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Roles of Human Peripheral Blood Leukocyte Protein Kinase C and G Proteins in Inflammatory Mediator Release by Isogenic *Escherichia coli* Strains

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The signal transduction pathway (protein kinase C [PKC], calcium influx, and G protein involvement) was studied with isogenic *Escherichia coli* strains expressing different types of adhesins (MSH$^{\pm}$ MS-Fim$^{\pm}$, P-MRH$^{\pm}$ P-Fim$^{\pm}$, and S-MRH$^{\pm}$ S-Fim$^{\pm}$) or varying only in the expression of *E. coli* alpha-hemolysin. As target cells, human polymorphonuclear granulocytes (PMN) and a lymphocyte-monocyte-basophil (LMB) cell suspension were used. The alpha-hemolysin-producing (Hly^{+}) strain *E. coli* K-12(pANN5211) induced calcium influx in a dose-dependent manner in both cell types. No calcium influx was detected after stimulation with the hemolysin-negative (Hly^{-}) *E. coli* bacteria independent of the type of fimbriae. With Hly^{+} bacteria, a dose-dependent activation of PKC was observed in both cell types. The Hly^{-} *E. coli* K-12 induced PKC to a lesser degree, expressing kinetics different from those of *E. coli* K-12(pANN5211) (Hly^{+}). *E. coli* MSH^{+} MS-Fim^{+} was the most potent activator for PKC. Membrane preparations from leukocytes stimulated with Hly^{+} *E. coli* K-12(pANN5211) showed increased binding of [^{3}H]guanylylimidodiphosphate, a nonhydrolyzable GTP analog, and increased GTPase activity compared with leukocytes stimulated with Hly^{-} *E. coli* K-12. The amounts of GTPase activation and [^{3}H]guanylylimidodiphosphate binding were similar for all Hly^{-} *E. coli* bacteria in human PMN as well as in human LMB; no activation was obtained for *E. coli* bacteria without any type of fimbriae. GTP-γ-S, a nonhydrolyzable GTP analog, inhibited the leukotriene B_4 (LTB_4) generation from human PMN by Hly^{-} bacteria, unlike *E. coli* K-12(pANN5211). However, in the presence of NaF, a predominant activator of G_i, LTB_4 generation by Hly^{+} and by Hly^{-} bacteria was significantly enhanced. For LMBs only LTB_4 generation by Hly^{+} bacteria was increased in the presence of GTP-γ-S. NaF decreased the chemiluminescence induced by all *E. coli* strains. Our results thus indicate that (i) Hly^{+} and Hly^{-} bacteria induce the activation of distinct G proteins, e.g., G_i, to different degrees, (ii) LTB_4 generation and chemiluminescence response are differently regulated, and (iii) in comparison with PMN, a different signal transduction pathway is activated by *E. coli* bacteria in LMBs.

Uropathogenic *Escherichia coli* strains generally exhibit several characteristics which contribute to their virulence, including the expression of hemolysin or the expression of mannose-sensitive and/or mannose-resistant fimbriae (7, 22, 23, 28). The fimbriae may have different functions depending on the route, stage, or type of infection. Mannose-sensitive fimbriae are expressed on nonpathogenic as well as pathogenic *E. coli* strains. Most of the *E. coli* strains isolated from patients with urinary tract infections carry mannose-resistant P fimbriae; the S fimbriae are present on many *E. coli* strains that cause neonatal sepsis or meningitis. In previous studies with human polymorphonuclear granulocytes (PMN), it was shown that bacteria can differ in their potency to trigger the cells for inflammatory mediator release depending on the receptor structure of their fimbriae as well as the distribution of receptors on the target cells (23). Hemolysin (7, 14) has been shown to be a potent virulence factor and a stimulus for mediator release from a variety of cells (22, 39, 50).

However, there is little known about the signal transduction pathway leading to the different cellular responses, e.g., chemiluminescence and leukotriene formation. In the past, the role of calcium has been frequently studied with regard to the function of the phagocytic cells, such as cell motility (2), phagocytosis, enzyme secretion (40), and release of oxygen radicals (27) or inflammatory mediators.

Substantial evidence was obtained showing that protein kinase C (PKC) plays an essential role in the cellular activation process (33), leading to reactive oxygen metabolites (11, 44) and mediators of inflammation such as leukotrienes (27). Controversial results were described when the involvement of the PKC in the activation of NADPH oxidase (38, 44) was studied by using a variety of inhibitors (51). The effect of many kinase inhibitors is frequently unspecific; several of them inhibit the NADPH oxidase. In our studies, isogenic *E. coli* strains with different types of adhesins and hemagglutination properties were analyzed; they differ markedly in the ability to induce a chemiluminescence response and inflammatory mediator release (23).

In addition to PKC, guanine nucleotide-binding proteins (G proteins) are involved in the complex events of cellular responses (1, 32). G proteins (13) participate in a number of receptor transduction systems, including the adenylate cyclase system (34, 41). G proteins appear to represent a family of regulatory proteins, all of which serve to transfer information from receptor to effector systems of the cell. We

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TABLE 1. Summary of strains under study

E. coli strain	Phenotype
536/21	Hly^- MSH^- MS-Fim^- MRH^- MR-Fim^-
536/21(pGB30int)	Hly^- MSH^+ MS-Fim^+ MRH^- MR-Fim^-
536/21(pANN801-4)	Hly^- MSH^- MS-Fim^- S-MRH^+ S-Fim^+
536/21(pANN921)	Hly^- MSH^- MS-Fim^- P-MRH^+ P-Fim^+
K-12	Hly^- MSH^+ MS-Fim^+ MRH^- MR-Fim^-
K-12(pANN5211)	Hly^+ MSH^+ MS-Fim^+ MRH^- MR-Fim^-

recently showed leukotriene formation (4) from PMN and from lymphocytes-monocytes-basophils (LMB) after preincubation with fluoride (5). Unlike fluoride, mannose-resistant *E. coli* strains induced leukotriene formation from human PMN (23), while human LMB became deactivated. G protein involvement in cellular responses induced by bacteria has not been studied until now.

It was the purpose of our study to analyze the components of the signal transduction cascade for human leukocyte activation (PMN and LMB) induced by adhesin-carrying *E. coli* strains (mannose-resistant and mannose-sensitive hemagglutination) and by an isogenic *E. coli* strain expressing alpha-hemolysin.

