

## G protein activation and mediator release from human neutrophils and platelets after stimulation with sodium fluoride and receptor-mediated stimuli

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### SUMMARY

Human polymorphonuclear granulocytes (PMN) and platelets were pre-activated with a receptor-mediated stimulus [formyl-methionyl-leucyl-phenylalanine (FMLP) or thrombin, respectively] and subsequently incubated with sodium fluoride (NaF). We investigated various cell responses, such as chemiluminescence by PMN, platelet aggregation and the release of lipid mediators [i.e. leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and its  $\omega$ -oxidation products from neutrophils and 12-hydroxy-eicosatetraenoic acid (12-HETE) from platelets]. As a marker of G protein involvement, the binding of [<sup>3</sup>H]guanylylimidodiphosphate (Gpp (NH) p) to the membrane fractions of stimulated cells was determined. PMN pre-stimulated with FMLP showed a synergistically enhanced generation of leukotrienes returning to control values with the time of preincubation. Platelets preliminary treated with thrombin followed by incubation with NaF resulted in a sub-additive and time-independent mediator generation. Neither chemiluminescence by PMN nor platelet aggregation showed a similar pattern compared to the mediator release: PMN preincubated with FMLP followed by NaF resulted in a second chemiluminescence response; the aggregation of platelets which were preincubated with thrombin was partially inhibited by the addition of NaF. Membrane fractions isolated from FMLP-pre-stimulated neutrophils showed a pattern in [<sup>3</sup>H]Gpp (NH) p-binding capacity that was comparable to the respective leukotriene release. With thrombin-prestimulated platelets, no similarities between Gpp (NH) p binding, aggregation or 12-HETE generation were observed. The sequential activation of different cell populations using the same kind of stimulation lead to different cell responses, indicating the diversity of G proteins and their control mechanisms.

### INTRODUCTION

Neutrophils as well as platelets participate in the regulation of inflammatory reactions. Platelet activation involves changes in the plasma membrane that lead to adhesion followed by aggregation of platelets and release of preformed vasoactive substances. Respective stimuli are epinephrine, thrombin or the platelet-activating factor (PAF). However, polymorphonuclear granulocytes (PMN) also show a characteristic biological profile (e.g. chemotactic migration, phagocytosis, or the genera-

tion of oxygen metabolites) towards specific activators such as formylated peptides, C5a or leukotriene B<sub>4</sub> (LTB<sub>4</sub>). In addition, both cell populations generate products of the arachidonic acid metabolism.<sup>1</sup> The cells have similar cyclo-oxygenase but strikingly different lipo-oxygenase pathways. Platelets generate the unstable peroxide 12-HPETE and 12-HETE via the 12-lipo-oxygenase pathway.<sup>2</sup> In contrast, neutrophils mainly convert arachidonic acid via the 5-lipo-oxygenase to leukotrienes and 5-HETE.<sup>3</sup> The products of both pathways are important mediators of inflammation. A biochemical interaction between the neutrophil 5-lipo-oxygenase and the platelet 12-lipo-oxygenase was described by Marcus *et al.*<sup>4</sup>

Abbreviations: FMLP, formyl-methionyl-leucyl-phenylalanine; Gpp (NH) p, guanylylimidodiphosphate; G proteins, guanine nucleotide-binding proteins; 12-HETE, 12-hydroxy-eicosa-6, 8, 11, 14-tetraenoic acid; 12-HPETE, 12-hydroperoxy-eicosa-6, 8, 11, 14-tetraenoic acid; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; NaF, sodium fluoride; PMN, polymorphonuclear granulocytes.

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Common to all physiological stimuli is their binding to specific receptors on the cell surface. The signal emitted from the occupied receptors is transduced through the plasma membrane by guanine nucleotide-binding proteins (G proteins).<sup>5</sup> G proteins are heterotrimeric complexes consisting of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. After receptor–ligand coupling, GDP, which has tightly bound to the  $\alpha$ -subunit, is substituted by GTP. As a result, the G protein complex dissociates and G $\alpha$ -GTP interacts

with the respective effector enzymes. An intrinsic GTPase activity hydrolyses GTP to GDP, thus terminating the G protein-mediated signal.

