INFECTION AND IMMUNITY, Aug. 1992, p. 3150–3155
0019-9567/92/083150-06$02.00/0

Vol. 60, No. 8

Induction of Inflammatory Mediator Release (Serotonin and 12-Hydroxyeicosatetraenoic Acid) from Human Platelets by *Pseudomonas aeruginosa* Glycolipid

BRIGITTE KÖNIG, UWE BERGMANN, AND WOLFGANG KÖNIG*

Lehrstuhl für Medizinische Mikrobiologie und Immunologie, Arbeitsgruppe für Infektabwehrmechanismen, Ruhr-Universität Bochum, Universitätsstrasse 150, 4630 Bochum, Germany

Received 18 July 1991/Accepted 5 May 1992

Purified glycolipid from *Pseudomonas aeruginosa* induced the generation of significant amounts of 12-hydroxyeicosatetraenoic acid (12-HETE) and serotonin release from human platelets. The release of serotonin was first observed 2 min after addition of the glycolipid and increased with time. Significant serotonin release was obtained at glycolipid concentrations above 5 μg/ml and increased dose-dependently up to 100% at glycolipid concentrations above 40 μg/ml. Glycolipid induced 12-HETE in a time- and dose-dependent manner. 12-HETE formation was first measured after 10 min of incubation and increased with time. Optimal 12-HETE formation was obtained at a glycolipid concentration of 50 μg/ml; higher concentrations of glycolipid led to a decrease in 12-HETE formation, indicating a cytotoxic effect. Stimulation of platelets with glycolipid (12-HETE formation and serotonin release) was accompanied by calcium influx, translocation of protein kinase C, activation of guanylylimidodiphosphate binding, and increased GTPase activity in platelet membranes within the same concentration range.

Pseudomonas aeruginosa is an opportunistic pathogen which causes either localized infections, such as pneumonia in patients with cystic fibrosis, or generalized septicemia in immunocompromised hosts who are suffering from severe burns or cancer or receiving immunosuppressive therapy (17, 23, 42, 47, 48, 56). Basically, the occurrence of such diverse clinical diseases may derive either from an altered host defense in these patients and/or a difference in virulence of taxonomically identical *P. aeruginosa* strains (1, 18, 30, 32, 41, 57).

Several pathogenicity factors of *P. aeruginosa* have been identified. Exotoxin A modulates lymphocyte functions; the heat-labile (phospholipase C) (3, 4, 36, 40) and the heat-stable (glycolipid) hemolysin (29) have been shown to induce inflammatory mediator release from human granulocytes as well as from mast cells (2). Alginate, the mucoid exopolysaccharide of *P. aeruginosa* which is mainly present in isolates from patients with cystic fibrosis (46), interferes with granulocyte functions, e.g., phagocytosis.

Little is known about the interaction of *P. aeruginosa* pathogenicity factors and human platelets. After stimulation with collagen, thrombin, or the Ca ionophore A23187, human platelets release mediators after degranulation of preformed constituents (e.g., serotonin) or after new generation (e.g., 12-hydroxyeicosatetraenoic acid [12-HETE]) (11, 12, 33, 38). The latter product is a potent chemotactic component for neutrophils and may mediate leukocyte diapedesis through the vascular endothelium (20, 51). It causes neutrophil degranulation and is involved in mucus secretion (10, 37, 52). Recently, we demonstrated (31) that 12-HETE alone is sufficient to induce the expression of heat shock proteins in human leukocytes, which may alter neutrophil responses to, e.g., bacterial products. However, platelet-derived 12-HETE can be rapidly incorporated into other cell populations (e.g., human leukocytes) and thereby act as a transcellular signal (6). Thus, platelet-derived mediators are involved in inflammatory processes such as occur in bacterial infections (34). Recently, Coutinho et al. (15) showed that the phospholipase C of *P. aeruginosa* induces the in vitro aggregation of human platelets. While phospholipase C induces leukotriene formation from human granulocytes, glycolipid proved to be inactive. However, glycolipid is a potent stimulus for histamine release from mast cells (2).

Platelet activation is transmitted via a complex signal transduction pathway, including an increase in intracellular calcium, activation of protein kinase C (PKC), and G-protein involvement (33). G-proteins represent a family of regulatory proteins (19), all of which serve to transfer information from the receptor to the effector system of the cell. The participation of GTP-binding proteins was demonstrated for receptor-linked activation (e.g., thrombin) or stimulation with fluoride ions (F^-), which mimick the gamma-phosphate of GTP at the GTP-binding site (5, 11, 26).

It was the purpose of our study to analyze the effect of glycolipid on inflammatory mediator release from platelets. In this regard, serotonin release and 12-HETE formation were studied. We demonstrate that glycolipid, an extracellular product of *P. aeruginosa*, activates human platelets for the release of inflammatory mediators (serotonin and 12-HETE) which may activate or alter the responsiveness of additional cells involved in the inflammatory host response. In this regard, platelet activation by *P. aeruginosa* extracellular products, e.g., the glycolipid, may contribute to the fatal outcome of *P. aeruginosa* infections in cystic fibrosis (10, 20, 37, 56). Since platelet activation is mediated via defined steps of the signal transduction pathway (33), studies of G-protein and PKC activation as well as calcium influx may help to understand signaling in glycolipid-stimulated human platelets. The knowledge of the precise signal transduction pathway as well as the cross-signaling between various cell types may lead to new concepts in the therapy of *P. aeruginosa* infections.

