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# Inhibition of Cytoskeletal Rearrangement by Botulinum C2 Toxin Amplifies Ligand-Evoked Lipid Mediator Generation in Human Neutrophils

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**SUMMARY**

Botulinum $C_2$ toxin, a binary toxin that ADP-ribosylates nonmuscle G-actin, was used as a selective tool to evaluate the role of actin-dependent cytoskeletal rearrangement in ligand-evoked lipid mediator generation. Human neutrophils (PMN) were preincubated with varying concentrations of the toxin for 30 min. Lipoxygenase products of arachidonic acid were measured by chromatographic techniques in the presence of exogenous arachidonic acid to probe PMN 5-lipoxygenase activity. Formation of platelet-activating factor (PAF) was assayed by the bioincorporation of [$^3$H]acetate. Stimulation was performed with the soluble chemotatic ligands formyl-methionyl-leucyl-phenylalanine (FMLP) and PAF, as well as opsonized zymosan. PMN pretreatment with $C_2$ toxin in the range between 200/400 and 800/1600 ng/ml $C_{2_{I/II}}$ caused a dose-dependent suppression of the basal F-actin content and of stimulus-induced actin assembly. Phosphoinositide hydrolysis (measured as liberated inositol phosphates) and PAF generation in response to FMLP and exogenous PAF were markedly increased at these toxin doses. Minor $C_2$ toxin concentrations (range, ≈25/50 to 200/400 ng/ml $C_{2_{I/II}}$) were sufficient to amplify stimulus-induced formation of leukotriene $B_4$ and its ω-oxidation products, nonenzymatic hydrolysis products of leukotriene $A_4$, and 5-hydroxyeicosatetraenoic acid (5-HETE). With increasing toxin doses, leukotriene generation declined and 5-HETE became the predominant metabolite. In contrast to the soluble ligands, the zymosan-effected generation of PAF and leukotrienes was dose-dependently inhibited by $C_2$ toxin concentrations of >200/400 ng/ml, paralleled by a loss of motile and phagocytotic functions in these cells. We conclude that selective inhibition of actin assembly amplifies PAF and 5-lipoxygenase product formation in response to soluble chemoattractants with distinct dose dependences. The augmentation of PAF generation may be linked to amplified second messenger levels at higher doses of $C_2$ toxin, whereas the sensitivity of the 5-lipoxygenase metabolism to low concentrations may indicate toxin effect on a small, functionally specified, actin pool. The present data support an important role of cytoskeletal rearrangement in temporal and/or spatial limitation of chemoattractant-evoked PMN activation.

Lipoxygenase products of AA, in particular LTs and 5-HETE, as well as PAF, are involved in a large variety of inflammatory processes (1, 2). PMN possess the capability of generating large amounts of these lipid mediators upon challenge with the calcium ionophore A23187 (1, 3–5). In contrast, naturally occurring inflammatory ligands such as FMLP and C5a, as well as the lipid chemoattractants PAF and $LTB_4$ themselves, are poor activators of PMN eicosanoid metabolism (5–7) and PAF generation (8). Eicosanoid formation in ligand-exposed PMN is, however, markedly increased upon simultaneous supply with exogenous precursor AA. Substantial quantities of $LTA_4$, $LTB_4$ and its metabolites, and 5-HETE are then released in response to both peptide and lipid chemoattractant challenge (5, 6).[1] These findings suggest activation of the 5-lipoxygenase pathway in ligand-activated PMN, in the absence of significant endogenous AA liberation. Ligand effects on the neutrophil 5-lipoxygenase pathway are, thus, suitably probed upon disposal of free exogenous AA.

PMN stimulation with soluble and particulate ligands is known to induce rapid cytoskeletal rearrangement; this was suggested to serve as a negative feedback loop, effecting temporal and/or spatial limitation of ligand-evoked cell activation (9–11). The sequential events of cytoskeletal reorganization are best characterized for FMLP receptor occupancy. They include short-lived initial actin disassembly and a subsequent sharp

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[1] F. Grimminger *et al.* Submitted for publication.

**ABBREVIATIONS:** AA, arachidonic acid; FMLP, formyl-methionyl-leucyl-phenylalanine; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; PMN, polymorphonuclear leukocyte(s); PAF, platelet-activating factor; PT, pertussis toxin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DMSO, dimethylsulfoxide; diHETE, dihydroxyeicosatetraenoic acid; $IP_1$, inositol monophosphate; $IP_2$, inositol diphosphate; $IP_3$, inositol triphosphate; HPLC, high performance liquid chromatography.

increase in F-actin formation in concert with filament cross-linking, promoting an actin network-related gel state in cytoplasmatic spaces. Evidence for a role of the cytoskeletal rearrangement in limiting PMN activation was obtained from investigations with the F-actin filament-disrupting agents cytochalasin B and D (12, 13), which increase PMN superoxide generation, degranulation, and diacylglycerol formation in response to FMLP challenge (14–16). Ligand-evoked calcium transients and inositol phosphate accumulation were, however, found to be not, or not uniformly, influenced by these agents (16–18). The very low PAF generation elicited upon neutrophil FMLP stimulation was only slightly increased by pretreatment with cytochalasins (8). The interpretation of cytochalasin B/D-related data is complicated by the fact that these agents may promote PMN activation by themselves (17, 19) and that several cytochalasin binding sites on the actin molecule exist (13, 20), resulting in complex interaction of these agents with the actin-based microfilamentous network.

A recently described specific tool for evaluating the impact of actin assembly on cell functions is the botulinum $C_2$ toxin. This is a binary toxin, consisting of the two components $C_{2_I}$ and $C_{2_{II}}$ (21, 22). $C_{2_{II}}$ promotes the translocation of component I into eukaryotic cells; $C_{2_I}$ selectively ADP-ribosylates G-actin (23, 24). Like diphtheria (25), pertussis (26), and cholera toxins (27), botulinum $C_2$ toxin thus belongs to a family of microbial agents that interfere with target cell function by specifically affecting regulatory proteins (28). ADP-ribosylation of non-muscle G-actin occurs at arginine-177 (29). This covalent G-actin modification leads to a drastically reduced rate of polymerization of isolated G-actin (23, 24). In addition, G-actin is converted into a capping protein, which binds to the barbed end of actin filaments and thereby inhibits the polymerization at this site, whereas the depolymerization at the pointed end is not affected; as a net effect, progressive cleavage of F-actin occurs (30).

