**Influence of Botulinum C2 Toxin on F-Actin and N-Formyl Peptide Receptor Dynamics in Human Neutrophils**

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**Abstract.** Stimulation of human neutrophils with the chemotactic N-formyl peptide causes production of oxygen radicals and conversion of monomeric actin (G-actin) to polymeric actin (F-actin). The effects of the binary botulinum C2 toxin on the amount of F-actin and on neutrophil cell responses were studied. Two different methods for analyzing the actin response were used in formyl peptide-stimulated cells: staining of F-actin with rhodamine-phalloidin and a transient right angle light scatter. Preincubation of neutrophils with 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin for 30 min almost completely inhibited the formyl peptide-stimulated polymerization of G-actin and at the same time decreased the amount of F-actin in unstimulated neutrophils by an average of ~30%. Botulinum C2 toxin preincubation for 60 min destroyed ~75% of the F-actin in unstimulated neutrophils. Right angle light scatter analysis showed that control neutrophils exhibited the transient response characteristic of actin polymerization; however, after botulinum C2 toxin treatment, degranulation was detected. Single components of the binary botulinum C2 toxin were without effect on the actin polymerization response. Fluorescence flow cytometry and fluorospectrometric binding studies showed little alteration in N-formyl peptide binding or dissociation dynamics in the toxin-treated cells. However, endocytosis of the fluorescent N-formyl peptide ligand–receptor complex was slower but still possible in degranulating neutrophils treated with botulinum C2 toxin for 60 min. The half-time of endocytosis, estimated from initial rates, was 4 and 8 min in control and botulinum C2 toxin–treated neutrophils, respectively.

**Neutrophils** play a central role in host defense and in processes of inflammation. Stimulation of neutrophils by the N-formyl peptide induces a series of coordinated cell responses such as directed migration, cell shape change, degranulation, and superoxide radical production. All of these cell responses seem to be influenced by the cytoskeletal transformation associated with changes in the state of actin polymerization during the stimulation (14). Cytoskeletal activation can be monitored with indirect spectroscopic methods, such as the determination of changes in right angle light scattering, or directly with cytometric assays by staining F-actin with rhodamine-phalloidin (21).

Botulinum C2 toxin belongs to a new class of bacterial ADP-ribosyltransferases that modifies nonmuscle G-actin (1, 2). The toxin is binary in structure and consists of two different components (13). Component I (50,000 mol wt) possesses ADP-ribosyltransferase activity (1), whereas component II (100,000 mol wt) is involved in the binding of the toxin to the cell membrane (13). Botulinum C2 toxin ADP-ribosylates nonmuscle G-actin at ARG-177 (26). This covalent modification blocks the ability of actin to polymerize (1), probably converting G-actin into a capping protein for the barbed end of the F-actin filament (28) and thereby inhibiting further growth of the actin filament.

Recently, we have shown that botulinum C2 toxin specifically ADP-ribosylates actin in intact neutrophils, enhances the superoxide radical production, and inhibits migration (12). Cytochalasins, which also inhibit the polymerization of actin (4), influence these cell responses in an analogous way and to a similar extent (12). It has been proposed and widely accepted that cytoskeletal events are involved in the regulation of the expression (4, 11) and the fate of the N-formyl peptide receptor (9). For instance, it has been reported that treatment of neutrophils with cytochalasin blocks the uptake of N-formyl peptide (10), enhances the expression of the N-formyl peptide receptor (3, 11), and appears to delay the formation of a GTPgammaS-insensitive slow-dissociating N-formyl peptide ligand–receptor complex (16). These data prompted us to study the effects of botulinum C2 toxin on the F-actin content and actin dynamics in human neutrophils and its relationship to the endocytosis, expression, and number of receptors for N-formyl peptide.


Materials and Methods

Neutrophils

Peripheral blood was drawn from normal human donors. Neutrophils were isolated by the gelatin sedimentation and elutriation method described elsewhere (24). The buffer for the experiments contained 137 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM glucose, and 10 mM Hepes.

Reagents

7-nitrobenz-2-oxa-1,3-diazole (NBD)-1-phalargin and rhodamine-phalloidin were obtained from Molecular Probes, Inc. (Junction City, OR). The N-formyl peptide receptor blocker t-butoxycarbonyl-phe-leu-phe-leu-phe was obtained from Vega Biotechnologies, Inc. (Tucson, AZ). Fluorescein-labeled N-formyl-norleu-leu-phe-norleu-tyr-lys was prepared as described (19). Botulinum C2 toxin was prepared and activated essentially as described (13).