* Corresponding author.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (5 Ci/mmol) and [^{3}H]serotonin (5-hydroxytryptamine, creatinine sulfate; 30.4 Ci/mmol) were purchased from New England Nuclear Corp. DE-52 cellulose was from Whatman. Synthetic leukotrienes were a generous gift from Merck-Frosst, Pointe Claire, Quebec, Canada. The *P. aeruginosa* glycolipid was kindly donated by G. Döring, Institut für Hygiene, Universität Tübingen, Tübingen, Germany.

P. aeruginosa bacteria were cultured in the medium described by Hauser and Karnovsky (24), containing 7% K_2HPO_4, 3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 1% $(NH_4)_2SO_4$, and 30% glycerin (94%), pH 7.4. The glycolipid was isolated from culture supernatants as described by Jarvis and Johnson (28). Purification was performed by the method of Johnson and Boese-Marrazo (29) with the following modification: the precipitate containing the glycolipid was solubilized in ethanol (98%) and was washed three to four times in the presence of charcoal. The crystallization process was performed as described by Johnson and Boese-Marrazo (29). The glycolipid preparation was free of additional substances other than sodium chloride; e.g., it was free of lipopolysaccharide (LPS) as verified by spectroscopy at the institute of biochemistry in Hannover, Germany, by Reinhard Kownatzki (unpublished data). Additional experiments (data not shown) were performed with rhamnolipids R1 and R3, donated by C. Syldatk, Technische Universität Braunschweig, Braunschweig, Germany (53); identical results were obtained.

P. aeruginosa LPS was prepared from *P. aeruginosa* PAC1R as described by Darveau and Hancock (16). The concentration of 2-keto-3-desoxyoctonate (KDO) was determined by the method of Osborn (45), and LPS concentrations were estimated by using a value of 2.4% (wt/vol) KDO in the LPS of *P. aeruginosa* PAC1R (13). The solvents used were of high-pressure liquid chromatography grade, obtained from local suppliers. All other chemicals were from Sigma, Deisenhofen, Germany.

Buffer. The buffer used for washing the platelets and for mediator release consisted of 137 mM NaCl, 8 mM Na_2HPO_4, 3 mM KCl, and 3 mM KH_2PO_4, pH 7.4 (modified Dulbecco's phosphate-buffered saline [PBS]). Buffer A consisted of 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA (ethylene glycol tetraacetic acid), 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

Preparation of cells. Platelets were prepared from venous blood of healthy donors. The blood was anticoagulated with EDTA (0.15%) and centrifuged at 200 × *g* for 25 min to obtain platelet-rich plasma (PRP). The PRP was centrifuged at 1,300 × *g* at 4°C. The platelets were washed twice and resuspended in PBS to a final concentration of 2×10^8 cells per ml. The purity of the platelets was 98%. Viability of the cells was assayed by light miroscopy with trypan blue exclusion, as well as by the analysis of lactate dehydrogenase (LDH) release from stimulated and nonstimulated cells. Analysis of LDH (EC 1.1.1.27) was carried out as described previously (33).

Serotinin release from human platelets. Washed platelets were resuspended at a concentration of 10^9/ml in PBS containing [^{3}H]serotonin (0.2 μCi/10^8 cells). The suspension was incubated for 30 min at 37°C; during this time, 75% of the [^{3}H]serotonin was incorporated. The labeled platelets were washed twice in PBS and finally suspended at a concentration of 2×10^8/ml. After stimulation at 37°C, the release of [^{3}H]serotonin was determined after mixing the cell suspension with 0.5 ml of paraformaldehyde (3% in PBS). The samples were centrifuged (1,200 × *g*, 10 min, 4°C), and the supernatants were analyzed by liquid scintillation spectrometry. The release of [^{3}H]serotonin was expressed as a percentage of the total bound [^{3}H]serotonin present within the platelets (33).

12-HETE generation from human platelets and 12-HETE analysis. Human platelets (10^8) were suspended in 0.5 ml of PBS buffer. For stimulation, $CaCl_2$ (final concentration, 0.8 mM), $MgCl_2$ (final concentration, 1.0 mM), and the glycolipid were added and incubated at 37°C. The supernatants of stimulated cells were analyzed for 12-HETE content as described previously (30, 33).

Loading cells with fura-2. The acetoxymethylester of fura-2 was added to 2×10^8 platelets in HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4]) at a final concentration of 3 μM. The cells were incubated at 37°C with intermittent mixing to allow complete uptake of the dye. After 30 min, the cells were washed in HEPES buffer.