In two preceding studies on $C_2$ toxin-pretreated human PMN, ADP-ribosylation of actin and suppression of ligand-evoked F-actin formation was demonstrated (31, 32). This interference with the cytoskeletal response resulted in a loss of motile cell functions and an enhancement of FMLP-elicited superoxide generation and degranulation. FMLP receptor binding and dissociation dynamics were, however, not altered, and the endocytosis of the ligand-receptor complex was only slightly retarded (32), favoring predominant impact of the bacterial agent on postreceptor events. Here we describe marked amplification and modulation of ligand-evoked eicosanoid and PAF synthesis in $C_2$ toxin-primed PMN, whereby dissociated dose-effect relationships suggest distinct efficacies of this experimental tool on the 5-lipoxygenase and the PAF synthesis pathways. These data support a significant role of the actin-based cytoskeleton in the regulation of chemoattractant-elicited PMN activation.

## Experimental Procedures

**Materials.** FMLP, PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), diethylenetriaminepentaacetic acid, HEPES, phorbol myristate acetate, the FMLP antagonist *N*-Boc-Phe-Leu-Phe-Leu-Phe, and phospholipids were obtained from Sigma (Deisenhofen, FRG). $LTC_4$, $LTD_4$, $LTE_4$, $LTB_4$, 20-OH- and 20-COOH-$LTB_4$, and the synthetic $LTA_4$-methyl ester were a generous gift from Dr. J. Rokach, Merck Frosst (Canada). Additional LTs were graciously supplied by Dr. Bartmann, Hoechst AG. AA, 5-, 8-, 9-, 11-, 12-, and 15-HETE, 5(*S*),12(*S*)-diHETE, 5,15-diHETE, and 12-HHT, as well as the non-enzymatic hydrolysis products of $LTA_4$ (6-*trans* diastereomeric pair of $LTB_4$ and 5,6-diHETES), were obtained from Paesel AG (Frankfurt, FRG). All LTs were checked for purity and quantified spectrophotometrically before use, as described (33). Tritiated LTs, used as internal standards, were obtained from NEN (Dreieich, FRG). Anti-$LTB_4$ antiserum was received from Dr. J. Salmon (Wellcome Research Laboratory, Beckenham, UK). The PAF receptor antagonist WEB 2086 was graciously supplied by Boehringer (Ingelheim, FRG). Highly purified *Bordetella* PT and its inactive B-oligomer were purchased from Calbiochem (La Jolla, CA). RPMI 1640 medium, medium 199, Hanks' balanced salt solution, and fetal calf serum were from GIBCO Laboratories (Karlsruhe, FRG), and Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). Tritiated inositol phosphates and [$^3$H]-PAF were obtained from Amersham (Dreieich, FRG). *myo*-[2-$^3$H]Inositol was purchased from New England Nuclear (Boston, MA). Chromatographic supplies included octadecylsilyl (5-$\mu$m, Hypersil) and silica gel (5 $\mu$m) column packing (Machery Nagel, Duren, FRG), HPLC-grade solvents distilled in glass (Fluka KG, Heidelberg, FRG), and C-18 Sep-pak cartridges (Waters Associates, Milford, MA). All other biochemicals were obtained from Merck (Darmstadt, FRG).

**Preparation of human granulocytes.** Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient (4, 34) to yield a PMN fraction of approximately 97% purity. Before experiments with measurement of LT or PAF synthesis, PMN were kept in RPMI 1640 medium with 10% calf serum for 2 hr. For analysis of phosphoinositide metabolism, preincubation with *myo*-[2-$^3$H]inositol were carried out in medium 199, with 2% calf serum, for 2 hr. For measurement of PT sensitivity, this agent (1.0 $\mu$g/ml), its B-oligomer (1.0 $\mu$g/ml), or a corresponding buffer volume was added to PMN suspended in medium 199 and was incubated for 2 hr. Immediately before stimulus application, all cells were washed twice and suspended in Hanks'-HEPES buffer. Cell viability, as assessed by trypan blue exclusion, ranged above 96%, and lactate dehydrogenase release was consistently below 3% at this time.

**Measurement of LTs.** LTs and HETEs were extracted from cell supernatants by octadecylsilyl solid-phase extraction columns, as described (33, 35). Conversion into methyl esters was performed by addition of freshly prepared diazomethane in ice-cold diethyl ether. Reverse phase HPLC of nonmethylated compounds was carried out on octadecylsilyl columns (Hypersil, 5-$\mu$m particles), with a mobile phase of methanol/water/acetic acid (72:28:0.16, pH 4.9) (33). In addition to the conventional UV detection at 270 nm (LTs) and 237 nm (HETEs), a photodiode array detector (Waters model 990) was used, which provided full UV spectra (190–600 nm) of eluting compounds and allowed checking for peak purity and subtraction of possible coeluting material. For further verification, samples were collected in 15-sec fractions in selected experiments and subjected to post-HPLC radioimmunoassay with anti-$LTB_4$, as described (33). Reverse phase HPLC of methylated compounds was performed isocratically (methanol:water:acetic acid 66:34:0.16, pH 4.9) for 5 min, followed by a linear gradient to 90:10:0.16 over 10 min (Gynkothek Gradient Former, model 250). Straight phase HPLC of methylated compounds was carried out using a modification of the method of Nadeau *et al.* (36). The mobile phase consisted of hexane/isopropanol/acetate (86:14:0.1), and the column was eluted isocratically at a flow rate of 1.0 ml/min. All data obtained by the different analytical procedures were corrected for the respective recoveries of the overall analytical procedure and are given in pmol throughout the experiments. Recovery was determined by separate recovery experiments using different quantities of the individual compounds in the appropriate concentration range. Factors for recovery were further confirmed by addition of 0.2 $\mu$Ci of [$^3$H]$LTB_4$ and [$^3$H]5-HETE to buffer medium as internal standards in selected experiments. For quantification of LTs and 5-HETE, correspondence of values calculated from UV absorbance in two different chromatographic procedures was demanded (deviation <10%).