Functional Assays

All experiments were performed at 37°C. Incubation of neutrophils with botulinum C2 toxin was performed in modified Hepes buffer containing 6.3 mg/ml cytochrome c. Oxygen radical production was determined using the chromophore parahydroxyphenylacetic acid assay as described by Hyslop and Sklar (8). Right angle light scattering measurements were performed on aliquots containing 106 cells/ml using a photon-counting spectrophotometer (8000; SLM Instruments, Inc., Urbana, IL) at 340 nm for excitation and emission (21).

Measurement of F-actin formation was performed essentially according to Howard and Meyer (7). Briefly, after stimulation, aliquots of cell suspensions were withdrawn at the indicated time intervals from the stirred sample compartment of the fluorometer (8000; SLM Instruments, Inc.) during light scattering measurement. Equal volumes of cells were fixed in a 7.4% formaldehyde buffer and mixed with the staining cocktail containing 7.4% formaldehyde, 0.33 μM NBD-phallacin or rhodamine-phalloidin, and 1 mg/ml lysophosphatidylcholine. The fluorescence intensity of F-actin was measured in a cytometer (FACS IV; Becton Dickinson & Co., Sunnyvale, CA) as described (21).

Analysis of formyl peptide binding and dissociation were performed using the fluorometric and cytometric methods described by Sklar et al. (19, 22). These measurements were performed on a photon-counting spectrophotometer (8000; SLM Instruments, Inc.). They made use of a fluorescein-labeled formyl peptide and a high-affinity antibody against fluorescein to discriminate between free and receptor-bound N-formyl peptide. To stabilize the fluorescence of the peptide ligand during the spectrofluorometric assays of ligand binding, dissociation, and endocytosis, the buffer was modified to contain 1 mg/ml BSA, 0.08 mg/ml bovine catalase, 0.08 mg/ml superoxide dismutase, 0.05 mg/ml chymostatin, and 2 mM PMSF, essentially as described earlier (22). For studies on degranulating neutrophils in the cytometer, we substituted 4 mM azide for PMSF to prevent the decay of fluorescent probes possibly associated with myeloperoxidase activity (29). In addition, 15 mM NH4Cl was present in cytometric binding studies to prevent acidification of intracellular endocytotic compartments. None of these reagents altered the actin-associated right angle light scatter response or the degranulation response.

Flow cytometric assay of binding and endocytosis of the N-formyl peptide was performed on a Facscan (Becton Dickinson & Co.) according to Sklar et al. (19). Briefly, neutrophils were exposed to different concentrations of N-formyl peptide, and sequential profiles of cellular fluorescence were acquired. The fluorescence of specific bound ligand was calculated from the mean fluorescence channel number as Δ M (t), which reflects the time dependence of ligand binding. Δ M (t) was calculated as Δ M (t) = M (t) − M(0), where M (t) and M(0) represent the mean fluorescence channel number of cells exposed to the same concentration of N-formyl peptide without and with the N-formyl peptide receptor antagonist t-boc-phe-leu-phe-leu-phe, respectively.

The analysis of endocytosis of the N-formyl peptide receptor was accomplished by adding HCl after the desired period of binding to quench the external ligand after a change in the pH from 7.45 to 4. The fraction of internalized fluorescence was analyzed by examining the mean fluorescence channel number before and after the pH change (see Fig. 7). The fraction of internalized fluorescence was calculated as the residual fluorescence extrapolated to the time of the pH change.

Results

Functional Aspects of Botulinum C2 Toxin

The influence of botulinum C2 toxin on the rate of the oxygen radical production in human neutrophils is shown in Fig. 1. Human neutrophils were incubated for 60 min without and with 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin and stimulated with 10 nM N-formyl peptide. The amount of the oxidant production in botulinum C2 toxin–treated neutrophils was increased by ~100%.

Fig. 2 shows the histogram of the fluorescence staining of F-actin by NBD-phallacin in untreated human neutrophils or cells treated for 60 min with botulinum C2 toxin. Control neutrophils show a single population of fluorescence. The mean fluorescence channel number was 83, ~20 times higher than fixed but unlabeled cells (mean channel number = 4). Neutrophils treated for 60 min with 400 ng/ml component I and 1,600 ng/ml component II of the botulinum C2 toxin show a narrow peak around channel number 5, representing an actin-depleted population of ~75% of the cells. A second smaller population of neutrophils had residual but reduced levels of F-actin. Longer incubation times with botulinum C2 toxin (90 min) reduced the second population further (data not shown).