Fluorescence measurements. Freshly loaded platelets were spun down and resuspended at a concentration of 2×10^7/ml in HEPES buffer supplemented with $CaCl_2$ (0.8 mM) and $MgCl_2$ (1.0 mM). A Perkin-Elmer spectrofluorometer was used with the excitation wavelength set at 340 nm, and the emission was measured at 510 nm (21, 55). Stimuli were added as indicated in the Results. Maximum fluorescence values (F_{max}) were obtained by adding digitonine up to a final concentration of 5 μg/ml, thus permeabilizing the cells for calcium entry and ensuring that essentially all fura-2 was complexed with calcium. Minimum fluorescence (F_{min}) was subsequently obtained by adding EGTA up to a final concentration of 6.6 mM, to chelate free calcium and to ensure that essentially no fura-2 was complexed with calcium.

Assay for PKC activity. Human platelets (2×10^8) were preincubated for 10 min at 37°C in PBS and subsequently stimulated with stimuli such as Ca ionophore A23187 or glycolipid for the indicated times. Activation of the cells was stopped by centrifugation at 1,000 × *g* for 10 min; the cells were then resuspended in 1 ml of bufer A and sonicated (amplitude, 50 μm; sonifier 250; Branson Power Company) twice for 10 s each. PKC activity was assayed as described previously (33).

Binding of Gpp(NH)p and GTPase activity. Human platelets (2×10^8) were incubated in the presence of glycolipid, NaF, or thrombin or in the absence of any stimulus for the indicated times at 37°C. The assay was performed as described previously (33). Guanylylimidodiphosphate [Gpp(NH)p] binding was expressed in counts per minute bound to the membranes (10 μg per assay) (9). Membrane-bound GTPase activity was reported as ^{32}P released from [γ-^{32}P]GTP, expressed in counts per minute.

Statistics. Data show mean values ± standard deviation (SD) for at least three individual experiments with cells from different donors. Significance was examined with Student's *t* test for independent means.

RESULTS

Glycolipid-induced mediator release. Since, among the hemolysins, the heat-stable glycolipid has proinflammatory activities (2), experiments were carried out to analyze the effect of purified glycolipid on serotonin release and 12-HETE formation. It is evident from Fig. 1A that glycolipid induced significant serotonin release in a dose-dependent

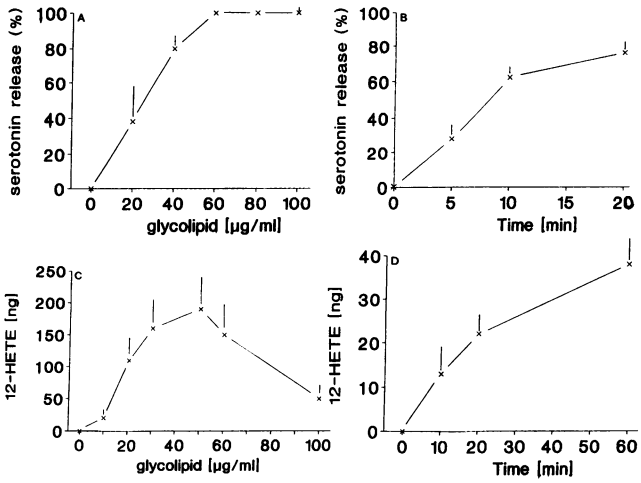

FIG. 1. Platelet activation by glycolipid from *P. aeruginosa*. (A) Dose-response of serotonin release. Glycolipid (0 to 100 µg/ml) was incubated with cells for 10 min at 37°C. (B) Kinetics of serotonin release. Platelets (10^8) were incubated with glycolipid (25 µg) for up to 20 min. (C) Dose-response of 12-HETE formation. Glycolipid (0 to 100 µg/ml) was incubated with cells for 60 min at 37°C. (D) Kinetics of 12-HETE formation. Platelets (10^8) were incubated with glycolipid (15 µg/ml) for up to 60 min at 37°C. Data represent mean values ± SD for four individual experiments. Values for unstimulated cells were subtracted from all other values for serotonin release and 12-HETE formation (see text).

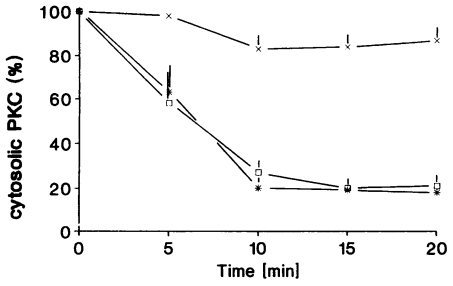

FIG. 2. Kinetics of the decrease in cytosolic PKC activity after stimulation with glycolipid (25 µg/ml) (□), Ca ionophore A23187 (10^{-7} M) (*), or PBS (×). Platelets (10^8) were incubated for up to 20 min at 37°C. PKC activation was determined as described in Materials and Methods. A decrease in cytosolic PKC activity represents activation of this enzyme. Data represent mean values ± SD for four individual experiments.

manner. At a glycolipid concentration of 20 µg/ml, serotonin release was approximately 40%. Concentrations above 40 µg/ml released 100% of serotonin, which may be the result of cytotoxicity. In additional experiments, platelets were stimulated with glycolipid at concentrations ranging from 0.1 to 10 µg/ml. Glycolipid concentrations as low as 5 µg/ml induced significant serotonin release (12% ± 4%) from human platelets (data not shown). As was apparent from kinetic studies, serotonin release from platelets had occurred after only 2 min of incubation with glycolipid (25 µg/ml) (16% ± 4%) (data not shown). In Fig. 1B it is shown that serotonin release increased steadily up to 62% ± 11% within 10 min, and prolonged incubation (20 min) led to 78% ± 6% serotonin release.