Characteristic receptor-mediated stimuli are thrombin for platelets and formylated peptides for neutrophils, respectively. The activated thrombin receptor interacts with two distinct G proteins,  $G_i$  inhibiting the adenylate cyclase and  $G_p$  activating the phosphoinositide metabolism.<sup>6,7</sup> However, it has been suggested that platelets have two distinct receptors for thrombin.<sup>8</sup> The most extensively studied neutrophilic receptor is that for N-formylated methionyl peptides. The formyl-peptide receptor seems to exist with two binding affinities, the low- and the high-affinity subsets, transmitting different biological responses.<sup>9</sup> It has been established that leucocytes contain a unique pertussis/cholera toxin-sensitive G protein that mediates chemoattractant-induced cell stimulation. G proteins are directly activated by fluoride ions bypassing receptor occupancy. Addition of NaF to human neutrophils or platelet suspensions results in the activation of the signal transduction pathway and various cellular responses.<sup>10-12</sup>

Previously, we reported the effects of NaF on the generation of lipo-oxygenase products from human neutrophils, mononuclear cells and platelets.<sup>13</sup> The studies showed that G protein activation is involved in subsequent leucotriene formation. The purpose of this study was to analyse whether the ligand-induced biological responses are modulated by direct G protein activation and to what extent the ligand-induced G protein activation may modulate the subsequent cellular response.

## MATERIAL AND METHODS

### Materials

LTB<sub>4</sub>, 20-OH-LTB<sub>4</sub> and 20-COOH-LTB<sub>4</sub> were kindly provided by Dr Rokach, Merck-Frosst, Pointe Claire, Canada. [<sup>3</sup>H]Gpp (NH) p (specific activity 185–550 GBq/mmol) was obtained from Amersham Buchler, Braunschweig, Germany. The reagents used were from the following sources: Ficoll 400 (Pharmacia, Uppsala, Sweden); Macrodex (6%) (Knoll, Ludwigshafen, Germany); sodium metrizoate solution (75%) (Nycomed, Oslo, Norway); heparin, FMLP, t-BOC-MLP, cytochalasin B, NaF, luminol, Gpp (NH) p, adenylylimidodiphosphate, ATP, ouabain, EGTA, leupeptin, dithiothreitol and thrombin (Sigma, Deisenhofen, Germany). All other chemicals were from Merck, Darmstadt, Germany.

### Preparation of cells

PMN were purified from heparinized blood (15 U/ml) of healthy donors. The neutrophils were isolated using a Ficoll-metrizoate gradient followed by dextran sedimentation, as described by Böyum.<sup>14</sup> Cells were suspended in Dulbecco's phosphate-buffered saline (PBS). Human platelets were isolated using EDTA anti-coagulant (1.5% EDTA in 0.9% NaCl, w/v) at a ratio of 9 parts blood to 1 part anti-coagulant. The bulk of erythrocytes and leucocytes was removed by sedimentation at 200 g for 25 min at 20° (Minifuge T, Heraeus Sepatech, Osterode, Germany). The platelets were centrifuged at 1300 g for 20 min at 4°, washed and resuspended in PBS (pH 7.4) up to a final concentration of 2 × 10<sup>8</sup> cells/ml. All isolation procedures were performed using plastic.