**Measurement of PAF.** PAF synthesis was quantified by incorporation of labeled acetate. PMN ($10^7$) were stimulated in the presence

of 50 μCi of [$^3$H]acetate (7.75 Ci/mmol) in a total volume of 1 ml, according to the method of Tessner *et al.* (37). Reactions were stopped by addition of 3 volumes of chloroform/methanol (1:2, v/v), and extraction was performed according to the method of Bligh and Dyer (38). The entire lipid extract was evaporated to dryness, redissolved in 60 μl of mobile phase, and subjected to straight phase HPLC separation. The column (25 × 0.46 cm) was packed with silica gel (5 μm) particles and eluted isocratically with acetonitrile/methanol/phosphoric acid (95.5:3.5:1), at a flow rate of 1.8 ml/min. Eluate fractions corresponding to appropriate standard retention times were collected and assayed for radioactivity by liquid scintillation counting. In selected experiments, the elution of radiolabel was monitored using a radiochromatogram-imaging system (5LS Raytest).

**Phosphoinositide metabolism.** The phosphoinositide turnover of stimulated neutrophils was investigated by measuring the accumulation of inositol phosphates, according to the method of Berridge *et al.* (39). For prelabeling of cellular phospholipid pools, PMN were resuspended, to 1 × $10^8$ cells/ml, in medium 199 containing 2% fetal calf serum plus 40 mM HEPES buffer, pH 7.4. *myo*-[$^3$H]Inositol (50 μCi/ml) was added, and cells were incubated at 37° for 2 hr on a shaking water bath. Before experimental use, cells were washed twice and resuspended in Hanks' balanced salt solution containing 20 mM HEPES and 10 mM LiCl (1 × $10^7$ PMN/ml). At the indicated times, samples were quenched with trichloracetic acid (final concentration, 7.5%), kept on ice for 15 min, and extracted four times with diethyl ether. The aqueous phase was neutralized with sodium tetraborate to pH 8.0 and processed to separate inositol phosphates on Dowex anion exchange columns, as described by Berridge *et al.* (39). The column was eluted sequentially with water (for free [$^3$H]inositol); 5 mM sodium tetraborate, 60 mM sodium formate (for glycerophospho[$^3$H]inositol); and 0.1 M formic acid, 1.0 M ammonium formate (for collection of $IP_1$, $IP_2$, and $IP_3$, measured as $IP_x$); samples were processed for liquid scintillation counting. All values were corrected for baseline levels (range, 700–1100 dpm) in nonchallenged PMN (indicated as net cpm).

**Quantification of cellular F-actin content.** F-actin content was determined as described (40). Briefly, cell incubations were fixed in 7.4% formaldehyde buffer and mixed with the staining cocktail containing 7.4% formaldehyde, 0.33 μM NBD-phallicidin, and 0.5 mg/ml lysophosphatidylcholine. The relative fluorescence intensity of methanol extracts from cells exposed to either control solvent (DMSO) (0.05%, v/v) or stimulus in DMSO was determined by fluorometry (excitation, 465 nm; emission, 535 nm) performed in an Aminco-Bowmann spectrofluorometer (Colora, Lorch, FRG). Relative F-actin content is expressed as the measured value at the specified time/control value at 0 sec.

## Results

**Cytoskeletal rearrangement.** PMN activation by the chemoattractants FMLP and PAF induced rapid actin polymerization, with maximum increase in cellular F-actin content within 1 min, as previously described (32). Preincubation of neutrophils with increasing concentrations of $C_2$ toxin reduced the FMLP- and PAF-evoked actin assembly in a dose-dependent manner (Fig. 1). At 400/800 ng/ml $C_{2_I}/C_{2_{II}}$, the ligand-evoked increase in F-actin was virtually completely suppressed. In addition, the baseline F-actin content was reduced by ≈20% at this toxin dose and by ≈70% at 800/1600 ng/ml $C_{2_I}/C_{2_{II}}$.

**Eicosanoid generation.** Neutrophil challenge with FMLP and PAF in the presence of exogenous AA caused substantial generation of $LTB_4$, with maximum metabolite levels at ≈5 min (Fig. 2). In addition, progressive formation of 20-OH-$LTB_4$ and 20-COOH-$LTB_4$, according to the established ω-oxidation capacity of human neutrophils (3), occurred (Fig. 3). PMN preincubation with low $C_2$ toxin doses (100/200 ng/ml $C_{2_{I/II}}$) caused marked augmentation of ligand-evoked LT generation. This was slightly more pronounced for cell activation by FMLP than by PAF. The time course of LT appearance was not altered in $C_2$ toxin-pretreated cells. Concomitantly, secretion of the LT precursor $LTA_4$ was amplified by toxin preincubation. Augmentation approached ≈50% in response to PAF and >100% in response to FMLP (Fig. 4). The capacity of $C_2$ toxin to enhance ligand-evoked eicosanoid synthesis was even more evident for 5-HETE (Fig. 5). This lipoxygenase product appeared in small quantities in PAF- and FMLP-challenged cells, with maximum levels being detected 5 min after stimulus application. The subsequent decline in 5-HETE concentration is attributable to rapid incorporation of this metabolite into membrane phospholipids (41, 42). Under the influence of low doses of $C_2$ toxin (100/200 ng/ml), the ligand-evoked generation of 5-HETE was increased >10-fold (FMLP) and >5-fold (PAF). Absolute quantities under these conditions approached those of $LTB_4$ and its ω-oxidation products. 12-HETE, as well as 5,12-diHETE, ranged below detection limits in all of the cell preparations, suggesting the absence of significant platelet contamination.

Studies on the dose dependence of $C_2$ toxin-evoked amplification of eicosanoid synthesis revealed marked differences between 5-HETE and the LT. Amplification of 5-HETE generation in response to FMLP (Fig. 6) and PAF (data not given in detail) commenced at 25/50 ng/ml $C_{2_I}/C_{2_{II}}$, was maximum at 200/400 ng/ml, and plateaued up to 800/1600 ng/ml. In contrast, $LTB_4$ synthesis in response to both ligands was maximal upon use of only 50/100 to 100/200 ng/ml $C_{2_I}/C_{2_{II}}$, with a sharp decline in efficacy being noted at higher toxin doses. Corresponding dose dependences were obtained for the nonenzymatic $LTA_4$ hydrolysis products (data not given in detail).

**PAF generation.** In control PMN, only minor quantities of PAF were elicited by FMLP and exogenously applied PAF. Marked amounts of this lipid mediator were, however, detected upon ligand activation of $C_2$ toxin-pretreated cells, with maximum PAF levels being detected 5–15 min after stimulus application. In contrast to the $C_2$ toxin-elicited enhancement of eicosanoid generation, maximal amplification of PAF generation required much higher toxin doses, plateauing between 400/800 and 800/1600 ng/ml (Fig. 6, 7).