The time dependency of botulinum C2 toxin action on the kinetics of actin polymerization is shown in Fig. 3A. Stimulation of control neutrophils caused a rapid polymerization of actin within 10 s. There was a doubling of the F-actin content followed by a more sustained polymerization and a recovery of initial F-actin amounts within 4 min. Preincubation of human neutrophils with botulinum C2 toxin for 30 min decreased the average F-actin content in unstimulated neutrophils by ~25% and largely inhibited the polymerization response, although a slight increase in the stimulated F-actin content (~20%) was still observed. Longer incubation times

Figure 1. Comparison of the relative rate of oxygen radical production in botulinum C2 toxin–treated with untreated human neutrophils. Neutrophils were treated with or without 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin for 60 min. 106 cells were stimulated with 10 nM N-formyl peptide. The absolute amounts were 8 and 16 nmol/106 neutrophils for control and botulinum C2 toxin–treated neutrophils, respectively. Data are representative of a single experiment performed in duplicate and repeated twice in this study (and previously, see reference 12) are shown.
with the botulinum C2 toxin further reduced the initial F-actin content. Preincubation of neutrophils with botulinum C2 toxin for 60 min reduced the mean F-actin content by ∼75%, and stimulated polymerization was not detected.

Right angle light scattering measurements were performed on the same sample (Fig. 3 B). In control cells, the typical transient reduction of the scattered light was observed after stimulation with N-formyl peptide (21). The decrease in scatter intensity of control neutrophils was small (10%), rapid, and reversible. This response apparently occurred without measurable delay. A minimum of light scatter intensity was observed after 10 s followed by a slow recovery of the initial intensity within 4 min. Toxin treatment for 15 min did not alter the onset time of the right angle light scatter response, but delayed the recovery of the intensity. The decrease in intensity of right angle scatter by neutrophils treated with botulinum C2 toxin (at least 30 min) was delayed for 1-2 s and was more pronounced (30%). There was no recovery of the scatter intensity. In cells treated either with botulinum toxin (not shown) or in previous studies with cytochalasin B, direct measurements have shown that degranulation kinetics, as measured by the release of elastase, coincide with this latter light scatter change (20).

As shown in Fig. 4, the effect of botulinum C2 toxin depended on the presence of both components. The separate application of component I or component II of botulinum C2 toxin did not alter the F-actin content. Neither the formyl peptide–stimulated actin polymerization nor the characteristics of the actin-associated right angle light scatter response was significantly changed in the presence of single components. These findings indicate the specificity of the botulinum C2 toxin action.

**Receptor Dynamics in Botulinum C2 Toxin–treated Cells**

Next we studied the influence of botulinum C2 toxin on the binding of the N-formyl peptide ligand to its receptor by cytometry. Neutrophils were incubated without and with 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin for 60 min and labeled with several concentrations of N-formyl peptide. Fluorescence profiles were acquired every 30 s for 5 min. As shown in Fig. 5, botulinum C2 toxin treatment did not alter the interactions of N-formyl peptide ligand with its receptor over the indicated period of time.

Using fluorimetric methods, we examined ligand dissociability with an ∼1-s time resolution (Fig. 6). Earlier studies of N-formyl peptide dissociation from cells have shown that receptors are converted from a fast (low-affinity) to a slow (high-affinity) form (22). Log plots of binding and dissociation kinetics showed that after 15 s of binding the dissocia-
tion of the receptor was heterogeneous. The data are consistent with two receptor forms: one form that dissociated with a half-time of \(\sim 10\) s and a second form that dissociated with a half-time of at least 2 min. After 2 min of binding, the receptors were predominantly in the slow-dissociating form. Botulinum C2 toxin treatment did not appear to alter these N-formyl peptide ligand–receptor dynamics.

In recent studies, it has been reported that the N-formyl peptide accumulation was blocked in cytochalasin-treated neutrophils, suggesting that the endocytosis of the N-formyl peptide ligand–receptor complex was inhibited (10). Neutrophils treated with botulinum C2 toxin for 60 min were used to examine the influence of actin on the early internalization of the N-formyl peptide ligand–receptor complex by real time flow cytometric methods. Control and botulinum C2 toxin–treated neutrophils were labeled with 10 nM fluorescent N-formyl peptide. Internalization was determined by reducing the pH from 7.45 to 4, which instantaneously

Figure 4. Influence of single components of botulinum C2 toxin on right angle light scatter and actin polymerization. Description is analogous to Fig. 3. Neutrophils were incubated for 30 min with 400 ng/ml of component I, 1,600 ng/ml component II, or 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin and compared with untreated cells. A is the measurement of F-actin and B shows the right angle light scatter response of neutrophils stimulated with 1 nM N-formyl peptide performed on the same cells. Representative data of one experiment performed twice in duplicate are shown. In A: control cells (C); cells treated with component I (O), component II (O), and components I and II (o). In B: control cells (---); cells treated with component I (•••), component II (---), and components I and II (••).