### Stimulation of the cells

PMN (1 × 10<sup>7</sup> cells/500 μl) or platelets (1 × 10<sup>8</sup> cells/500 μl) were preincubated in the presence of calcium (0.9 mM) and magnesium (0.5 mM) for the indicated time periods at 37° with the respective stimuli (20 mM NaF, 10<sup>-6</sup> M FMLP, 10<sup>-6</sup> M t-BOC-MLP or 2 U/ml thrombin). Prior to activation with FMLP, an incubation of the cells with cytochalasin B (5 μg/ml) was performed for 2 min at 37°. The reaction was stopped by centrifugation (with PMN, 300 g for 10 min at 4°; with platelets 1300 g for 15 min at 4°). The cells were resuspended in PBS and incubated with the indicated agents in the presence of calcium and magnesium for further 20 min at 37°. The incubations were terminated by centrifugation as described above. For leukotriene analysis, 2 ml of methanol/acetonitrile (50:50, v/v) were added to the resulting supernatants, overlaid with argon and stored at -70°. For membrane preparations, the cell pellets were resuspended in Tris (0.05 M)/sucrose (0.25 M) buffer, pH 7.5, and stored at -70°.

### Analysis of chemiluminescence

Chemiluminescence was studied by preincubating PMN (1 × 10<sup>6</sup> cells/50 μl) in PBS containing calcium (1.8 mM) and magnesium (1 mM) and luminol (13 μM) for 15 min at 37°. Subsequently, the stimuli NaF (20 mM) and FMLP (2 × 10<sup>-7</sup> M) were added. Chemiluminescence was monitored with a Lumacounter M 2080 (Lumac, Schaesberg, The Netherlands).

### Platelet aggregation

Platelet suspensions (2 × 10<sup>8</sup> cells/ml) were transferred into aggregometer cuvettes and prewarmed at 37°. Aggregation was monitored after the addition of calcium (0.9 mM) and magnesium (0.5 mM) and the respective stimuli (20 mM NaF and 2 units/ml thrombin) upon stirring using a Whole-Blood Aggrometer (Chrono-Log Corporation, Havertown, PA).

### Binding of [<sup>3</sup>H]Gpp (NH) p

Purified neutrophils were resuspended at a concentration of 1 × 10<sup>7</sup> cells/ml in Tris buffer (0.05 M, pH 7.5) supplemented with sucrose (0.25 M), EDTA (1 mM), EGTA (1 mM), dithiothreitol (1 mM) and leupeptin (100 μg/ml) were added and cell disruption was carried out by sonication in three periods over 10 seconds (energy output 40 W, Branson sonifier 250W). Light microscopy revealed a complete cell breakage. Suspensions were centrifuged first at 10,000 g for 20 min (J2-21, rotor JA-20, Beckman, Palo Alto, CA) followed by centrifugation of the respective supernatant at 100,000 g for 60 min (Beckman centrifuge L8-70, rotor SW60Ti). The 100,000 g pellet was characterized as a microsomal fraction by biochemical analysis.<sup>15</sup> The protein content was assayed according to the method of Lowry *et al.*<sup>16</sup>

The binding of [<sup>3</sup>H]Gpp (NH) p was measured as previously described.<sup>17</sup> The membrane fraction (10 μg protein) was incubated in 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM adenylylimidodiphosphate, 0.25 mM ouabain and 0.5 μM [<sup>3</sup>H]Gpp (NH) p. After 60 min at room temperature incubations were terminated by filtration through cellulose ester membranes (pore size 0.45 μm, Millititer HA filters, Millipore, Eschborn, Germany). The filters were washed four times with Tris buffer (20 mM) containing 0.25 mM MgCl<sub>2</sub> and 50 μM EGTA. The filters were measured for radioactivity by liquid scintillation counting (LKB Rack Beta 1209, Turku, Finland). The non-specific

binding component was determined in the presence of a 1000-fold molar excess of non-labelled Gpp (NH) p. The specific binding was calculated by subtracting the non-specific binding component from total binding. The specific binding component was  $63.4 \pm 18.0\%$  (mean  $\pm$  SD,  $n = 10$ ) with unstimulated PMN membranes and  $67.5 \pm 18.8\%$  ( $n = 8$ ) with unstimulated platelet membranes, respectively.