For comparison, the time kinetics of 12-HETE formation with 15 µg of glycolipid per ml are presented in Fig. 1D (the value for unstimulated platelets, 12 ± 8 ng/10^8 cells, was subtracted). Glycolipid-induced 12-HETE formation showed a linear increase, which reached 13 ± 4 ng of 12-HETE after incubation for 10 min. With prolonged incubation, a flattening of the curve is shown, with values of 22 ± 7 ng of 12-HETE after 20 min, 38 ± 7 ng after 60 min (Fig. 1D), and 43 ± 6 ng after 120 min of incubation (data not shown). Increasing amounts of glycolipid induced 12-HETE formation in a dose-dependent manner (Fig. 1C) (value for unstimulated platelets was subtracted). At a glycolipid concentration of 20 µg/ml, 150 ± 28 ng of 12-HETE was released; at 50 µg/ml, generation increased up to 180 ± 42 ng within 60 min. A further increase in the glycolipid concentration reduced the amount of 12-HETE formed to 50 ± 7 ng, suggesting cytotoxic damage of the cells. Lactate dehydrogenase release above 5% ± 3% was not detected.

To rule out mediator release by glycolipid-associated LPS, additional experiments were performed with purified *P. aeruginosa* LPS at concentrations ranging from 10 ng/ml to 10 µg/ml. A high dose (10 µg/ml) of LPS induced the formation of 30 ± 10 ng of 12-HETE per 10^8 cells, compared with 12 ± 8 ng/10^8 cells in unstimulated platelets. At low doses of LPS, no significant 12-HETE formation was observed in human platelets. It is obvious that such high amounts of LPS would be detectable by spectroscopy.

Analysis of signal transduction. Obviously, the activation of platelets for inflammatory mediator release (serotonin and 12-HETE) proceeds via defined pathways of the signal transduction cascade (33). For this purpose, the activation of PKC was studied. PKC is found in the cytosol in its inactive form; after activation, the PKC is translocated to the membrane. Therefore, the activation of PKC can be measured as a decrease in cytosolic PKC.

In subsequent experiments, glycolipid and the Ca ionophore A23187 were studied for comparison. For this purpose, human platelets were incubated with glycolipid (25 µg/ml) or A23187 (10^{-7} M) for up to 30 min. As is evident from Fig. 2, both stimuli, the glycolipid and the Ca ionophore A23187, induced a decrease in cytosolic PKC activity after 15 min of incubation down to 80% ± 12%. No further decrease was obtained with incubation for up to 20 min. The activation of the PKC in the presence of buffer was only marginal over time. To rule out PKC inactivation by the glycolipid, partially purified PKC from unstimulated platelets was incubated with the glycolipid or the Ca ionophore A23187. Inactivation of PKC was not obtained (data not shown). As a further control, cytosolic preparations from untreated cells were mixed with cytosolic preparations from untreated or glycolipid-treated cells, incubated, and then assayed for PKC activity; no differences were observed. Our data show that a decrease in cytosolic PKC activity is observed in parallel with 12-HETE formation and with an increase in serotonin release.

G-proteins are essential regulators of ligand- and non-ligand-induced cell activation (19). Sodium fluoride (NaF) has been shown to be a potent stimulus of mediator release (12-HETE and serotonin) by its action on G-proteins (5, 11, 33). The next set of experiments were carried out to study the involvement of G-proteins in the platelet cellular response to purified glycolipid.

Binding of Gpp(NH)p to platelet membranes. The binding of Gpp(NH)p, a nonhydrolyzable GTP analog, indicates one parameter for G-protein activation (39). Gpp(NH)p binding was determined after stimulation of intact cells with purified glycolipid. Cells stimulated with NaF (12.5 or 20 mM), a

TABLE 1. Stimulation of Gpp(NH)p binding and GTPase activity[a]

Stimulus	Incubation time (min)	Gpp(NH)p binding (cpm)	GTPase activity (cpm)
Thrombin			
2 U/ml	10	3,765 ± 200*	24,563 ± 460*
3 U/ml	10	4,108 ± 430*	28,565 ± 510*
NaF			
12.5 mM	10	2,700 ± 120*	27,870 ± 430*
20 mM	10	4,300 ± 380*	29,547 ± 370*
PBS	5	211 ± 29	2,390 ± 110
	10	245 ± 15	3,428 ± 100
	20	231 ± 15	2,900 ± 260
Glycolipid			
5 μg/ml	10	757 ± 18*	10,601 ± 90*
25 μg/ml	5	2,590 ± 159*	21,680 ± 340*
	10	3,424 ± 220*	21,007 ± 270*
	20	3,389 ± 190*	21,340 ± 430*