MATERIALS AND METHODS

Materials. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden; 6% (wt/vol) Macrodex was from Knoll, Ludwigshafen, Germany; and 75% (wt/vol) sodium metrizoate solution was from Nycomed, Oslo, Norway. Brain heart infusion broth was obtained from Oxoid. [γ-^{32}P]ATP (5 Ci/mmol) was purchased from New England Nuclear; DE-52 cellulose was from Whatman. Synthetic leukotrienes were a generous gift from J. Rokach (Merck-Frosst, Pointe-Claire, Quebec, Canada. Acetonitrile (high-pressure liquid chromatography [HPLC] grade) was purchased from Baker Chemicals (Gross-Gerau, Germany), and methanol, EDTA, dipotassium hydrogen phosphate, and phosphoric acid were from Riedel de Häen (Seelze, Germany). All other chemicals were purchased from Sigma, Deisenhofen, Germany.

Buffer solutions. The buffer used for washing the peripheral leukocytes and for mediator release was phosphate-buffered saline (PBS) (0.137 M NaCl, 0.008 M Na_2HPO_4, 0.003 M KCl [pH 7.4]). $CaCl_2$ (0.6 mM) and $MgCl_2$ (1 mM) were added shortly before the cells were stimulated. Buffer A consisted of 20 mM Tris HCl (pH 7.5), 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

Preparation of the cells. Human blood leukocytes were obtained from heparinized blood of healthy donors, which was separated on a Ficoll-metrizoate gradient followed by dextran sedimentation (3). The purified cell fraction contained more than 95% intact PMN. The LMB fraction consisted of lymphocytes (84% ± 4.6%), monocytes (14.2% ± 4.1%), and basophilic granulocytes (1.2% ± 0.5%). The PMN and LMB were resuspended to a final concentration of 2×10^7 cells per ml in PBS.

Bacterial strains. *E. coli* 536, isolated from a patient with a urinary tract infection, was the parent strain for the *E. coli* 536 variants. Strain 536/21 is a spontaneous mutant of strain 536 that has lost the ability to produce hemolysin, the MRH phenotype, and the MSH phenotype. *E. coli* 536/21 was transformed with recombinant plasmids to *E. coli* 536/21 variants expressing different types of adhesins (Table 1). Cloning and functional characterization of the plasmid-encoded determinants were performed at the Institut für Genetik und Mikrobiologie, Universität Würzburg, Würzburg, Germany.

Bacterial growth. Brain heart infusion broth (10 ml) was inoculated with 100 μl of an overnight culture; bacterial growth proceeded for 3.5 h at 37°C on a shaker (150 rpm) (36). To obtain bacterial suspensions with the desired constant cell concentrations but different hemolytic activities, isogenic bacteria from *E. coli* K-12 (Hly^-) and K-12 (pANN5211) (Hly^+) from the same growth phase were mixed.

Hemolysin assay. The production of hemolysin was tested on sheep blood agar plates. A quantitative hemolysin assay was performed as described previously (22).

Assay for the translocation of PKC activity. Human PMN (4×10^7) and human LMB (4×10^7) in 2 ml of buffer were preincubated for 10 min at 37°C in buffer A. The cells were subsequently activated by the addition of NaF and washed *E. coli* bacteria as indicated in the legends to the figures. Activation of the cells was stopped by centrifugation at 1,200 rpm for 10 min; the cells were then resuspended in 1 ml of buffer A and subsequently sonicated (amplitude, 50 μm; 2 × 10 s) (Sonifier 250; Branson Power Co.). The crude homogenate was centrifuged at 13,000 rpm for 10 min in an Eppendorf centrifuge. The cytosolic fractions were chromatographed on DEAE-cellulose columns (0.5 by 3 cm), equilibrated with buffer A, and washed with 3 ml of buffer A. PKC was eluted with 1.5 ml of buffer A containing 100 mM NaCl.

PKC assay. The reaction mixture (190 μl) contained 20 mM Tris HCl (pH 7.5), 10 mM magnesium acetate, 500 μg of histone type IIIS per ml, 10 μM [γ-^{32}P]ATP (10^5 cpm/nmol), 2.5 μg of diolein, 25 μg of phosphatidylserine, 1 mM $CaCl_2$, and the enzyme preparation under study. All reagents were dissolved in distilled water. The quantitation of background phosphotransferase activity was performed by analyzing histone phosphorylation in the absence of calcium and phospholipid. After 10 min at 30°C, the reaction was stopped by the addition of 0.75 ml of 25% trichloroacetic acid. Acid-precipitable materials were collected on membrane filters with a pore size of 0.45 μm (Millititer HA filtration plates; Millipore Corp., Eschborn, Germany). The filters were washed twice with 1 ml of 10% trichloroacetic acid. The dried filters were transferred into scintillation vials, distilled water was added, and the radioactivity was measured by Cerenkov radiation (Rack beta 1209; LKB, Turku, Finland). PKC activity was calculated by subtracting background activities; the values are reported in counts per minute.

Loading cells with fura-2. The acetoxymethylester of fura-2, fura-2AM, was added to 5×10^7 PMN or LMB in 1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, and 10 mM HEPES (pH 7.4) at a final concentration of 3 μM (46). The cells were incubated at 37°C with intermittent mixing to allow complete uptake of the dye. After 30 min, the cells were washed with HEPES buffer.

Fluorescence measurements. Freshly loaded cells were spun down and resuspended at a concentration of 2×10^6 cells per ml in HEPES buffer supplemented with $CaCl_2$ (1 mM) and $MgCl_2$ (0.8 mM). A Perkin-Elmer spectrofluorometer was used with the excitation wavelength set at 340 nm

and emission measured at 510 nm. The stimuli were added as indicated in Results.

Chemiluminescence. Chemiluminescence was measured at 37°C in a Lumacounter M 2080 (Lumac). Samples for chemiluminescence were obtained by adding a PMN or LMB suspension (50 μl, 10^6 cells) to polypropylene tubes containing PBS (300 μl) and luminol (20 μl, 0.25 mM) (39).

Leukotriene release from human PMN and LMB. Human PMN (2×10^7) and human LMB (2×10^7) were suspended in 1 ml of PBS. For stimulation, bacterial cell suspension (100 μl, 10^9 bacteria) or the indicated stimulus was added to the cells and incubated in the presence of calcium (0.8 mM) and magnesium (1 mM) as described in Results. In experiments in which the stimulus was combined with NaF, the stimulus was added simultaneously to the cells. When GTP-γ-S was studied, the cells (10^7/500 μl) were incubated for 10 min (unless stated otherwise) with GTP-γ-S (10^{-6} M) at 37°C or the same volume of PBS (50 μl). After preincubation, the stimulus, either NaF, the various washed bacteria, or the same volume of PBS, was added to the cell suspension, and incubation proceeded for an additional period of time as indicated. The supernatants of stimulated cells (1,000 μl) were analyzed for leukotrienes by HPLC.