**Phosphoinositide hydrolysis.** $C_2$ toxin pretreatment caused marked amplification of the FMLP-elicited generation of inositol phosphates ($IP_3$, $IP_2$, and $IP_1$; termed $IP_x$ in Table 1). Studies on the dose dependence of this effect revealed maximum response in the concentration range between 200/400 and 800/1600 ng/ml.

**Phagocytosis-related generation of lipid mediators.** Incubation of neutrophils with 2 mg/ml opsonized zymosan resulted in the progressive formation of $LTB_4$ and its ω-oxidation products (total sum, ≈100 pmol/$10^7$ PMN within 30 min), as well as PAF (data not given in detail). As anticipated, the ingestion of zymosan was dose-dependently inhibited by $C_2$ pretreatment and was completely suppressed upon preincubation with 400/800 ng/ml toxin for 30 min. Concomitantly, the phagocytosis-related lipid mediator generation was inhibited in a dose-dependent manner. Both the $LTB_4$ and PAF responses were decreased by nearly half at 200/400 ng/ml $C_{2_I}/C_{2_{II}}$; at 400/800 ng/ml, all lipid mediator generation was completely suppressed.

**Control experiments and influence of selected inhibitors.** Use of component I in the absence of $C_{2_{II}}$ did not influence

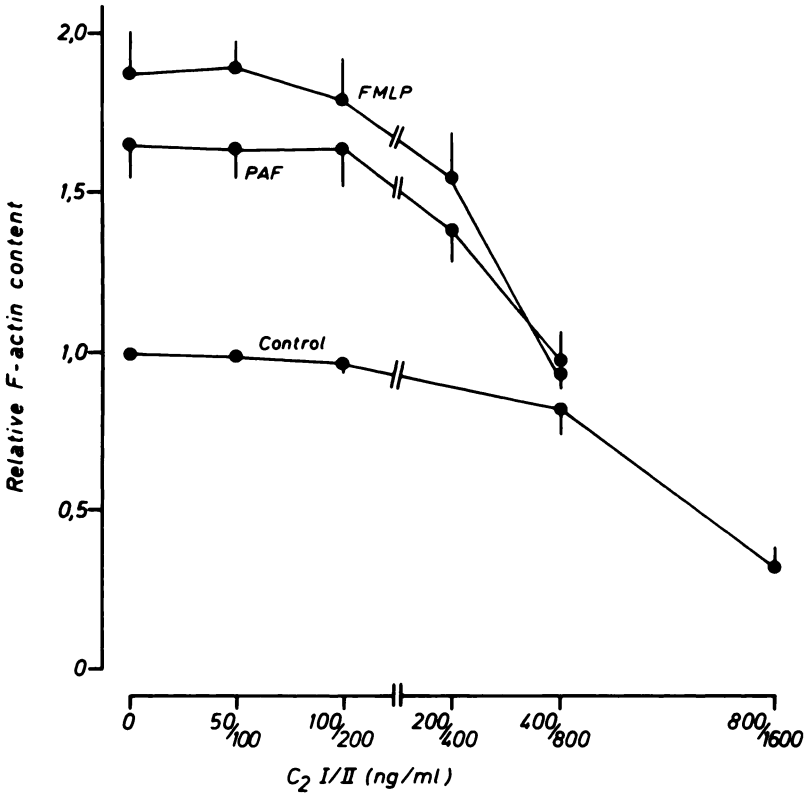


**Fig. 1.** Dose-dependent inhibition of ligand-evoked actin polymerisation by pretreatment with $C_2$ toxin. PMN ($10^7$) were preincubated with increasing concentrations of $C_{2_I}$ for 30 min. Stimulation was performed with 0.1 μM FMLP or 1 μM PAF; controls received vehicle only. The F-actin content, measured as rhodamine-phalloidin fluorescence 30 sec after stimulus or vehicle application, is expressed relative to baseline values of non-toxin-preincubated and nonstimulated cells. All data points represent mean ± standard error of five independent experiments.

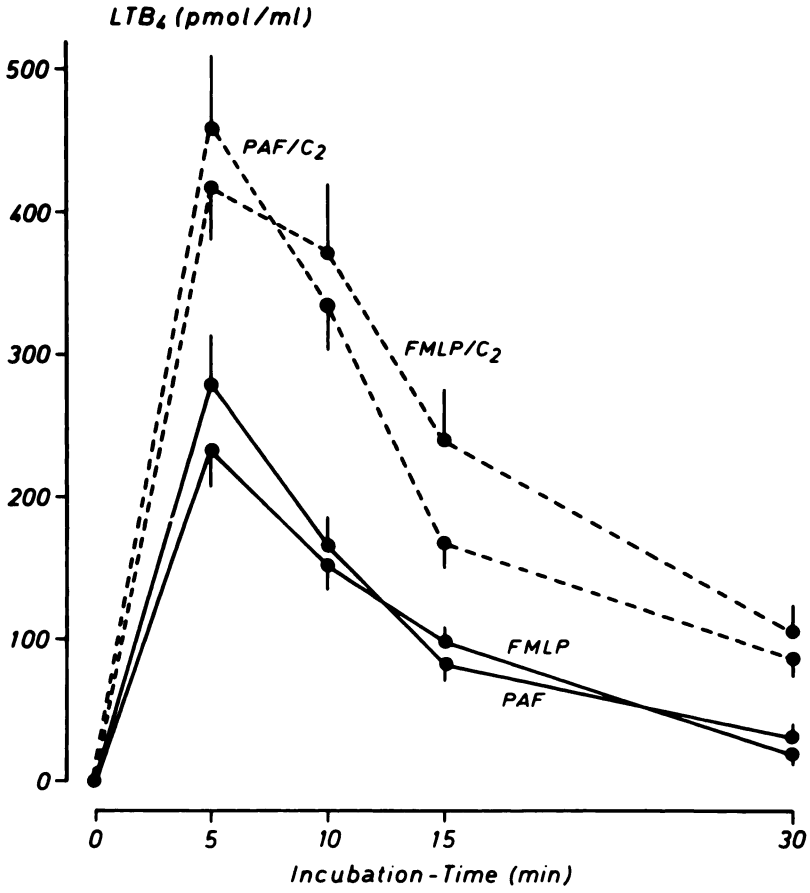


**Fig. 2.** Augmentation of ligand-evoked $LTB_4$ generation by $C_2$ toxin pretreatment. PMN ($10^7$) in 1-ml buffer volume were preincubated with 100/200 ng/ml $C_{2_I}$ for 30 min (– – –) or sham-incubated for the same time period (——). Stimulation was performed with 0.1 μM FMLP or 1 μM PAF in the presence of 10 μM AA, and the time course of $LTB_4$ appearance is given. All data points represent mean ± standard error of six independent experiments.