[a] Values are means ± SD for at least three independent experiments. *, Significantly different from buffer control ($P < 0.05$).

direct G-protein activator, with thrombin (2 or 3 U/ml), or with buffer (PBS) served as controls. The incubation proceeded for 5, 10, or 20 min at 37°C. Gpp(NH)p binding was maximal after 10 min of incubation. At a glycolipid concentration of 25 μg/ml, the values for Gpp(NH)p binding were 2,590 ± 159, 3,424 ± 220, and 3,389 ± 190 cpm after 5, 10, and 20 min of incubation, respectively. As is apparent from Table 1, thrombin, NaF, and purified glycolipid induced an increase in Gpp(NH)p binding in a dose-dependent manner for up to 10 min of incubation.

GTPase activity. In parallel to the binding of Gpp(NH)p to platelet membranes, the activity of GTPase (8, 39), which terminates the binding of GTP to the G-proteins, was determined. The glycolipid, thrombin, and NaF induced a significant increase in GTPase activity compared with the basal level (buffer control) (Table 1). After only 5 min of stimulation, maximal enzyme activity was detected in glycolipid (25 μg/ml)-stimulated platelets. Dilution of the stimulus concentration led to a reduction in GTPase activity. Our data thus emphasize that the glycolipid affects G-protein activities.

Induction of Ca^{2+} influx. An important prerequisite for mediator induction is the influx of Ca^{2+}, which is regarded as a second messenger for cellular activation. In subsequent experiments, the effects of thrombin (2 U/ml) and glycolipid at various concentrations were analyzed. It is apparent from Fig. 3 that glycolipid induced an increase in Ca^{2+} influx in a dose-dependent manner. Actually, we did not quantitate the intracellular Ca^{2+} concentrations (Ca^{2+}_i); therefore, digitonine was added to determine the maximal increase in Ca^{2+}_i. Similar to thrombin, low doses of glycolipid (1, 5, and 25 μg/ml) caused a short-lived calcium influx, whereas a high dose (125 μg/ml) caused a sustained influx. We suggest that the short-lived Ca^{2+} influx indicates a reversible process, while the sustained influx leads to inactivation of cellular responsiveness and consequently to cell death.

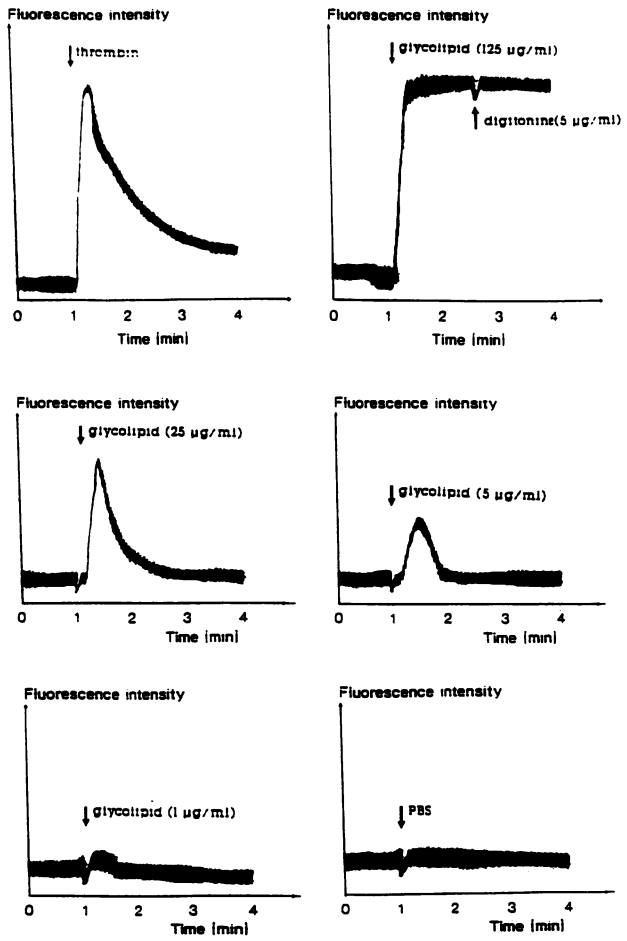

FIG. 3. Calcium influx after platelet activation with thrombin, glycolipid (125, 25, 5, and 1 μg/ml), and PBS (control). Experiments were carried out as described in Materials and Methods. The traces show representative determinations performed on at least three experiments.

DISCUSSION

Recently, we have shown that *P. aeruginosa* induces inflammatory mediator release from human granulocytes and mast cells in correlation to expression and secretion of the heat-labile hemolysin (phospholipase C) (2). The heat-stable hemolysin, the glycolipid, proved to be a potent stimulus for the release of the preformed mediator histamine from mast cells, but it did not induce leukotriene generation (a newly formed mediator). Owing to these discrepancies, we analyzed the effect of glycolipid on mediator release from human platelets, e.g., preformed serotonin as well as newly formed 12-HETE. Our experiments, performed with purified glycolipid, indicate that concentrations of 5 to 20 μg/ml are effective for significant 12-HETE formation. One may speculate that 5-lipoxygenase, the essential enzyme in leukotriene formation in human granulocytes, is differently regulated than platelet 12-lipoxygenase, which is responsible for the formation of 12-HETE (34). In addition, these results clearly show that the sensitivity of the cells (granulocytes and platelets) to glycolipid may differ. To support this hypothesis, additional experiments are necessary.

