively blocked the Hly-evoked superoxide generation, as anticipated for a protein kinase C–dependent toxin mechanism. Inhibition of leukotriene and 5-hydroxyeicosatetraenoic acid generation as well as use of a specific PAF antagonist did not affect the Hly-induced inositol phosphate release. Secondary loops of autocrine PMN activation due to generation of these lipid mediators (51, 52) can thus be excluded as major contributing events under the presently studied conditions. Thus, initiation of the PtdIns hydrolysis-related stimulus transmission pathway appears to be the predominant underlying mechanism, triggering metabolic and secretory PMN responses to Hly in subcytolytic concentrations.

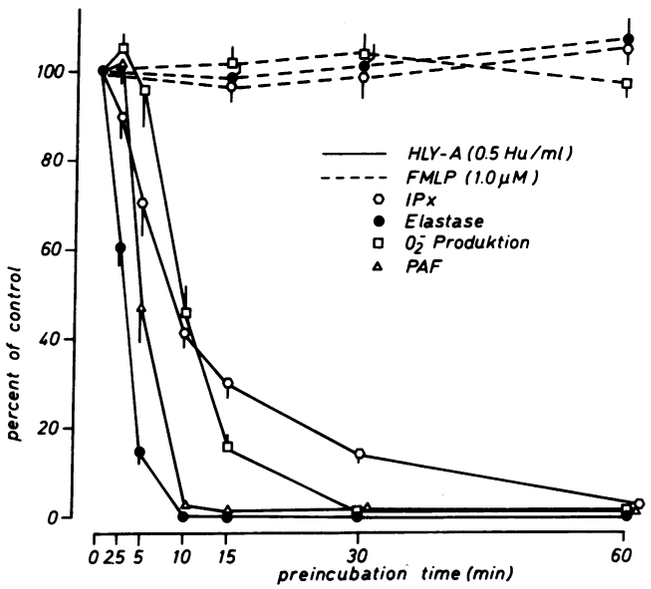

Figure 9. Loss of Hly activity upon incubation at 37°C. Before application to 10^7 PMN, Hly was incubated at 37°C for various time periods. IP_x was measured at 5 min, PAF at 10 min, and superoxide and elastase at 15 min after stimulus application. All data are given as percentage of control values obtained from parallel experiments with fully active Hly. In contrast to the rapid "aging" of Hly at 37°C, no significant decrease in activity was observed upon preincubation of FMLP (used at 0.1 [data not given] and 1 μM). All values represent mean±SEM of four independent experiments.

*Table II. Influence of Different Experimental Agents on Hly-induced PMN Stimulation**

	Elastase	O_{2^-}	IP_x
	% total	*nmol/5 × 10^7 cells*	*net cpm*
Hly	66.8±4.2	72.4±10.2	5,504±676
Hly + H7 (25 μM)	69.3±6.9	31.6‖±4.4	5,811±588
Hly + WEB (1 μM)	72.5±7.2	68.4±9.2	5,439±614
Hly + L663-536‡ (1 μM)	63.4±4.1	74.0±6.6	5,364±618
Hly after PMA priming§	51.6‖±5.3	138.4‖±11.6	4,112‖±442

* 10^7 PMN were incubated with 0.5 HU/ml Hly (measurement of elastase and IP_x) and 5×10^6 PMN with 0.1 HU/ml (superoxide generation) in the absence or presence of different experimental agents. All agents were preincubated for 3 min. Inositol phosphates (IP_x) were quantified 5 min, and elastase and superoxide 15 min after neutrophil stimulation. Means±SEM of four or five independent experiments each are given.

‡ In the presence of the 5-lipoxygenase inhibitor L 663-536, the Hly-induced LTB_4 and 5-hydroxyeicosatetraenoic acid generation was inhibited by > 90%.

§ Cells were incubated with PMA (3 nM) for 3 min before challenge with 0.1 HU/ml Hly (measurement of superoxide) or 0.5 HU/ml Hly (measurement of elastase and IP_x).

‖ $P < 0.01$ (two-tailed t test for unpaired samples obtained in the absence and presence of experimental agent).

There is compelling evidence that the PMN activation was evoked by the bacterial exotoxin itself and not by contaminating LPS. A toxin preparation with markedly depleted endotoxin content was used (residual contamination amounting only to ≈ 3 ng of LPS/μg protein). Rapid loss of toxin activity occurred upon "aging" of the preparation at 37°C, a feature repeatedly described for the hemolysin, but not compatible with established endotoxin characteristics (20, 21). Preincubation of PMN with endotoxin is known to exert "priming" effects in these cells, but direct initiation of acute secretory responses has not been described (53–56). Accordingly, no PMN responses were evoked upon use of *E. coli* endotoxin up to 100 ng/ml within the stimulation periods of the present study (data not given in detail). However, these findings do not exclude cooperative interaction of remaining LPS molecules and Hly peptides in eliciting metabolic and secretory responses in the neutrophils.

How is the hemolysin effect linked with the initiation of PMN PtdIns turnover and second messenger induction? Pore-forming bacterial agents certainly evoke a variety of cellular responses via facilitation of passive calcium flux into the cells (19, 57–61). However, optimal concentrations of the calcium ionophore A23187 induce only moderate and delayed-onset inositol phosphate formation in neutrophils, which may actu-

ally be due to the marked generation of LTB_4 and PAF with secondary (autocrine) PMN activation. Moreover, staphylococcal α-toxin induces transmembrane calcium flux in neutrophils (60), but does not evoke any significant PtdIns turnover in these cells. It has previously been noted that inositol phosphate formation by PIP_2- and PIP-specific phospholipase C is independent of cytoplasmatic Ca^{++} elevation (62–64). Thus, it is presently unclear whether Hly-elicited transmembrane calcium flux is involved in triggering the described metabolic responses. The significant PT sensitivity of PtdIns turnover and related metabolic and secretory responses may indicate enrollment of G proteins in Hly-evoked PMN stimulation (51, 65–66). Cell surface receptors for Hly have hitherto not been characterized, and further experimental approaches are necessary to delineate the molecular mechanisms by which this bacterial exotoxin triggers key events of PMN regulation with outstandingly strong potency.

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