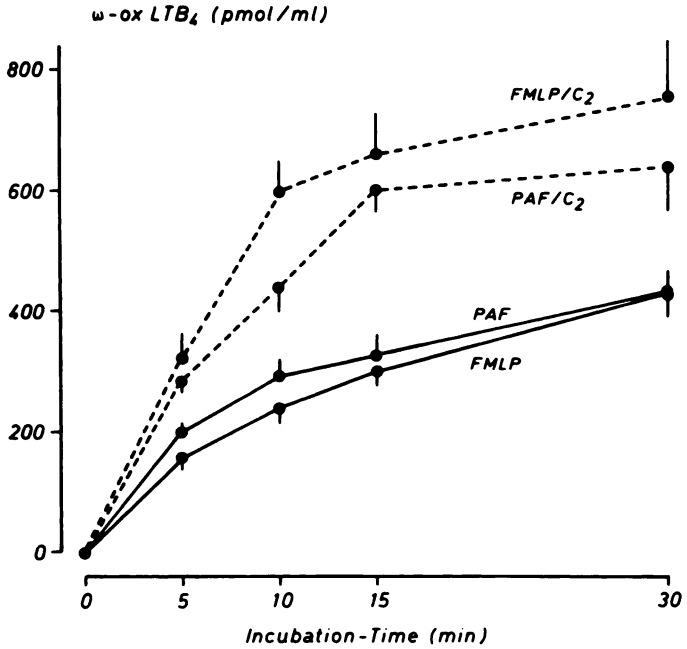


**Fig. 3.** Influence of $C_2$ toxin pretreatment on ligand-evoked generation of $LTB_4$ ω-oxidation products. PMN ($10^7$) in 1-ml buffer volume were preincubated with 100/200 ng/ml $C_{2_I/II}$ for 30 min (– – –) or sham-incubated for the same time period (——). Stimulation was performed with 0.1 μM FMLP or 1 μM PAF in the presence of 10 μM AA. The time course of appearance of 20-OH- and 20-COOH-$LTB_4$ (*ω-ox-LTB4*) is given. All data points represent mean ± standard error of six independent experiments.

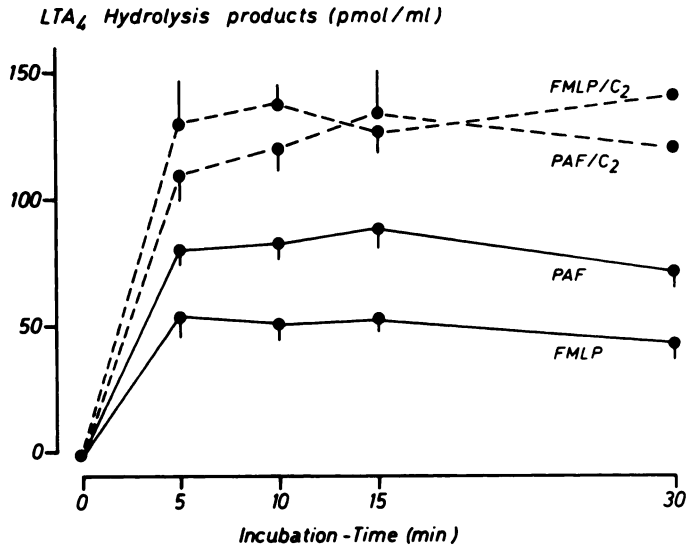


**Fig. 4.** Influence of $C_2$ toxin pretreatment on ligand-evoked liberation of $LTA_4$ hydrolysis products. PMN ($10^7$) in 1-ml buffer volume were preincubated with 100/200 ng/ml $C_{2_I/II}$ for 30 min (– – –) or sham-incubated for the same time period (——). Stimulation was performed with 0.1 μM FMLP or 1 μM PAF in the presence of 10 μM AA. The time-dependent appearance of the nonenzymatic $LTA_4$ hydrolysis products (6-*trans*-diastereomeric pair of $LTB_4$ and 5,6-diHETEs), indicative of $LTA_4$ secretion, is depicted. All data points represent mean ± standard error of six independent experiments.

ligand-evoked $LTB_4$, 5-HETE, and PAF generation (Table 2). Modest efficacy with respect to $LTB_4$ and 5-HETE formation was noted upon use of the isolated component II, which is most probably attributable to some minor $C_{2_I}$ contamination of this preparation. In the absence of ligands, neither the isolated

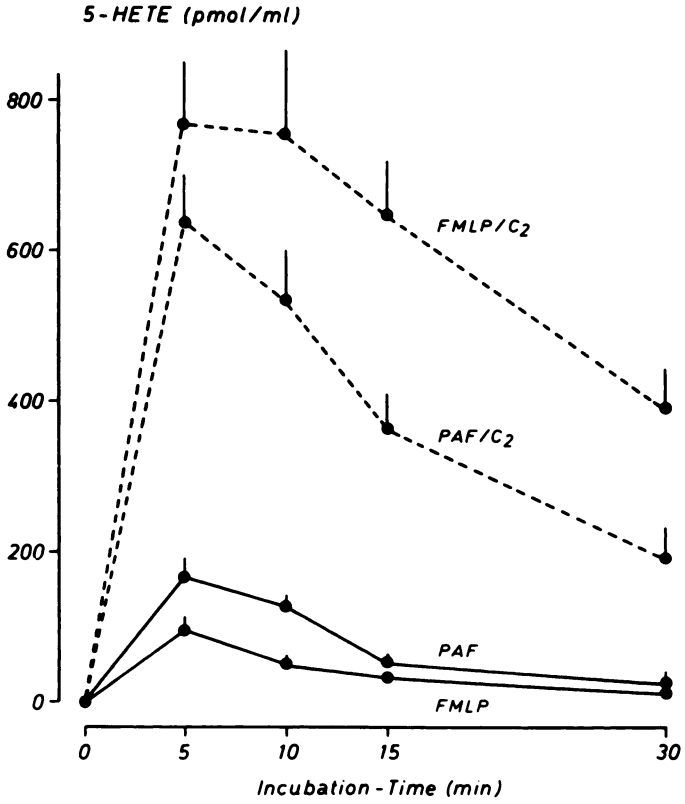


**Fig. 5.** Augmentation of ligand-evoked 5-HETE generation by $C_2$ toxin pretreatment. PMN ($10^7$) in 1-ml buffer volume were preincubated with 100/200 ng/ml $C_{2_I/II}$ for 30 min (– – –) or sham-incubated for the same time period (——). Stimulation was performed with 0.1 μM FMLP or 1 μM PAF in the presence of 10 μM AA, and the time course of 5-HETE production is given. All data points represent mean ± standard error of six independent experiments.

compounds nor the combined $C_2$ toxin exerted any effect on the investigated variables.