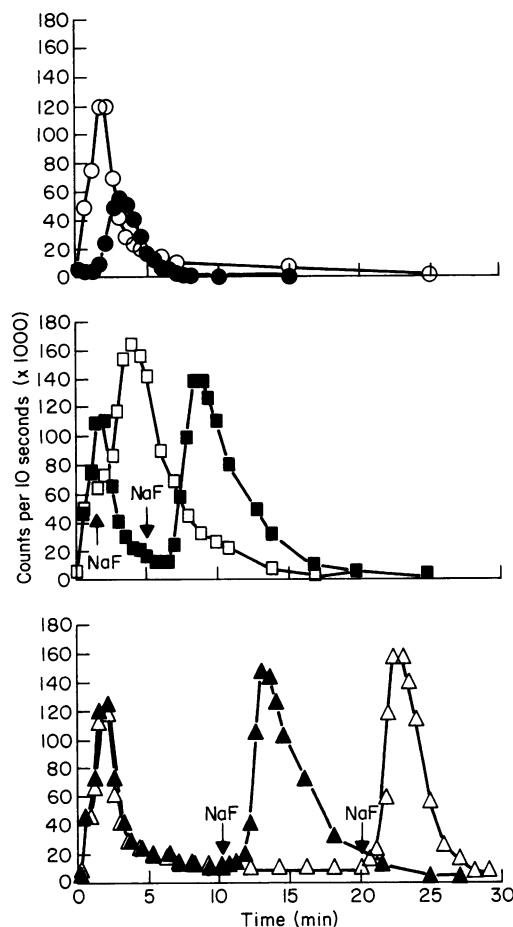
#### Analysis of lipid mediators

After centrifugation at 1900 *g* for 15 min (Cryofuge 6-4, Heraeus Christ, Osterode, Germany) the supernatants were evaporated to dryness by lyophilization (Modulyo, Edwards-Kniese, Marburg, Germany). The residues were dissolved in 600  $\mu$ l of methanol/water (30:70, v/v) and 200  $\mu$ l were analysed by reversed-phase high-pressure liquid chromatography.<sup>18</sup> The column (4.6  $\times$  200 mm) was packed with Nucleosil C<sub>18</sub> (particle size 5  $\mu$ m; Macherey-Nagel, Düren, Germany). HPLC equipment consisted of a CM4000 pump, a SM4000 detector (both Laboratory Data Control/Milton Roy, Hasselroth, Germany), and an automatic sample injector (WISP 710B, Waters, Eschborn, Germany). Peak integration was carried out by using a chromatographic software (system 2600, Nelson Analytical, Cupertino, CA). Leukotrienes were analysed using a mobile phase consisting of methanol/water/acetonitrile/phosphoric acid (48:24:28:0.03, v/v) including 0.04% EDTA and 0.15% K<sub>2</sub>HPO<sub>4</sub>, pH 5.0. The flow rate was maintained at 0.9 ml/min, leukotrienes were detected at 270 nm. Overall recoveries were between 81% and 86%. 12-HETE was analysed using a mobile phase consisting of methanol/water/acetonitrile/phosphoric acid (42:28:30:0.03, v/v) containing 0.02% EDTA and 0.1% K<sub>2</sub>HPO<sub>4</sub> in the aqueous phase (pH 5.0). The flow rate was maintained at 0.9 ml/min. 12-HETE was detected at 235 nm.