In addition to 12-HETE, the preformed mediator serotonin is released from human platelets after stimulation with the glycolipid. Despite the fact that serotonin release occurs more rapidly, the kinetics of 12-HETE formation and serotonin release are similar. At glycolipid concentrations above 60 μg/ml, 12-HETE formation decreased, perhaps owing to a cytotoxic effect, while the release of the preformed serotonin

increased up to 100%, which does not contradict a cytotoxic effect at a high dose of glycolipid.

Only a few data exist as to the signal-transducing events in platelet activation caused by defined pathogenicity factors of *P. aeruginosa*. Human platelets respond to agonists by the release of dense granule contents (serotonin) or by generating free arachidonic acid, the precursor of thromboxane A2 and 12-HETE (22, 33, 51). Recent studies emphasize the role of PKC in platelet activation and regulation (33, 44, 58). It has been suggested that PKC mediates several responses of human platelets after activation with a variety of stimuli (43, 49, 50, 54). The direct activation of PKC by phorbol 12-myristate 13-acetate leads only to minute serotonin release; 12-HETE formation, however, is not induced. In contrast, the purified glycolipid induced PKC activation and mediator release (12-HETE and serotonin) in a dose-dependent manner. In addition to PKC activation, a rise in intracellular calcium levels was observed in platelets stimulated with the glycolipid.

Although the regulatory mechanisms which underlie the arachidonic acid release process are not clearly understood, a crucial role for calcium is indicated by the ability of the Ca ionophore A23187 to generate 12-HETE and serotonin release from human platelets. These data are consistent with earlier observations (33) that serotonin release might not be due to the activation of PKC alone. Furthermore, it became evident from our own work (33) as well as from that of other investigators (12, 38, 58) that under appropriate conditions, PKC activation and calcium mobilization also act synergistically to elicit a physiological response of the platelets, e.g., serotonin release and 12-HETE formation. Our data show that glycolipid activates PKC, leads to an increase in $Ca^{2+}{}_i$, and induces 12-HETE generation and serotonin release from human platelets. One may assume that the synergistic action of PKC activation and calcium mobilization is correlated to mediator release. Nonetheless, the knowledge that PKC exists in at least seven isoforms raises the possibility that not all of these forms of the enzyme are equally involved in platelet activation by the various stimuli. Furthermore, it has been reported that the isoenzymes of PKC exhibit differences in activation by fatty acids, diacylglycerol, and phorbol esters (14, 25, 27, 35).

In the past, evidence has been presented that G-proteins are involved in mediator formation following receptor-linked cell activation (11, 39). In our study, three parameters of G-protein involvement were considered by the different assays (5, 39). An increase in Gpp(NH)p binding represents an increased exchange of GTP for GDP at the GTP-binding sites of the G-proteins. An increase in GTPase activity indicates an accelerated inactivation of GTP into the inactive GDP at the GTP-binding site (8). The role of G-protein activation is represented by stimulation with sodium fluoride (NaF) (5). With fluoride, a direct G-protein activator of predominantly the inhibitory G proteins (G_i), or thrombin as the stimulus, the release of serotonin and the formation of 12-HETE were observed in parallel with an increase in Gpp(NH)p binding and increased GTPase activity (11). With purified glycolipid, an increase in Gpp(NH)p binding and GTPase activity was observed in a dose-dependent manner concomitant with an enhanced induction of mediators from human platelets. However, our data do not allow discrimination of which of the G-proteins are involved in 12-HETE formation or serotonin release (7, 26).

Our results support a role for G-proteins, PKC, and calcium influx in the regulation of inflammatory mediator release from human platelets stimulated by purified glycolipid. Further experiments will be directed to analyzing the fine tuning of the cellular responses.

ACKNOWLEDGMENTS

W.K. was supported by Deutsche Forschungsgemeinschaft, and B.K. and U.B. were supported by Bundesminister des Inneren.