Analysis of leukotriene release. For analysis of leukotriene release, the supernatants of the stimulated cells were deproteinized, evaporated to dryness, and resuspended in 400 μl of methanol-water (30:70, vol/vol) for reverse-phase HPLC (23).

Binding of guanylylimidodiphosphate [Gpp(NH)p]. Cells were incubated in the presence of NaF (25 mM), *E. coli* K-12 (10^9), and *E. coli* K-12(pANN5211) (10^9) or in the absence of any stimulus (buffer control) for 40 min at 37°C. The cell suspensions were washed, suspended in 0.05 M Tris–0.34 M sucrose buffer (pH 7.5), and EGTA (1 mM), dithiothreitol (1 mM), and leupeptin (100 μg/ml) were added. Cell disruption was carried out by sonication (Sonifier 250; Branson Power Co.). The sonicates were centrifuged at 300 × *g* for 10 min, and the resulting postnuclear supernatant was further differentiated by centrifugation at 10,000 × *g* for 20 min (J2-21 centrifuge with a JA-20 rotor; Beckman, Palo Alto, Calif.) and at 100,000 × *g* for 60 min (L8-70 ultracentrifuge with an SW 60Ti rotor; Beckman). The 100,000 × *g* pellet was resuspended in Tris-sucrose buffer, assayed for protein content by the method of Bradford (4), and stored at −70°C.

The binding of [^{3}H]Gpp(NH)p was determined by a modified procedure of Matsumoto et al. (29). The membrane fraction (10 μg of protein) was incubated in 20 mM Tris HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, and 0.5 μM [^{3}H]Gpp(NH)p. Nonspecific binding was defined as the amount of [^{3}H]Gpp(NH)p bound in the presence of a 1,000-fold molar excess of nonlabeled Gpp(NH)p. Incubations were terminated after 120 min at room temperature by rapid filtration through cellulose ester membranes with a pore size of 0.45 μm (Millititer HA filtration plates; Millipore Corp.). The filters were washed four times with 20 mM Tris buffer containing 0.25 mM $MgCl_2$ and 50 μM EGTA. The dried filters were measured for radioactivity by liquid scintillation counting.

Cell viability. The release of lactate dehydrogenase as a marker for cell damage was determined as previously described (23).

Statistics. If not stated otherwise, all data were calculated as means ± standard deviations of three independent experiments.

TABLE 2. PKC activation by various *E. coli* strains

Stimulus	Characteristic(s)	Cytosolic PKC activity (% of control)[a]	
		PMN	LMB
E. coli 536/21		93 ± 9	75 ± 6
(pANN801-4)	S-MRH$^+$ S-Fim$^+$	79 ± 7	61 ± 4
(pANN921)	P-MRH$^+$ P-Fim$^+$	43 ± 6	21 ± 7
(pANN801-1)	S-MRH$^+$ S-Fim$^-$	62 ± 8	42 ± 4
(pGB30int)	MSH$^+$ MS-Fim$^+$	33 ± 5	18 ± 6
E. coli K-12 (pANN5211)	50% Hemolysis	38 ± 8	42 ± 8
	80% Hemolysis	17 ± 6	27 ± 4

[a] The decrease in cytosolic PKC activity parallels activation of the enzyme.

RESULTS

PKC activation. Primarily, experiments were carried out to study PKC activation in PMN and LMB after stimulation with the various bacterial strains (Table 1). As a control, PMN and LMB (2×10^7/ml) were incubated with phorbol myristic acid (10^{-8} M) for 10 min at 37°C. With a decrease of PKC from the cytosolic fraction by 60% ± 7%, a concomitant increase in membrane-associated PKC activity, which represents the active form of the enzyme, was observed (135% ± 15% versus 100% of the control, i.e., unstimulated, cells). In further experiments, the activation of PKC was measured as a decrease in cytosolic PKC activity. Human PMN (2×10^7) and LMB (2×10^7) in 1 ml of PBS with calcium (1 mM) and magnesium (0.8 mM) were incubated with the different *E. coli* strains (5×10^8) for various time periods. The remaining cytosolic PKC activities were quantitated. In PMN, the decrease in cytosolic PKC activity was detectable within 5 min and returned to a basal level within 30 min after the addition of bacteria (data not shown). The values in Table 2 represent the maximal PKC activations induced by the various *E. coli* strains. The hemolysin-positive *E. coli* K-12(pANN5211) led to a decrease in cytosolic PKC activity, depending on the level of hemolysin activity. At a hemolysin activity level of 80%, a decrease in cytosolic PKC activity to 17% of that of the PBS control was obtained after 5 min. A decrease in cytosolic PKC activity to 38.8% was shown at a hemolysin activity level of 50%. *E. coli* 536/21(pANN801-4) induced an intermediate decrease in cytosolic PKC activity by 20% ± 10%. With *E. coli* 536/21(pANN921), *E. coli* 536/21(pANN801-1), and *E. coli* 536/21(Gb30int) as stimuli, remaining cytosolic PKC activities of about 43% ± 6%, 62% ± 8%, and 33% ± 5%, respectively, compared with that of the buffer control (100%), were measured (Table 2). In LMB, the decrease in cytosolic PKC activity was detectable within 10 to 20 min; basal levels were reached after 40 to 50 min of stimulation. The activation pattern was similar to that obtained with PMN (Table 2). As is shown in Fig. 1, in neutrophils stimulated with 12.5 mM NaF, the activation event was characterized by a lag period of 5 min and returned to a basal level after 40 min. The maximal loss of cytosolic PKC activity was about 60%. Activation of neutrophils with 25 mM NaF was characterized by a lag period of less than 5 min and a maximal loss of cytosolic PKC activity of 90% and showed a prolonged duration (Fig. 1a). In LMB stimulated with 12.5 and 25 mM NaF, respectively, the activation process was similar to but less pronounced than that in neutrophils (Fig. 1B). LMB stimulated with 12.5 mM NaF showed a maximal decrease in cytosolic PKC activity of about 50% after 20 min. After the