## RESULTS

### Biological responses of neutrophils and platelets induced by receptor-mediated stimuli and NaF

Experiments were carried out to study the chemiluminescence by PMN and the ability of platelets for aggregation following ligand-induced cell activation and sequential stimulation with NaF. Chemiluminescence of neutrophils was detected after preloading the cells with luminol at 37°. PMN ( $1 \times 10^6$  cells) were then incubated with FMLP ( $2 \times 10^{-7}$  M) or NaF (20 mM) in the presence of 1 mM calcium and 0.5 mM magnesium (Fig. 1). FMLP induced an immediate response with a maximal rise in chemiluminescence within 2 min; the NaF-induced chemiluminescence response was observed after its characteristic lag phase, with the maximum at 4 min. In subsequent experiments neutrophils were pre-stimulated with FMLP over different time periods followed by the addition of NaF. As is apparent, the addition of NaF resulted in a second chemiluminescence peak. Treatment of the neutrophils with the consecutive stimulus (e.g. FMLP followed by FMLP or NaF followed by NaF) showed no second chemiluminescence response (data not shown). Preincubation of the cells with NaF totally inhibited the subsequent FMLP-induced chemiluminescence response. These data suggest a cellular desensitization by FMLP as the result of receptor down-regulation, whereas stimulation with NaF causes a block distal of the G proteins which inhibits further production of oxygen radicals.

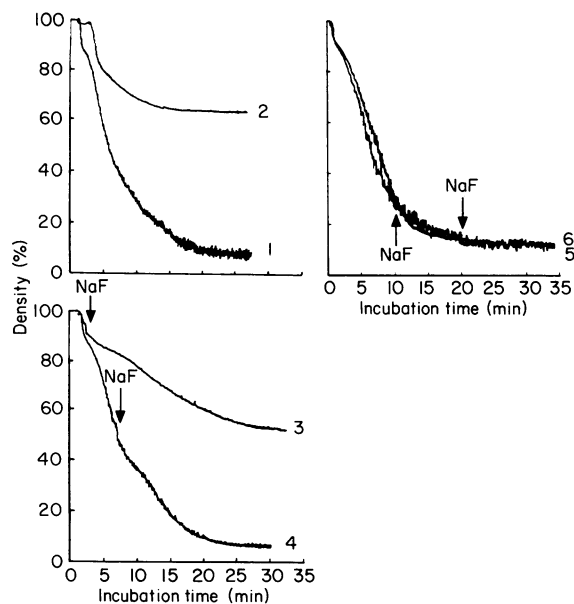


**Figure 1.** Chemiluminescence response by human neutrophils. Luminol-preloaded PMN ( $1 \times 10^6$  cells) were stimulated with FMLP ( $2 \times 10^{-7}$  M;  $\circ$ ) or NaF (20 mM;  $\bullet$ ) or pre-stimulated with FMLP for 1 min ( $\square$ ), 5 min ( $\blacksquare$ ), 10 min ( $\blacktriangle$ ) and 20 min ( $\triangle$ ) followed by the addition of NaF. The ordinate shows the integration of counts for 10 seconds. A representative experiment out of three is shown.

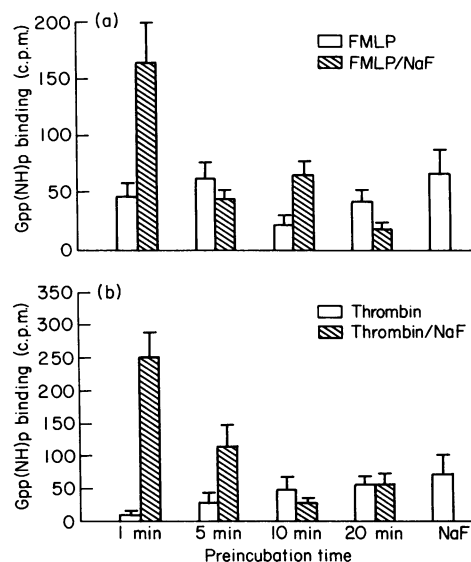
Figure 2 shows the effects of the receptor-mediated stimulus thrombin (2 U/ml) and NaF (20 mM) on platelet aggregation. Compared with the pattern induced by thrombin, NaF showed only a weak and delayed aggregation. Addition of NaF to platelets which were pretreated with thrombin for 1 or 5 min partially inhibited the subsequent aggregation of the cells. However, there is a difference between the regulatory mechanisms involved in the neutrophilic chemiluminescence response and the thrombin-induced platelet aggregation.