REFERENCES

1. **Arturson, G.** 1985. Neutrophil granulocyte functions in severely burned patients. Burns **11**:309–319.
2. **Bergmann, U., J. Scheffer, M. Köller, W. Schönfeld, G. Erbs, F. E. Müller, and W. König.** 1989. Induction of inflammatory mediators (histamine and leukotrienes) from rat peritoneal mast cells and human granulocytes by *Pseudomonas aeruginosa* strains from burn patients. Infect. Immun. **57**:2187–2195.
3. **Berka, R. M., G. L. Gray, and M. L. Vasil.** 1981. Studies on phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect. Immun. **34**:1071–1074.
4. **Berka, R. M., and M. L. Vasil.** 1982. Phopsholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J. Bacteriol. **152**:239–245.
5. **Bigay, J., P. Deterre, C. Pfister, and M. Chambre.** 1987. Fluoride complexes of aluminium or beryllium act on G-proteins as reversibly bound analogues of the gamma-phosphate of GTP. EMBO J. **6**:2907–2911.
6. **Bigby, T. D., and N. Meslier.** 1989. Transcellular lipoxygenase metabolism between monocytes and platelets. J. Immunol. **143**:1948–1954.
7. **Bokoch, G. M., and C. A. Parkes.** 1988. Identification of novel GTP-binding proteins in the human neutrophil. FEBS Lett. **227**:66–70.
8. **Bourne, G. M., D. A. Sanders, and F. McCormick.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (London) **348**:125–132.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
10. **Bray, M. A.** 1986. Leukotrienes in inflammation. Agents Actions **19**:87–99.
11. **Brom, C., M. Köller, J. Brom, and W. König.** 1989. Effect of sodium fluoride on the generation of lipoxygenase products from human polymorphonuclear granulocytes, mononuclear cells, and platelets—indication for the involvement of G-proteins. Immunology **68**:240–246.
12. **Charo, J. F., R. D. Feinman, and T. C. Detwiler.** 1976. Inhibition of platelet secretion by an antagonist of intracellular calcium. Biochem. Biophys. Res. Commun. **72**:1462–1467.
13. **Chester, J. R., and P. M. Mecedow.** 1975. Heterogeneity of the lipopolysaccharide from *Pseudomonas aeruginosa*. Eur. J. Biochem. **85**:273–282.
14. **Coussens, L., J. Parker, L. Rhee, T. L. Yang-Feng, E. Chen, M. D. Waterfield, U. Francke, and A. Ullrich.** 1986. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in celular signalling pathways. Science **233**:859–866.
15. **Coutinho, I. R., R. S. Berk, and E. Mammen.** 1988. Platelet aggregation by phospholipase C from *Pseudomonas aeruginosa*. Thromb. Res. **51**:495–505.
16. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for the isolation of bacterial lipopolysaccharide from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
17. **Döring, G.** 1989. Host response to *Pseudomonas aeruginosa*. Acta Paedriatr. Scand. Suppl. **313**:37–39, discussion 40.
18. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210–230.
19. **Gilman, A.** 1987. G-proteins: transducers of receptor generated signals. Annu. Rev. Biochem. **56**:615–649.
20. **Goetzl, J., M. Woods, and R. R. Gorman.** 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. J. Clin. Invest. **59**:179–183.
21. **Grzegorz, G., M. Poenie, and R. Tsien.** 1985. A new generation