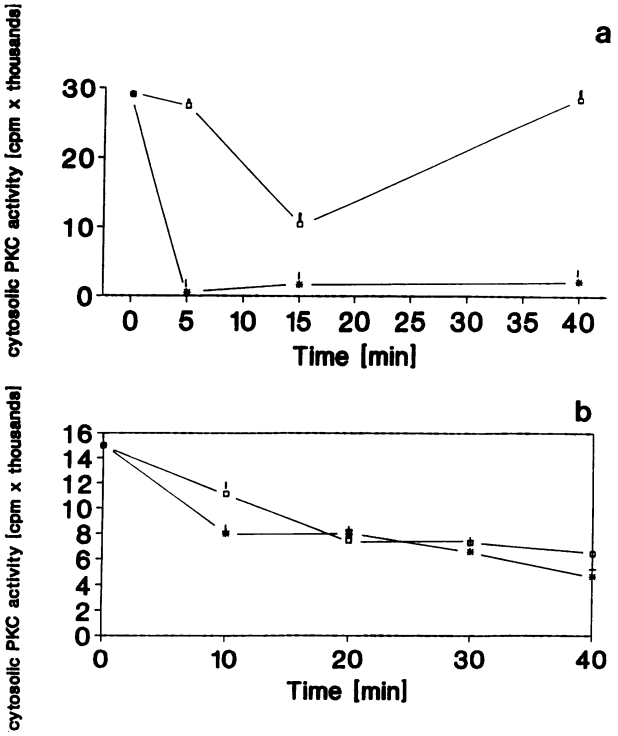

FIG. 1. Time- and dose-dependent activation of cytosolic PKC by NaF in human PMN and LMB. PMN (a) or LMB (b) (2×10^7/ml) were treated with 12.5 (□) or 25 (*) mM NaF for various times. Cytosolic PKC activity was analyzed as described in Materials and Methods. Results are means ± SD from three independent experiments.

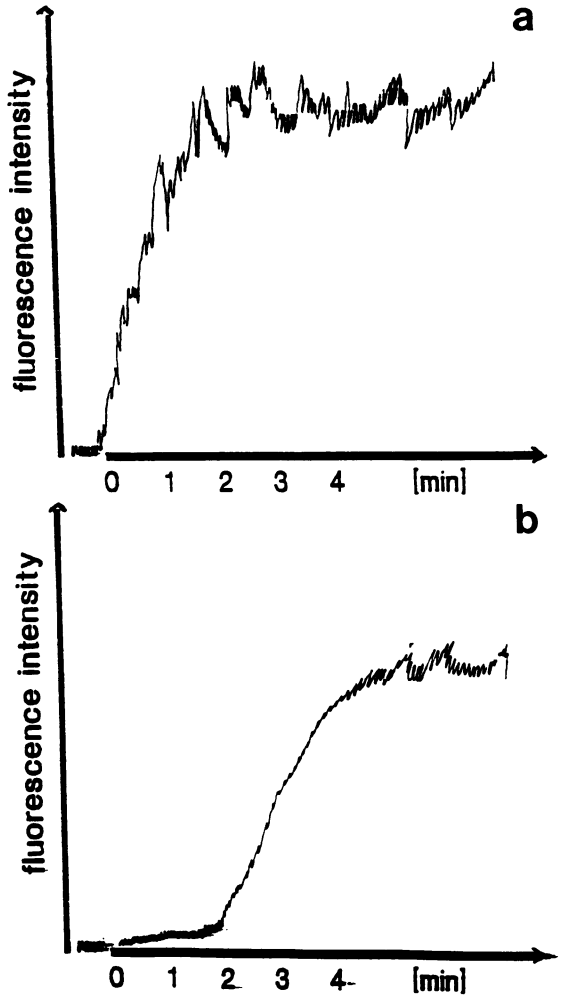

FIG. 2. Calcium influx induced by *E. coli* K-12(pANN5211) and NaF. A typical fluorescence tracing after addition of *E. coli* K-12(pANN5211) (80% hemolysis) (a) and NaF (25 mM) (b) to human PMN is shown. Experiments were carried out as described in Materials and Methods.

addition of 25 mM NaF, the decrease in cytosolic PKC activity started after 1 to 2 min and reached a maximum of about 50% within 10 min. To study the role of G proteins in bacterium-induced PKC activation, the simultaneous addition of NaF (12.5 mM) and the different *E. coli* bacteria (see Table 1) was then studied with regard to PKC activation. The presence of NaF had no effect on PKC activation induced by the *E. coli* strains (data not shown). To rule out PKC inactivation by hemolysin itself, partially purified PKC from unstimulated PMN or LMB was incubated with hemolysin-positive bacteria as well as with hemolysin-containing culture supernatant. Inactivation of PKC was not obtained (data not shown).

Effect of NaF and bacteria on $Ca^{2+}{}_i$. Ca^{2+} influx has been shown to be a second messenger for mediator release. In subsequent experiments, cells were loaded with the calcium-sensitive fluorescence dye fura-2 and the $Ca^{2+}{}_i$ was measured. *E. coli* K-12(pANN5211) (80% hemolysis) induced a rapid, substantial increase of intracellular calcium ($Ca^{2+}{}_i$) in PMN (Fig. 2a); the hemolysin-negative *E. coli* bacteria failed to do so independently of their type of fimbriae (data not shown). In LMB, the calcium increase induced by Hly^+ bacteria was similar to that in PMN. Further experiments were carried out to compare the effects of *E. coli* bacteria on calcium influx with those of NaF, a direct G protein activator. In PMN, calcium influx caused by sodium fluoride was characterized by a relatively long lag period of 2 to 7 min at NaF concentrations of 25 to 2.5 mM, respectively. Figure 2b shows a typical fluorescent tracing after stimulation of PMN with 25 mM NaF. These data emphasize that the increases in $Ca^{2+}{}_i$ caused by *E. coli* hemolysin and NaF proceed via different mechanisms, in the former case, predominantly by a direct calcium influx and, in the latter case, through a G protein-mediated pathway which takes a longer time. In contrast to the calcium increase caused by *E. coli* K-12 (pANN5211) (Hly^+), no calcium increase was detected in LMB after stimulation with NaF at concentrations of 2.5 to 25 mM (data not shown). Subsequently, the influence of activated G proteins on calcium influx by *E. coli* bacteria was studied. The simultaneous stimulation of PMN with NaF (12.5 mM) and the hemolysin-negative *E. coli* strains did not affect the increase of $Ca^{2+}{}_i$ induced by NaF alone (data not shown). Similarly, when PMN were treated simultaneously with *E. coli* K-12(pANN5211) (80% hemolysis) and NaF (12.5 mM), the Ca^{2+} uptake was the same as with the hemolysin-positive bacteria [*E. coli* K-12(pANN5211)] alone (data not shown). In LMB, the simultaneous stimulation with NaF and the respective stimuli did not alter the response induced by the various stimuli without the addition

TABLE 3. G protein activation by NaF, *E. coli* K-12, and *E. coli* K-12(pANN5211) in human PMN and human LMB

Stimulus	PMN		LMB	
	Binding of Gpp(NH)p (cpm)	GTPase activity (cpm)	Binding of Gpp(NH)p (cpm)	GTPase activity (cpm)
None	5,366 ± 144	3,084 ± 122	1,204 ± 212	2,128 ± 213
NaF (25 mM)	7,375 ± 214	18,235 ± 403	5,197 ± 198	15,021 ± 318
E. coli K-12	6,111 ± 189	21,073 ± 213	5,017 ± 102	14,321 ± 223
E. coli K-12(pANN5211)	6,408 ± 87	21,213 ± 308	5,405 ± 122	18,023 ± 125

of NaF (data not shown). Thus, the *E. coli* hemolysin and NaF interact with LMB via different mechanisms.