### Generation of lipid mediators by neutrophils and platelets

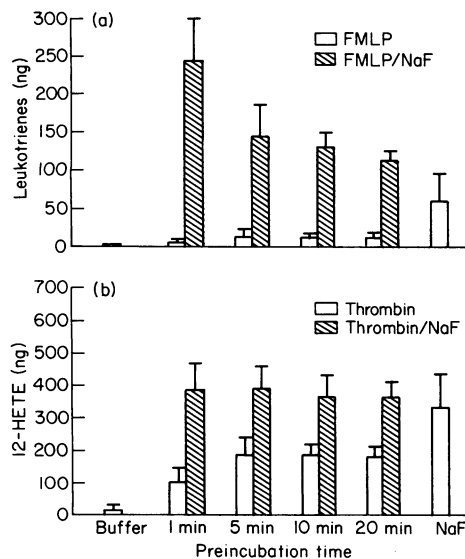
Incubation of human PMN ( $1 \times 10^7$  cells/0.5 ml) with FMLP ( $10^{-6}$  M) or NaF (20 mM) resulted in the generation of leukotrienes (defined as the sum of LTB<sub>4</sub>, 20-OH-LTB<sub>4</sub> and 20-COOH-LTB<sub>4</sub>). Additional products of the leukotriene pathway (the non-enzymatic LTB<sub>4</sub> metabolites, 5S, 12S-DiHETE or the cysteinyl-leukotrienes) were negligible compared to the amounts of LTB<sub>4</sub> and its  $\omega$ -oxidized products. PMN were then preincubated with FMLP for 1–20 min and subsequently stimulated with NaF for further 20 min. The respective controls were cells preincubated in the presence of PBS and then



**Figure 2.** Aggregation of human platelets. Platelets ( $2 \times 10^8$  cells/ml) were stimulated with thrombin (2 U/ml, curve 1) or NaF (20 mM, curve 2) or pre-stimulated with thrombin for 1 min (curve 3), 5 min (curve 4), 10 min (curve 5) and 20 min (curve 6) followed by the addition of the second activator NaF (20 mM). A representative experiment out of four is shown.



**Figure 4.** Binding of [ $^3$ H]Gpp (NH) p to isolated membrane fractions of pre-stimulated neutrophils or platelets. PMN (a) or platelets (b) were stimulated with the receptor-mediated stimuli and/or NaF as was described in the legend of Fig. 3. The isolated membrane fractions (protein content 10  $\mu$ g) were analysed for the capacity of [ $^3$ H]Gpp (NH) p binding (0.5  $\mu$ M). The ordinate shows the specific binding of [ $^3$ H]Gpp (NH) p in c.p.m.; the data were corrected with regard to the respective basal binding component of unstimulated cells. Represented values are means  $\pm$  SD from four independent experiments.



**Figure 3.** Mediator generation by pre-stimulated neutrophils or platelets. (a) PMN ( $1 \times 10^7$  cells) were pretreated with FMLP ( $10^{-6}$  M) for the indicated time periods and subsequently stimulated with buffer or NaF (20 mM) for further 20 min. The ordinate shows the release of LTB<sub>4</sub> and its  $\omega$ -oxidized products in ng. (b) Platelets ( $1 \times 10^8$  cells) were pretreated with thrombin (2 U/ml) for the indicated time periods and subsequently stimulated with buffer or NaF (20 mM) for further 20 min. The ordinate shows the release of 12-HETE in ng. The bars on the left side represent the formation of leukotrienes of 12-HETE by unstimulated cells, the bars on the right side the NaF-induced mediator generation after 20 min. All values were means  $\pm$  SD from four independent experiments.

stimulated with NaF for 20 min as well as cells pretreated with FMLP for the indicated time periods and further incubated with buffer for 20 min. All experiments were carried out in the presence of calcium (1 mM) and magnesium (0.5 mM) at 37°. The incubations with FMLP were performed in the presence of cytochalasin B, which enhanced the low generation of leukotrienes induced by FMLP. Cytochalasin B alone did not affect the formation of leukotrienes (data not shown). As is shown in Fig. 3a, the formation of leukotrienes induced by the receptor-mediated stimulus was synergistically enhanced after the addition of NaF. A maximal response was observed already by 1 min after pretreatment with FMLP. Prolonged preincubation, e.g. more than 5 min, reduced the leukotriene generation.