All responses to FMLP and PAF in $C_2$ toxin-treated PMN were sensitive to PT inhibition (Table 3). As anticipated, the FMLP receptor antagonist *N*-Boc-Phe-Leu-Phe-Leu-Phe efficiently suppressed the FMLP-evoked responses in control PMN, as well as in toxin-pretreated PMN. The PAF antagonist WEB 2086 did not substantially influence eicosanoid generation in $C_2$ toxin- and FMLP-exposed PMN, rendering a major contribution of putative PAF-related autocrine cell activation improbable (43, 44). However, moderate inhibition of PAF synthesis was noted in these cells. The PAF-evoked lipid mediator generation in $C_2$ toxin-preincubated PMN was virtually completely suppressed in the presence of WEB 2086.

## Discussion

Dose-dependent inhibition of the FMLP-induced actin polymerization was achieved by $C_2$ toxin concentrations of >200/400 ng/ml, which corresponds to previously published data (32). In addition, the PAF-evoked cytoskeletal rearrangement was found to be suppressed with corresponding dose dependence. The actin assembly in response to this lipid chemoattractant is known to occur with a similar time course but reduced extent and duration, compared with the FMLP-elicited response (45, 46). At $C_2$ toxin concentrations of >400/800 ng/ml, additional reduction of the basal cellular F-actin content by >20% occurred; at the highest toxin dose used (800/1600 ng/

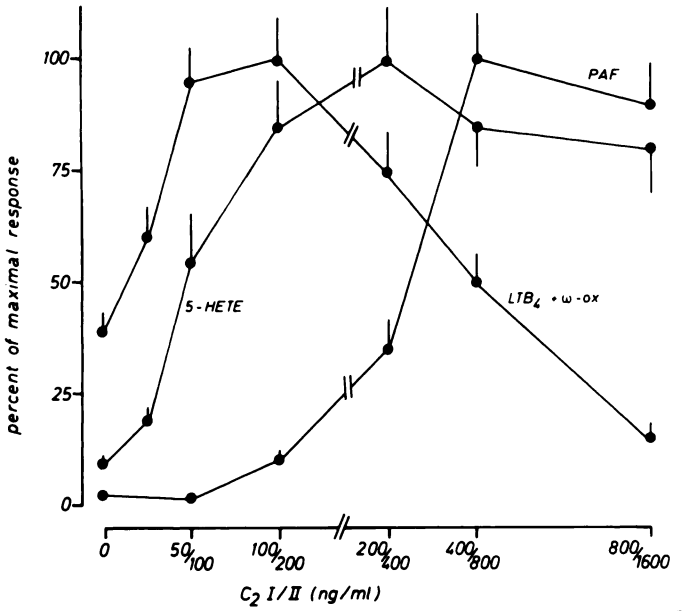


**Fig. 6.** Dose-dependent augmentation of ligand-evoked lipid mediator generation by pretreatment with $C_2$ toxin. PMN ($10^7$) were preincubated with increasing concentrations of $C_{2_{I/II}}$ for 30 min. Stimulation was performed with 0.1 μM FMLP in the presence of 10 μM AA. $LTB_4$ and its ω-oxidation products ($LTB_4$ + ω-ox), 5-HETE, and PAF were detected 10 min after FMLP application. All data are given as percentage of the maximal responses evoked after pretreatment with different $C_2$ toxin doses, with respect to the different mediators. Means ± standard errors of six independent experiments are indicated.

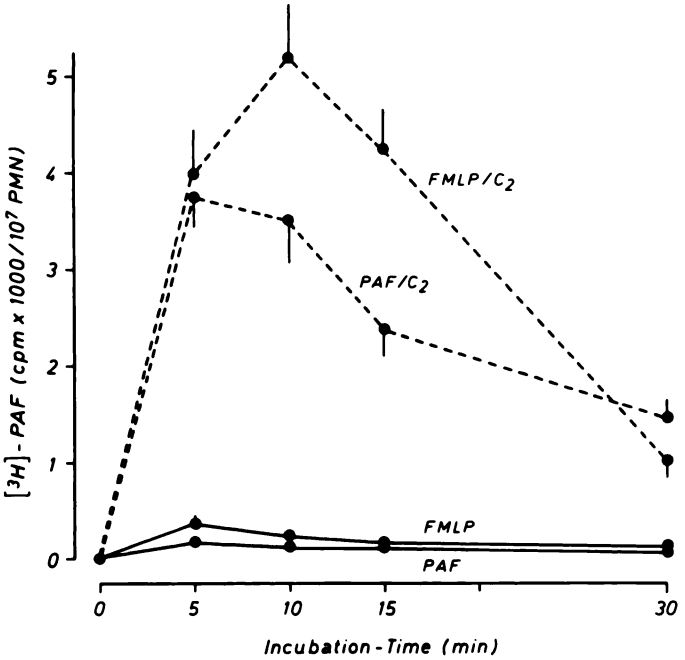


**Fig. 7.** Amplification of ligand-evoked PAF generation by $C_2$ toxin pretreatment. PMN ($10^7$) in 1-ml buffer volume were preincubated with 400/800 ng/ml $C_{2_{I/II}}$ for 30 min (– – –) or sham-incubated for the same time period (——). Stimulation was performed with 0.1 μM FMLP or 1 μM PAF after a 1-min incubation period with [$^3$H]acetate. Secreted and cell-bound labeled PAF was lipid extracted and separated by HPLC, and the eluate at appropriate retention time was collected for liquid scintillation counting. The time course of PAF generation is indicated; data points represent mean ± standard error of six independent experiments.

ml), even the maximum F-actin amounts elicited by chemoattractant challenge ranged markedly below the baseline levels of control cells. Together, these data confirm the feasibility of $C_2$ toxin as a tool to achieve far-reaching suppression of actin assembly in quiescent and, in particular, in activated PMN.