Studies on G protein activation by *E. coli* bacteria. The role of G protein involvement was assessed by the binding of the nonhydrolyzable GTP analog [^{3}H]Gpp(NH)p and the determination of the GTPase activity. Intact leukocytes (2×10^7/ml) were incubated in the absence of a stimulus (PBS control) or in the presence of NaF (25 mM), *E. coli* K-12(pANN5211) (80% hemolysis), or the hemolysin-negative bacteria (Table 1). After incubation times of 10 min for PMN and 20 min for LMB at 37°C, the reaction was stopped. The membrane fraction was prepared as described above, and the specific binding of [^{3}H]Gpp(NH)p (0.5 μM) was analyzed. It is apparent from Table 3 that PMN membranes revealed an increased binding capacity for Gpp(NH)p after stimulation with *E. coli* K-12(pANN5211) and *E. coli* K-12 compared with unstimulated cells. *E. coli* bacteria with mannose-resistant hemagglutination properties expressed an activity similar to that of *E. coli* K-12 (data not shown). The enhanced binding capacity for [^{3}H]Gpp(NH)p was in the same range as that obtained with NaF (25 mM) as the stimulus. The G protein-associated GTPase activity was also measured after incubation of the membrane preparations (protein content, 10 μg) stimulated with NaF, *E. coli* K-12(pANN5211), the hemolysin-negative *E. coli* bacteria with different adhesive properties, and the PBS control. NaF, *E. coli* K-12(pANN5211), and the hemolysin-negative *E. coli* bacteria showed a 10-fold greater activation of GTPase than the PBS control. The data obtained for NaF, *E. coli* K-12(pANN5211), and *E. coli* K-12 are shown in Table 3. With LMB as target cells, a similar activation pattern for GTPase and similar levels of [^{3}H]Gpp(NH)p binding were obtained (Table 3).

Involvement of PKC and G protein activation in the chemiluminescence response. In previous publications (22, 23), we demonstrated that hemolysin-positive as well as hemolysin-negative *E. coli* bacteria induced a chemiluminescence response in human PMN and LMB. Cells (2×10^6) were incubated with 2.5×10^8 bacteria over a time period of 30 min at 37°C. As shown in Fig. 3, with PMN as target cells, *E. coli* 536/21 (MRH$^-$ MSH$^-$ Fim$^-$) and *E. coli* 536/21 (pANN801-4) (S-MRH$^+$ S-Fim$^+$) led to low to intermediate chemiluminescence responses and *E. coli* 536/21(pGb30int) (MSH$^+$ MS-Fim$^+$), *E. coli* 536/21(pANN801-1) (S-MRH$^+$ S-Fim$^-$), and *E. coli* 536/21(pANN921) (P-MRH$^+$ P-Fim$^+$) were potent stimuli of the chemiluminescence response ($P = 0.01$). A steady increase was obtained over the time period (30 min) analyzed. The concomitant expression of hemolysin led to the following results: *E. coli* 536/21(pANN5211) (40% hemolysis) induced a chemiluminescence response which significantly exceeded that of hemolysin-negative bacteria ($P < 0.01$) (Fig. 3b). With LMB as target cells, a similar pattern was obtained (data not shown). NaF was studied as a direct G protein activator. In PMN, the time course of the chemiluminescence response was characterized by a prolonged lag period; it ranged from 5 min at an NaF concentration of 25 mM to 20 min at an NaF concentration of 5 mM (data not shown). A similar pattern of the chemiluminescence response was obtained with LMB as target cells. Further experiments were carried out to analyze the modulatory

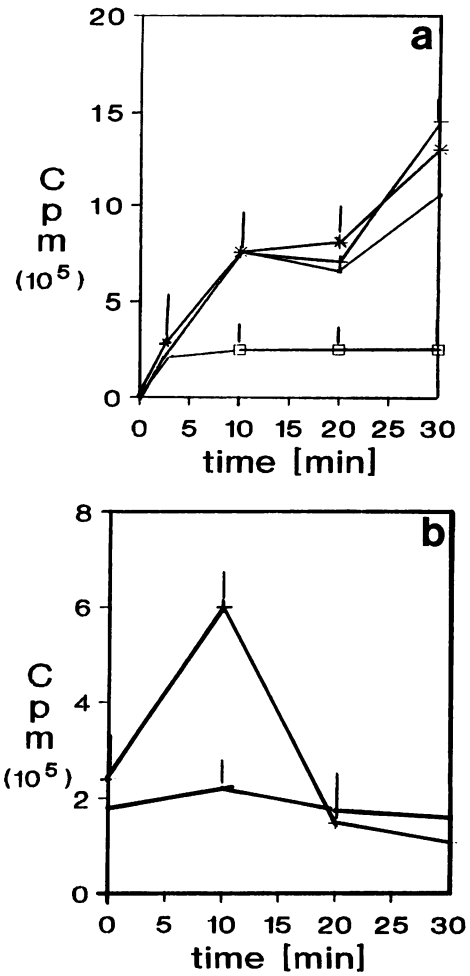

FIG. 3. Kinetics of the chemiluminescence response by *E. coli* strains with various adhesive properties and hemolysin production. Human PMN (2×10^6) were stimulated with washed bacteria (2.5×10^8) at 37°C. (a) *E. coli* strains with different adhesive properties. Symbols: ——, 536/21(pGb30int); —+—, 536/21(pANN921); *, 536/21 (pANN801-1); □, 536/21(pANN801-4). (b) *E. coli* 536/21 (without adhesins) (——) and *E. coli* K-12(pANN5211) (Hly$^+$) (—+—). The chemiluminescence response was monitored at different times for 10 s. The results are expressed in counts per minute. The experiment was performed four times, and the mean values are shown ($n = 4$).