In addition, purified platelets ( $1 \times 10^8$  cells/0.5 ml) were stimulated with the receptor-mediated stimulus thrombin (2 U/ml) or the direct G protein activator NaF (20 mM) and the formation of the lipid mediator 12-HETE was analysed (Fig. 3b). No conversion products of 12-HETE were observed by HPLC analysis under the experimental conditions. The generation of 12-HETE induced by thrombin was detected within 1 min of incubation, whereas after 5 min of incubation no further increase in mediator release was observed. When platelets were incubated with NaF, 12-HETE was released in a time-dependent manner, but a lag phase of about 5 min was required (data not shown). According to the experimental design described for neutrophils, platelets were first stimulated with thrombin over the indicated time periods followed by a subsequent incubation with NaF for 20 min. The amounts of the released 12-HETE (about 380 ng) were independent of the duration used to preactivate the platelets with thrombin. It is apparent that the

value was below the sum of the respective control values which amounted to 550 ng.

### Involvement of G proteins

Since NaF, thrombin, as well as FMLP, stimulate cells via G proteins, the binding of [<sup>3</sup>H]Gpp (NH) p was measured (Fig. 4). Intact PMN ( $4 \times 10^7$  cells/2 ml buffer) or platelets ( $6 \times 10^8$  cells/3 ml buffer) were preincubated with the receptor-mediated stimulus for the indicated time periods at 37°. The cells were subsequently activated with NaF (20 mM) for further 20 min. FMLP as well as NaF activate intact PMN and show an increased Gpp (NH) p-binding. Pre-treatment of PMN with FMLP for only 1 min showed an enhanced binding of [<sup>3</sup>H]Gpp (NH) p. A prolonged preincubation resulted in a reduced binding which then returned to control values (Fig. 4a). In correlation to experiments in which leukotriene generation was studied, FMLP was also used in the presence of cytochalasin B. Incubation of cells with cytochalasin B alone showed no effects on Gpp (NH) p-binding. In addition, a synergistic enhanced binding capacity was only observed after preincubation of platelets with thrombin for 1 min (Fig. 4b). After prolonged preincubation periods the values obtained after simultaneous stimulation with thrombin and NaF were similar compared to the control values.

To exclude artificial effects induced during membrane preparation, FMLP was substituted by equimolar concentrations of the non-formylated peptide t-BOC-MLP. No synergistic effects with respect to leukotriene generation and Gpp (NH) p-binding capacity were observed (data not shown).

### DISCUSSION

This study analyses the mutual interaction of ligand- and G protein-mediated activation with regard to the chemiluminescence response of human neutrophils and platelet aggregation as immediate biological responses, as well as the generation of potent lipid mediators. Whereas agonists have their effects on the receptor outside the cell, fluoride ions are direct activators of multiple G proteins bypassing the receptor/G protein interaction. Previously, the involvement of the inositol phosphate pathway, calcium mobilization, protein kinase C activation or effects on adenylate cyclase have been described.<sup>10,19</sup> However, it cannot be excluded that fluoride ions act on a signalling element that is not a G protein. Previously, it was suggested that NaF also stimulates cytosolic phospholipase C followed by association of the enzyme with the membrane. It was concluded that NaF activates a soluble G protein.<sup>20</sup> Furthermore, an unspecific effect of fluoride ions was excluded, substituting NaF by equimolar concentrations of sodium bromide or sodium iodide. In these experiments no effects were obtained (own unpublished observations).