In line with its previously noted capacity to enhance PMN secretory responses (degranulation, superoxide generation) (31, 32), $C_2$ toxin is reported here to amplify ligand-evoked lipid mediator generation in human neutrophils. This effect was more pronounced upon FMLP than PAF challenge, which might indicate hitherto poorly defined differences in cytoskeleton dependence of the respective receptor kinetics and/or postreceptor events (47).

The $C_2$ toxin effects on eicosanoid generation and PAF synthesis markedly differed in their dose dependences, thereby suggesting distinct underlying events. The amplification of PAF formation was maximal at 400/800 to 800/1600 ng/ml $C_{2_{I/II}}$, doses that similarly cause maximal enhancement of respiratory burst and degranulation (31, 32) and that result in virtually complete suppression of ligand-evoked actin assembly within the given preincubation time of 30 min. Inasmuch as the regulation of expression and fate of chemoattractant receptors has been linked to cytoskeletal events (15, 48, 49), $C_2$ toxin might be operating at this level to elicit extended and prolonged cell activation. A detailed analysis of FMLP receptor binding and dissociation dynamics, however, did not detect significant differences between control and $C_2$-exposed PMN, and the endocytosis of the ligand-receptor complex was only slightly retarded (32). These data were obtained in lipopolysaccharide-primed neutrophils, and the currently used techniques for PMN isolation and toxin incubation similarly result in lipopolysaccharide exposition. Predominant impact of the $C_2$ toxin on postreceptor events is thus suggested, and the present finding of increased accumulation of inositol phosphates does support this notion. A more detailed analysis of ligand-evoked phosphoinositide hydrolysis and second messenger events under the influence of $C_2$ toxin indicates predominant increase in $IP_1$ accumulation, concomitant with severalfold increased diacylglycerol generation and sustained elevation of cytosolic $Ca^{2+}$ levels.[2] These results emphasize an important role of cytoskeletal rearrangement, linked to ligand-evoked remodeling of membrane phosphoinositides, in temporal and/or spatial limitation of chemoattractant-evoked PMN activation. In particular, actin-binding proteins such as gelsolin and profilin are assumed to serve as "shuttles" between plasma and plasma membrane, effecting disassembly and subsequent polymerization of actin (sol-gel transition) and, thereby, limiting the cellular response (11). $C_2$ toxin interference with actin assembly will, thus, augment and prolong phosphoinositide turnover. The presently noted amplification of PAF synthesis may easily be linked to such findings of increased and prolonged signal transduction events. Activated protein kinase C and elevated cytosolic calcium levels act synergistically to amplify the biosynthesis of PAF. $Ca^{2+}$ is assumed to be regulatory by stimulation of phospholipase $A_2$-mediated production of lyso-PAF from the membrane PAF precursor, by activation of the subsequent acetyltransferase step, and by inhibition of the countercurrent acyltransferase activity (1, 50). Protein kinase C may phosphorylate and, thereby, modify phospholipase-inhibitory proteins

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TABLE 1

**Dose-dependent amplification of ligand-evoked inositol phosphate generation by pretreatment with $C_2$ toxin**

PMN ($10^7$/ml) (prelabeled with [$^3$H]inositol as detailed in Experimental Procedures) were pretreated with increasing concentrations of $C_2$ toxin for 30 min. Stimulation was performed with FMLP, PAF, or vehicle only for 5 min. The inositol phosphates $IP_1$, $IP_2$, and $IP_3$, comprising $IP_x$, are given as net cpm/$10^7$ PMN. Means ± standard errors of six independent experiments each are indicated.

| Stimulus | $IP_x$ | | | |
|---|---|---|---|---|
| | 0[a] | 100/200[a] | 200/400[a] | 400/800[a] |
| | cpm/$10^7$ PMN | | | |
| FMLP (0.1 μM) | 581 ± 63 | 827 ± 111 | 2416 ± 269 | 2884 ± 311 |
| PAF (1.0 μM) | 474 ± 55 | 525 ± 48 | 1036 ± 123 | 1408 ± 139 |
| Control | <100 | <100 | <100 | <100 |

[a] $C_2$ toxin concentration (ng/ml $C_{2I}/C_{2II}$).

TABLE 2

**Influence of isolated versus combined $C_2$ toxin components on ligand-evoked lipid mediator generation in human neutrophils**

| | $LTB_4$ + ω-oxidation products[a] | 5-HETE[a] | PAF[b] |
|---|---|---|---|
| | pmol/ml | pmol/ml | dpm/$10^7$ cells |
| FMLP (0.1 μM) | | | |
| 0 | 390.4 ± 34.5 | 40.5 ± 3.7 | 269 ± 30 |
| $C_I$ | 408.7 ± 40.4 | 38.4 ± 2.9 | 284 ± 28 |
| $C_{II}$ | 421.9 ± 32.9 | 75.6 ± 8.0 | 274 ± 34 |
| $C_{I/II}$ | 969.0 ± 81.7 | 775.3 ± 87.2 | 5111 ± 631 |
| PAF (1.0 μM) | | | |
| 0 | 463.8 ± 29.8 | 131.8 ± 11.3 | 139 ± 12 |
| $C_I$ | 439.6 ± 38.4 | 139.1 ± 14.7 | 152 ± 17 |
| $C_{II}$ | 521.4 ± 57.8 | 188.6 ± 21.5 | 138 ± 12 |
| $C_{I/II}$ | 882.9 ± 90.2 | 556.7 ± 48.6 | 3582 ± 402 |
| Control | | | |
| 0 | <15 | <20 | <100 |
| $C_I$ | <15 | <20 | <100 |
| $C_{II}$ | <15 | <20 | <100 |
| $C_{I/II}$ | <15 | <20 | <100 |

[a] For measurement of eicosanoids ($LTB_4$ and its ω-oxidation products and 5-HETE), PMN ($10^7$/ml) were pretreated with 100 ng/ml $C_{2I}$, 200 ng/ml $C_{2II}$, 100/200 ng/ml $C_{2I/II}$, or vehicle only for 30 min. Subsequent stimulation was performed with 0.1 μM FMLP or 1 μM PAF for 10 min. Means ± standard errors of four or five independent experiments each are given.