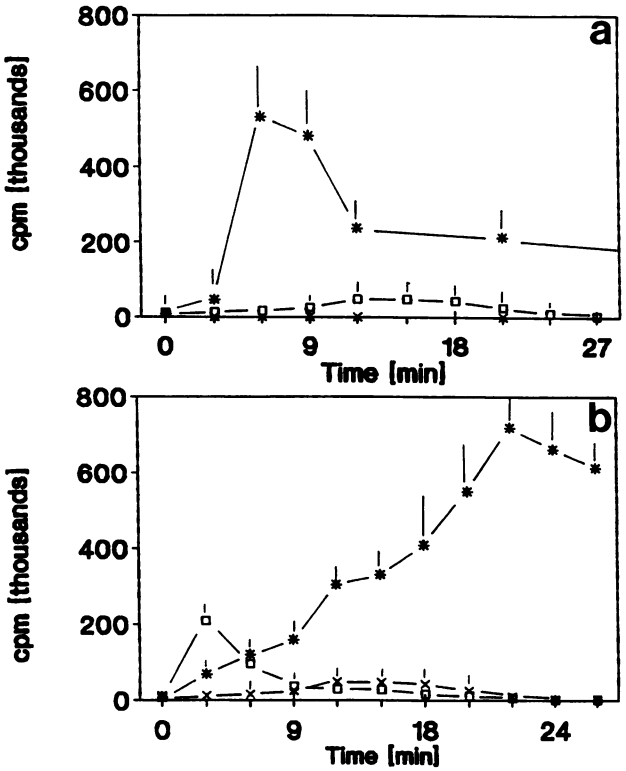

FIG. 4. Modulatory effects of NaF on the chemiluminescence response of stimulated PMN. Purified cells (2×10^6) were incubated with *E. coli* K-12(pANN5211) (a) and *E. coli* 536/21(pANN801-1) (b) in the absence (*) and presence (□) of NaF (12.5 mM). The chemiluminescence response by NaF (12.5 mM) alone (×) is also shown. The data are expressed as means ± standard deviations (n = 3).

effect of NaF in combination with the various bacterial strains (Table 1). For this purpose, cells (2×10^6) were incubated simultaneously with NaF (12.5 mM) and the respective *E. coli* strains for 30 min at 37°C. As shown in Fig. 4 for *E. coli* K-12(pANN5211) and *E. coli* 536/21 (pANN801-1), the combined incubation of NaF and the different *E. coli* bacteria with human neutrophils showed an inhibitory effect on the chemiluminescence response in comparison with bacterial stimulation of the cells in the absence of NaF. *E. coli* K-12(pANN5211) induced a fast chemiluminescence response which reached a maximum after 5 to 10 min of incubation with the cells. At later times, a sharp decline occurred. In comparison, NaF revealed a low chemiluminescence response with a maximum at 10 min of incubation. The simultaneous incubation of NaF and *E. coli* K-12(pANN5211) completely suppressed the chemiluminescence response. *E. coli* 536/21(pANN801-1) induced a strong chemiluminescence response which showed an increase up to an incubation time of 20 min. The combination of NaF and *E. coli* 536/21(pANN801-1) induced a slight increase in the chemiluminescence response up to an incubation time of 5 min; subsequently, the chemiluminescence response was nearly abolished. With the remaining hemolysin-negative strains, an inhibitory effect similar to that of NaF was obtained (data not shown). When LMB were used as target cells, a similar inhibition pattern was obtained (data not shown). It has been established that GTP-γ-S modulates G protein functions in permeabilized cells. To determine the role of G proteins in the chemiluminescence response, cells (10^7) were preincubated with GTP-γ-S (10^{-6} M) or PBS (control) for 5, 10, or 20 min at 37°C and subsequently stimulated with hemolysin-positive or -negative *E. coli* strains for another 30 min. In comparison with the control and in the absence of GTP-γ-S (data not shown), the chemiluminescence response increased to 20%. GTP-γ-S by itself induced no chemiluminescence response.

Effect of G proteins and PKC on LTB_4 generation by bacteria. It was previously shown that mannose-resistant *E. coli* as well as hemolysin-positive *E. coli* bacteria induced leukotriene B_4 (LTB_4) formation from PMNs. With LMB as target cells, mannose-resistant, hemolysin-negative *E. coli* bacteria decreased the LTB_4 formation after subsequent stimulation of the cells with the Ca ionophore A23187 or opsonized zymosan. Experiments were then carried out to analyze the role of G proteins in LTB_4 formation in the presence of the various *E. coli* strains. For this purpose, leukocytes (10^7) were simultaneously stimulated with NaF, a potent LTB_4 activator for PMN, and the Hly^+ and the Hly^- *E. coli* strains. The simultaneous incubation of NaF with *E. coli* K-12(pANN5211) (Hly^+) induced a pronounced increase in LTB_4 release from human PMN over that induced by incubation with *E. coli* K-12(pANN5211) (Hly^+) or NaF separately. The increase in LTB_4 formation was dependent on the NaF concentration (from 2.5 to 25 mM) as well as on the different hemolysin activities (10, 50, and 80%); the bacterial concentration was not varied (see Materials and Methods). With hemolysin-negative bacteria, low amounts of LTB_4 were released. The simultaneous incubation of NaF and hemolysin-negative bacteria showed a two- to threefold increase in LTB_4 generation over that caused by the stimulation of the cells with NaF alone. Figure 5 shows the time course for *E. coli* K-12 in the presence of two different NaF concentrations (2.5 and 12.5 mM). At an NaF concentration of 2.5 mM, an LTB_4 release of 24 ± 4 ng was detected after 15 min of incubation with *E. coli* K-12 compared with 3 ± 2 ng without *E. coli* K-12. Only negligible amounts of LTB_4 were released from human PMNs by *E. coli* K-12. Stimulation with 12.5 mM NaF resulted in a threefold increase in LTB_4 formation in the presence of *E. coli* K-12. *E. coli* strains expressing the various types of adhesins showed no significant differences in their modulatory capacities (data not shown). When human LMB were used as target cells, no modulatory effect of NaF on the LTB_4 generation by the various *E. coli* strains was detected (data not shown). In subsequent experiments, the effect of GTP-γ-S pretreatment on LTB_4 formation induced by NaF and the *E. coli* strains listed in Table 1 were analyzed. The cells (10^7) were preincubated with GTP-γ-S (10^{-6} M) or PBS (as a control) for 10 min at 37°C. Incubation was then continued with the indicated stimuli for various time periods (for PMN, 5, 10, and 15 min; for LMB, 5, 15, and 25 min). The data obtained by HPLC analysis from three independent experiments are expressed in Fig. 6 for NaF (12.5 mM), *E. coli* K-12 (pANN5211), and *E. coli* 536/21(pANN801-4), representative of the hemolysin-negative *E. coli* strains. GTP-γ-S by itself at a concentration ranging from 10^{-4} to 10^{-8} M in the absence of any additional stimuli was not sufficient to induce leukotriene generation either in PMN or in LMB. Pretreatment of PMN with GTP-γ-S significantly decreased the subsequent LTB_4 generation induced by NaF (Fig. 6a) as well as by *E. coli* 536/21(pANN801-4) (Fig. 6b). In contrast, the GTP-γ-S pretreatment of PMN had no effect on leukotriene generation induced by the hemolysin-positive *E. coli* K-12(pANN5211) (Fig. 6c). When GTP-γ-S-pretreated LMB