of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260**:3440–3450.
22. **Hamberg, M., and B. Samuelsson.** 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proc. Natl. Acad. Sci. USA **71**:3400–3404.
23. **Hansbrough, J. F., W. B. Carroll, R. L. Zapata-Sievent, B. R. Reller, and J. A. Boswick.** 1985. Identification and antibiotic susceptibility of bacterial isolates from burned patients. Burns **11**:393–403.
24. **Hauser, G., and M. L. Karnovsky.** 1954. Studies on the production of glycolipid by *Pseudomonas aeruginosa*. J. Bacteriol. **68**:645–654.
25. **Housey, G. M., C. A. O'Brian, M. D. Johnson, P. Kirschneier, and J. B. Weinstein.** 1987. Isolation of cDNA clones encoding protein kinase C: evidence for a protein kinase C-related gene family. Proc. Natl. Acad. Sci. USA **84**:1065–1069.
26. **Houslay, M. D., D. Bulanic, D. Gawler, S. O'Hagan, and A. Wilson.** 1986. Thrombin, unlike vasopressin, appears to stimulate two distinct guanine nucleotide regulatory proteins in human platelets. Biochem. J. **238**:109–113.
27. **Huang, F. L., Y. Yoshida, H. Nakabayashi, J. L. Knopf, W. S. Young, and K.-P. Huang.** 1987. Immunochemical identification of protein kinase C isoenzymes as products of discrete genes. Biochem. Biophys. Res. Commun. **149**:946–952.
28. **Jarvis, F. G., and M. J. Johnson.** 1949. A glycolipid produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. **71**:4124–4126.
29. **Johnson, M. K., and D. Boese-Marrazzo.** 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect. Immun. **29**:1028–1033.
30. **Knöller, J., W. Schönfeld, M. Köller, T. Hensler, and W. König.** 1988. Arachidonic acid metabolites from polymorphonuclear leukocytes of healthy donors, severely burned patients, and children with cystic fibrosis. Routine monitoring by high performance liquid chromatography. J. Chromatogr. **427**:199–208.
31. **Köller, M., and W. König.** 1991. 12-Hydroxyeicosatetraenoic acid (12-HETE) induces heat shock proteins in human leukocytes. Biochem. Biophys. Res. Commun. **175**:804–809.
32. **Köller, M., W. König, J. Brom, et al.** 1989. Studies on the mechanism of granulocyte dysfunctions in severely burned patients—evidence for altered leukotriene generation. J. Trauma **29**:435–445.
33. **König, B., W. Schönfeld, J. Scheffer, and W. König.** 1990. Signal transduction in human platelets and inflammatory mediator release induced by genetically cloned hemolysin-positive and -negative *Escherichia coli* strains. Infect. Immun. **58**:1591–1599.
34. **König, W., W. Schönfeld, M. Raulf, M. Köller, J. Knöller, J. Scheffer, and J. Brom.** 1990. The neutrophil and leukotrienes—role in health and disease. Eicosanoids **3**:1.
35. **Kosaka, Y., K. Ogita, K. Ase, H. Nomara, U. Kikkawa, and Y. Ishizuka.** 1988. The heterogeneity of protein kinase C in various rat tissues. Biochem. Biophys. Res. Commun. **151**:973–981.
36. **Kurioka, S., and P. V. Liu.** 1967. Effect of *Pseudomonas aeruginosa* on phosphatides and on phospholipase C activity. J. Bacteriol. **93**:670–674.
37. **Marom, Z., J. H. Shelhamer, and M. Kaliner.** 1981. Effects of arachidonic acid monohydroxyeicosatetraenoic acid and prostaglandins on the release of mucous glycoproteins from human airway in vitro. J. Clin. Invest. **67**:1695–1702.
38. **Massini, P., and E. F. Lüscher.** 1976. On the significance of the influx of calcium ions into stimulated human blood platelets. Biochim. Biophys. Acta **436**:652–663.
39. **Matsumoto, T., T. F. P. Molski, Y. Kanaho, E. L. Becker, and R. I. Sha'afi.** 1987. G-protein dissociation, GTP-GDP exchange and GTPase activity. in control and PMA treated neutrophils stimulated by fMet-Leu-Phe. Biochem. Biophys. Res. Commun. **143**:489–498.
40. **Meyers, D. J., and R. S. Berk.** 1990. Characterization of phopsholipase C from *Pseudomonas aeruginosa* as a potent inflamatory agent. Infect. Immun. **58**:659–666.
41. **Moran, K., and A. Munster.** 1987. Alterations of the host defense mechanism in burned patients. Surg. Clin. North. Am. **67**:47–56.
42. **Morrison, A. J., and R. P. Wenzel.** 1984. Epidemiology of infections due to *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6**(S3):627–642.
43. **Naka, M., M. Nishikawa, R. S. Adelstein, and H. Hidaka.** 1983. Phorbolester induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. Nature (London) **306**:490–492.
44. **Nishizuka, Y.** 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature (London) **308**:693–698.
45. **Osborn, M. J.** 1963. Studies on the gram-negative cell wall. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **50**:499–507.
46. **Pedersen, S. S., A. Kharazmi, F. Espersen, and N. Hoiby.** 1990. *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. Infect. Immun. **58**:3363–3368.
47. **Pier, P. B.** 1985. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. **151**:575–580.
48. **Pruitt, B. A., Jr., R. B. Lindberg, W. F. McManus, and A. D. Mason.** 1983. Current approach to prevention and treatment of *Pseudomonas aeruginosa* infection in burned patients. Rev. Infect. Dis. **5**(S5):889–897.
49. **Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka.** 1983. A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. J. Biol. Chem. **258**:2010–2013.
50. **Schächtele, C., R. Seifert, and H. Osswald.** 1988. Stimulus-dependent inhibition of platelet aggregation by the protein kinase C inhibitors polymyxin B, H7, and staurosporine. Biochem. Biophys. Res. Commun. **151**:542–547.
51. **Spector, A. A., J. A. Gordon, and S. A. Moore.** 1988. Hydroxyeicosatetraenoic acids (HETEs). Prog. Lipid Res. **27**:271–323.
52. **Stenson, W. F., and C. W. Parker.** 1980. Monohydroxyeicosatetraenoic acids (HETEs) induce degranulation of human neutrophils. J. Immunol. **124**:2100–2104.
53. **Syldatk, C., S. Lang, and F. Wagner.** 1985. Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM2874 grown on *n*-alkanes. Z. Naturforsch. **40c**:51–60.
54. **Taplay, P. M., and A. W. Murray.** 1985. Evidence that treatment of platelets with phorbol ester causes proteolytic activation of Ca^{2+}-activated, phospholipid-dependent protein kinase. Eur. J. Biochem. **151**:419–423.
55. **Tsien, R. Y., T. Pozzan, and T. J. Rink.** 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new intracellular trapped fluorescence dye. J. Cell Biol. **94**:325–334.
56. **Wood, R. E., T. F. Boat, and C. F. Doershuk.** 1976. Cystic fibrosis. Am. Rev. Respir. Dis. **113**:833–878.
57. **Woods, D. E., M. S. Schaffer, H. R. Rabin, G. D. Campbell, and P. A. Sokol.** 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. **24**:260–264.
58. **Yaminishi, J., Y. Takai, K. Kaibuchi, K. Sano, M. Castagna, and Y. Nishizuka.** 1983. Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. Biochem. Biophys. Res. Commun. **112**:778–789.