[b] For measurement of PAF, PMN were pretreated with 400 ng/ml $C_{2I}$, 800 ng/ml $C_{2II}$, 400/800 ng/ml $C_{2I/II}$, or vehicle only for 30 min. Stimulation with 0.1 μM FMLP or 1 μM PAF was performed after a 1-min preincubation period with [$^3$H] acetate. Labeled liberated and cell-associated PAF was quantified 10 min after stimulus application. Means ± standard errors of four or five independent experiments each are given.

like lipocortin, and it modulates acetyltransferase activity (1). Thus, augmented second messenger levels, arising due to suppression of cytoskeletal rearrangement normally coupled to receptor occupancy, may underly the $C_2$ toxin-effected marked enhancement of PAF synthesis in response to ligands, which do not evoke substantial formation of this lipid mediator in nonprimed neutrophils (8). The data support the general notion that the microfilamentous network is involved in spatial and temporal limitation of the cascade of events occurring with neutrophil activation (9–11). The PT sensitivity of ligand-evoked events in $C_2$ toxin-pretreated PMN, which corresponds to that in control cells, indicates unchanged signal transduction via GTP-binding proteins (51) under the influence of this bacterial agent.

Compared with PAF, the generation of $LTA_4$-derived products was maximally increased at markedly lower toxin concentrations (range, 50/100 to 100/200 ng/ml $C_{2I/II}$). This was true for both $LTB_4$ and its ω-oxidation products and for the nonenzymatic hydrolysis products of $LTA_4$. The amplification of eicosanoid synthesis occurred below the threshold of detectable $C_2$ toxin influence on total cellular actin assembly and in the presence of only very moderately increased inositol phosphate accumulation. One possible explanation might be a prolonged availability of the exogenously applied precursor AA, which is known to be rapidly incorporated into membrane phospholipids under normal conditions (52–55). Alternatively, 5-lipoxygenase activity might be modified by a $C_2$ toxin effect on a small, functionally specific, actin pool. Such a pool, supposedly located near the plasma membrane (56), may be more easily accessible to the bacterial toxin than is the mass of cytosolic actin. Translocation of the 5-lipoxygenase from cytosol to cellular membrane is postulated to be a critical early activation step (57, 58), the enzyme is highly $Ca^{2+}$ dependent, and a complex

TABLE 3

**Influence of selected inhibitors on ligand-evoked lipid mediator generation in $C_2$ toxin-pretreated PMN**

PMN ($10^7$/ml) (in the presence of [$^3$H]acetate for PAF detection) were pretreated with 100/200 ng/ml $C_{2I/II}$ (eicosanoid generation) or 400/800 ng/ml toxin (PAF measurement) for 30 min. The FMLP receptor antagonist *N*-Boc-PLPLP, the PAF antagonist WEB 2086, or vehicle (control) was admixed 5 min before stimulus application. Pretreatment with PT was performed for 2 hr. Stimulation was performed with 0.1 μM FMLP and 1 μM PAF for 10 min. Eicosanoids ($LTB_4$ and its ω-oxidation products and 5-HETE) are given in pmol/$10^7$ PMN; labeled liberated and cell-associated PAF is in dpm/$10^7$ cells. Means ± standard errors of four to six independent experiments each are given.

| | $LTB_4$ + ω-oxidation products | 5-HETE | PAF |
|---|---|---|---|
| | pmol/ml | pmol/ml | dpm/$10^7$ cells |
| FMLP (0.1 μM) | | | |
| Control | 942.8 ± 86.0 | 739.6 ± 99.5 | 5309 ± 504 |
| *N*-Boc-PLPLP (50 μM) | 79.5 ± 8.9 | <20 | 439 ± 56 |
| WEB (1.0 μM) | 896.4 ± 76.5 | 709.2 ± 82.1 | 3885 ± 427 |
| PT (1 μg/ml) | 228.6 ± 21.5 | 109.3 ± 8.7 | 1239 ± 145 |
| PAF (1.0 μM) | | | |
| Control | 841.2 ± 84.6 | 529.3 ± 51.4 | 3714 ± 426 |
| WEB (1.0 μM) | 67.5 ± 8.3 | <20 | <100 |
| PT (1 μg/ml) | 388.6 ± 43.4 | 244.7 ± 31.2 | 619.0 ± 74.3 |

interaction with a recently identified membrane protein ("5-lipoxygenase-activating protein") (59, 60), as well as soluble, hitherto only partially purified, protein fractions (57, 61), is required for enzymatic activity. One or several of these events involved in the unique multicomponent regulatory complex of 5-lipoxygenase may be under the control of a specialized $C_2$ toxin-accessible actin pool. Further support for this view, albeit indirect, is obtained from the finding of a progressive change in the 5-lipoxygenase metabolite profile upon increases in toxin dosage. Augmentation of 5-HETE generation commences in parallel with that of LT synthesis at toxin doses of >25/50 ng/ml, and maximal amplification of HETE release is noted in the entire range between 200/400 and 800/1600 ng/ml, whereas a marked decline in LT synthesis occurs upon use of such toxin concentrations. The ratio of 5-HETE to $LTB_4$ and its $\omega$-oxidation products is, thus, changed from ≈0.1 in control cells to >5 in the presence of 800/1600 ng/ml $C_2$ toxin. Alterations in putatively cytoskeleton-linked spatial arrangements of substrates and 5-lipoxygenase and $LTA_4$ synthase activities within the multicomponent enzyme complex, with a resultant shift of 5-HETE metabolism to the hydroperoxidase pathway, may underly this $C_2$ toxin effect. Further experimental approaches are needed to verify this assumption.

Exposure of neutrophils to opsonized zymosan, with attendant phagocytosis, has previously been reported to elicit marked prolonged generation of $LTB_4$ and PAF (62, 63). In contrast to the soluble ligand-evoked synthesis of lipid mediators, the response to the particulate stimulus was dose-dependently inhibited by $C_2$ toxin pretreatment. This toxin effect is readily explained by the loss of actin-dependent motile cell functions, including migration and phagocytosis, as described in a preceding study on $C_2$-pretreated PMN (31).

In conclusion, selective inhibition of chemoattractant-evoked cytoskeletal rearrangement by botulinum $C_2$ toxin causes marked amplification of PMN lipid mediator generation, and at least two toxin effects can be dissociated. At high toxin doses, marked amplification of PAF synthesis occurs, putatively linked to increased second messenger levels under these conditions. At low toxin doses, efficacy on PMN 5-lipoxygenase activity is noted, which might be related to specialized, putatively spatially confined, actin pools. These results emphasize a role of cytoskeletal rearrangement in temporal and/or spatial limitation of chemoattractant-evoked PMN activation.

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