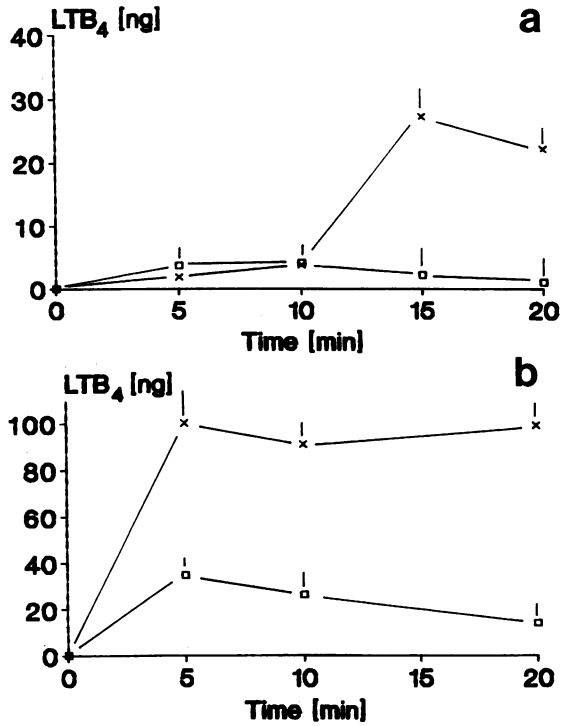

FIG. 5. Modulatory effect of NaF on LTB_4 generation from stimulated neutrophils. Purified cells (10^7) were incubated with NaF (2.5 mM [a] or 12.5 mM [b]) in the absence (□) and presence (×) of *E. coli* K-12 for the indicated time periods at 37°C. The data are expressed as means ± standard deviations (n = 3).

were studied, the NaF-induced (Fig. 6d) and *E. coli* 536/21 (pANN801-4)-induced (Fig. 6e) LTB_4 responses were not affected. In contrast, pretreatment of LMB with GTP-γ-S significantly augmented the *E. coli* K-12(pANN5211)-induced LTB_4 generation (Fig. 6f). NaF by itself is a weak stimulus for LTB_4 formation in LMB.

DISCUSSION

Our results show that hemolysin-producing and hemolysin-negative *E. coli* bacteria differ in their interactions with components of the signal transduction cascade, e.g., PKC, calcium influx, and G proteins.

Thus, Hly^+ as well as Hly^- *E. coli* strains expressing different types of adhesins activate PKC to various degrees. Calcium may also contribute to the regulation of PKC by inducing its translocation from the cytosol to the plasma membrane (20, 31). Furthermore, it has been reported that PKC and calcium may act together in a synergistic manner to produce responses such as LTB_4 generation (37). Human PMN and LMB stimulated with *E. coli* K-12(pANN5211) (Hly^+) induced a rapid increase in intracellular calcium. No calcium increase was observed after stimulation with hemolysin-negative bacteria. It is well established that a bacterium-cell interaction induces G protein activation as is shown by increased Gpp(NH)p binding and altered GTPase activity. G proteins are a heterogeneous family of GTP-binding proteins which differ in their supportive or inhibitory roles in inflammatory cell responses. Our data show that hemolysin-producing *E. coli* bacteria and to a lesser degree Hly^- bacteria, independently of their type of fimbriae, induced enhanced Gpp(NH)p binding and increased GTPase activity in PMN and LMB. However, the experiments do not allow discrimination among the G proteins involved. Therefore, the two substances, NaF, a direct G protein activator of predominantly G_i, and GTP-γ-S, a nonhydrolyzable GTP analog, were included in our studies.

Stimulation of PKC has been suggested to induce the activation of NADPH oxidase and subsequently to lead to superoxide formation. The role of PKC (11) in the neutrophil respiratory burst has been demonstrated. Earlier studies with PKC inhibitors did not elucidate the role of PKC in the activation of NADPH oxidase (51). Our data show a clear correlation between PKC activation and the chemiluminescence response in human PMN and LMB. *E. coli* 536/21 MSH^+ $MS\text{-}Fim^+$ was the most potent stimulus for the chemiluminescence response and also an activator of PKC, followed by *E. coli* 536/21 $P\text{-}MRH^+$ $P\text{-}Fim^+$ and *E. coli* 536/21 $S\text{-}MRH^+$. With hemolysin-producing *E. coli* bacteria, a dose-dependent activation of PKC and the chemiluminescence response were observed in human PMN and LMB.

Bacterial adherence obviously represents the first step in the activation of PMN and LMB. Until now, the role of G protein activation in the chemiluminescence response, which represents an early step in the signal transduction pathway compared with PKC translocation, has not been elucidated. It was recently established that fluoride ions (F^-) are useful tools for the activation of G proteins coupled to adenylate cyclase or to phospholipase C in the absence of receptor occupancy. NaF, an activator of predominantly G_i, by itself leads to only a moderate chemiluminescence response. This response was abolished when PMN or LMB were simultaneously activated with the Hly^+ or Hly^- bacteria and with NaF. These results indicate that G_i activation inhibits the chemiluminescence response. Experiments with GTP-γ-S further support the notion that the bacterium-induced chemiluminescence response is regulated by G proteins. Activation of G proteins occurs when, during ligand-receptor interactions, the bound GDP is exchanged by GTP (29). The α subunit [G(α)-GTP] dissociates from the β chain [G(β,γ)] and modifies the activity of the effector enzymes. Nonhydrolyzable nucleotide analogs of GTP such as GTP-γ-S induce persistent activation of the alpha subunits of the activated G protein. Simultaneous incubation of PMN and LMB with GTP-γ-S led to an enhanced chemiluminescence response. Thus, our data demonstrate that the chemiluminescence response of Hly^+ bacteria as well as of Hly^- bacteria in PMN and LMB seems to be regulated via the same G proteins, which are different from those predominantly activated by NaF.

The role of PKC in mediator release such as LTB_4 formation is not well understood. The direct activation of PKC by phorbol myristic acid (8, 9) does not lead to leukotriene generation. In PMN and LMB, hemolysin-producing *E. coli* K-12(pANN5211) activates PKC and induces LTB_4 generation in a dose-dependent manner. With hemolysin-negative strains, the release of inflammatory mediators from human PMN is dependent on the type of adhesins. In PMN, the highest level of leukotriene generation was obtained with *E. coli* 536/21 $S\text{-}MRH^+$ $S\text{-}Fim^+$; the absence of S fimbriae decreased leukotriene generation in human LMB. Our results show that PKC activation does not correlate with LTB_4 generation from human PMN or LMB.