Our results show that pre-stimulation of PMN with the receptor-mediated stimulus FMLP and subsequent addition of NaF led to a second chemiluminescence pattern. However, preliminary treatment with NaF inhibited the response towards the subsequent stimulus of FMLP. In addition, this inhibition was also observed when the cells were consecutively stimulated with first FMLP and then FMLP or first NaF and then NaF. These results indicate a reduced biological response towards FMLP as a consequence of receptor down-regulation or an

inhibition of receptor/G protein interaction. However, cellular activation with NaF seems to inhibit the chemiluminescence response distal the G protein level. Aggregation of platelets induced by short-term incubation with the receptor-mediated stimulus thrombin was prevented by the addition of the second activator NaF. This inhibition was not observed when thrombin was substituted by the calcium ionophore (unpublished observations). Therefore, we conclude that the addition of NaF modulates the interaction between the thrombin receptor and the G proteins. It was postulated that two GTPases were stimulated by thrombin in membranes of human platelets. However, it may be also possible that different signal transduction pathways, including different G-proteins, are involved in the cell activation by NaF and thrombin.<sup>6,11</sup>

With regard to mediator generation our data demonstrate that preincubation of PMN with FMLP followed by stimulation with NaF shows a significant synergism or an additive leukotriene formation. In contrast, sequential stimulation of platelets with thrombin/NaF resulted in only sub-additive effects when the generation of 12-HETE was studied.

The response of neutrophils or platelets towards two different agonists may exceed the arithmetic sum of each response to the individual agonist alone. This synergism implicates a positive feedback amplification of the external signals. In this regard, stimulation of platelets results in the release of, for example, ADP or serotonin, which may support the initial activation of the cell.<sup>21</sup> Moreover, arachidonic acid metabolites such as prostaglandins, thromboxanes or leukotrienes are able to amplify a suboptimal stimulatory response.<sup>22</sup> Furthermore, activation of protein kinase C and the mobilization of calcium synergistically potentiates diverse cell functions, including platelet aggregation and secretion.<sup>23</sup> With human neutrophils as target cells, an enhanced generation of leukotrienes was observed following cell stimulation with phorbol myristate acetate and FMLP or calcium ionophore A23187 at various concentrations.<sup>24</sup>

G proteins are essential prerequisites to transduce cell biological signals. In this regard, the binding of the non-hydrolysable GTP analogue Gpp (NH) p to isolated membranes was enhanced after cell stimulation. These effects correlated with the respective synergism of leukotriene generation, suggesting a causal relationship; cellular activation affected the binding of Gpp (NH) p and by this means modulated G protein involvement. It may be possible that NaF acts on a regulatory factor which enhances the exchange of GDP for GTP, as was recently isolated and characterized for rasP21 proteins.<sup>25</sup> Furthermore, there have been implications that arachidonic acid might be released from the cell membranes independently of inositol phosphate generation, possibly due to a direct coupling of a receptor to phospholipase A<sub>2</sub> by a G protein.<sup>26,27</sup> The time-course of leukotriene generation correlated with Gpp (NH) p binding in activated PMN and showed a maximal synergism within 1 min of preincubation with FMLP. Prolonged incubation with FMLP revealed that the cells became deactivated towards a subsequent stimulus. We suggested an uncoupling mechanism of G proteins and of the effector enzyme (see also Wilde *et al.*)<sup>28</sup> resulting in a cellular state which does not allow a synergistic response. In contrast, no correlation was observed between the pattern of 12-HETE generation and the respective Gpp (NH) p binding. The different cellular responses of neutrophils and platelets using the same kind of stimulation

indicate a different set of G proteins in these cell populations. The respective heterogeneity of the G protein family was described previously.<sup>29</sup> A further explanation for the inhibitory effects using platelets may be due to the activation of negative feedback mechanisms (e.g. the involvement of protein kinase C or changes in cAMP levels) after the addition of potent stimuli.

The comparison of diverse cell responses observed with human neutrophils or platelets suggests multiple pathways of cellular activation.<sup>30</sup> In this way, G proteins do not only mediate and amplify external signals, but show a regulatory and modulatory role.

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