The identification of at least three forms of PKC (10, 17, 18, 24, 35) and the knowledge that PKC can be activated in either a diacylglycerol-dependent or -independent manner after proteolysis raise the possibility that not all of these forms of the enzyme are equally involved in leukotriene

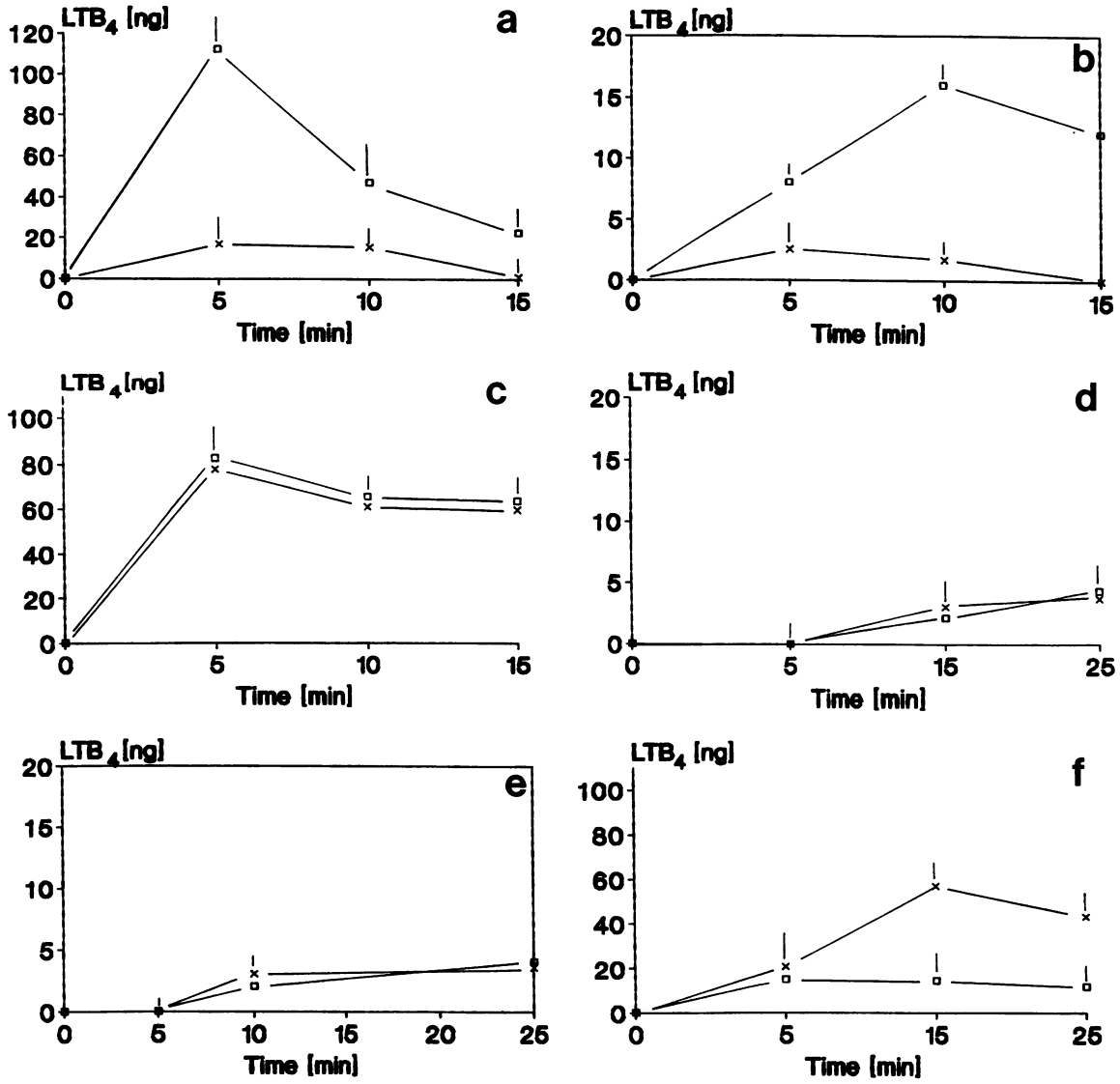

FIG. 6. Effects of GTP-γ-S pretreatment on leukotriene generation. Cells (10^7) were preincubated with GTP-γ-S (10^{-6} M) (×) or PBS (control) (□) for 5 min at 37°C; incubation was then continued with NaF (12.5 mM) (a and d), *E. coli* K-12(pANN5211) (c and f), *E. coli* K-12(pANN801-4) (b and e), or PBS for the time periods indicated. (a, b, and c) PMN; (d, e, and f) LMB. The data represent the means ± standard deviations of three independent experiments.

generation (16, 21, 25, 30, 31). It is likely that the chemiluminescence response and leukotriene generation are mediated predominantly by only one or two of the above-described active species of PKC. Furthermore, it has been reported that isoenzymes of PKC exhibit quantitative differences in the activation by fatty acids, diacylglycerol, and phorbol esters.

Our results support a role of G proteins in the regulation of leukotriene generation from human PMN and LMB stimulated by Hly^+ as well as by Hly^- bacteria. In contrast to human LMB, human neutrophils can be activated by fluoride ions for arachidonic acid release and significant leukotriene release. In combination with a bacterial stimulus, either Hly^- or Hly^+ bacteria, NaF exhibited modulatory effects such as enhanced formation of leukotrienes. Thus, G proteins which are predominantly activated by NaF play a stimulatory role for leukotriene generation from PMN.

Incubation of PMN with the nonhydrolyzable GTP analog GTP-γ-S inhibited the LTB_4 generation after subsequent stimulation with Hly^- *E. coli* bacteria. Thus, Hly^- bacteria apparently activate G proteins which are different from those activated by NaF and Hly^+ bacteria [*E. coli* K-12 (pANN5211)]. The decrease in NaF-induced LTB_4 generation by GTP-γ-S can be explained by an altered ratio of activated G_i to additional activated G proteins.

When LMB were used as target cells, significant LTB_4 release was observed only with *E. coli* K-12(pANN5211) as the stimulus. In LMB, NaF exhibited no modulatory role in LTB_4 generation. Pretreatment of LMB with GTP-γ-S only enhanced the LTB_4 release induced by *E. coli* K-12 (pANN5211) (Hly^+).

Our studies clearly demonstrate that stimulatory as well as inhibitory G proteins may be activated after stimulation with the various bacteria ($Hly^\pm$). Furthermore, our results sug-

gest that hemolysin-negative and -positive bacteria interact with PMN and LMB via different membrane biochemical events. The data may explain our observations that PMN and LMB, after stimulation with *E. coli* bacteria, respond in different ways with regard to the formation of leukotrienes.

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