Figure 5. Concentration dependence of N-formyl peptide binding in human neutrophils. Neutrophils were incubated with (solid symbols) or without (open symbols) 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin for 60 min and stimulated with 10 (■ and ○), 3 (▲ and ◦), and 1 (● and ◦) nM N-formyl peptide. Fluorescence detected in the presence of tBoc-phe-leu-phe-leu (nonspecific binding) is shown for 10 (○), 3 (●), and 1 (○) nM fluoresceinated hexapeptide. Each channel of specific binding represents \(\sim 1,100\) fluoresceinated hexapeptides using a fluorescent calibration standard as described previously (23). Data are means with standard deviation of one representative experiment performed in duplicate and repeated six times.

Figure 6. Comparison of N-formyl peptide binding and dissociation in control with botulinum C2 toxin–treated cells. Measurements were performed on cells in suspension (\(5 \times 10^6\)/ml) using the antibody to fluorescein as described in Materials and Methods. The data reflect an analysis of the amount of ligand bound at 15 and 120 s and the dissociation thereafter. Neutrophils were incubated for 60 min with (--) or without (---) 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin and stimulated with 1 nM N-formyl peptide. Representative data of one experiment performed in duplicate and repeated five times are shown. In some experiments there was a loss of 10% of the peptide fluorescence in toxin-treated as compared with control cells, probably associated with the release of myeloperoxidase.
Figure 7. Real-time analysis of binding and internalization of fluorescent N-formyl peptide by flow cytometry. Neutrophils were incubated for 60 min with 400 ng/ml component I and 1,600 ng/ml component II of botulinum C2 toxin. After binding measurement using flow cytometry (see Fig. 5), the pH of the cell suspension was reduced from 7.45 to 4. Data are displayed as dot plot of side scatter vs. fluorescent formyl peptide. (A) Control neutrophils after a 105-s binding. (B) Control neutrophils after a 120-s binding and 10-s pH change. (C) Botulinum C2 toxin-treated neutrophils after a 105-s binding. (D) Botulinum C2 toxin-treated neutrophils after a 120-s binding and 10-s pH change. (E) Botulinum C2 toxin-treated neutrophils after a 120-s binding and 10-s pH change in the presence of 4 x 10^{-7} M tBoc-phe-leu-leu-phe. Representative data of one experiment performed in duplicate repeated twice are shown. Each channel of specific fluorescence represents ~225 molecules of hexapeptide per cell.

was 451. After 2 min, the pH was changed and the sample was reanalyzed at 10-s intervals (the first of which is shown in Fig. 7 B). We observed a residual fluorescence of 167 channels. Fig. 7, C and D, shows botulinum C2 toxin-treated neutrophils labeled and quenched in the same manner. The mean fluorescence channel number was 459, similar to control neutrophils. After the pH change, a residual mean fluorescence of 116 was detected. Addition of the receptor antagonist tBoc-phe-leu-phe-leu-phe before labeling with 10 nM N-formyl peptide prevented binding or internalization of the fluorescence peptide (Fig. 7 E). The fluorescence mean channel number was 46, similar to the autofluorescence of unstimulated neutrophils (data not shown; mean channel number = 42).

In control neutrophils, typically 50% of occupied receptors are internalized after 4 min of binding. In botulinum C2 toxin-treated neutrophils, an estimated 30% of the occupied receptors were internalized. In both cases, the internalized receptor was quenched by the extracellular pH change with a similar although not identical half-time (Fig. 8 A).

A comparison of the early time course of internalization of control with botulinum C2 toxin-treated neutrophils is shown in Fig. 8 B. The estimated half-time of endocytosis of the N-formyl peptide ligand–receptor complex in neutrophils treated with botulinum C2 toxin was ~8 min, while the half-time of control neutrophils was ~4 min. In parallel we verified that botulinum C2 toxin diminished the F-actin content. The side scatter signal in cytometry, verified to be indicative of degranulation, was reduced ~30% in the toxin-treated population (Fig. 7, C and D) compared with the control cells (Fig. 7, A and B).

Discussion

The Cytoskeleton in Neutrophil Function

Change of F-actin content in eukaryotic cells is associated with changes in cell shape and motility (18). N-formyl peptide–stimulated neutrophils show a very rapid polymerization of G-actin to F-actin. Preincubation of the neutrophils with botulinum C2 toxin decreased the content of F-actin by ~75% in a time-dependent manner. However, the fluorescence histogram plotted vs. cell number showed two different populations of neutrophils, one completely depleted of F-actin and a second with some residual F-actin. These findings indicate that botulinum C2 toxin destroys the F-actin network in intact neutrophils as reported for chicken embryo cells (17). In addition the N-formyl peptide–stimulated conversion of G-actin to F-actin was almost completely inhibited after a 30-min incubation with botulinum C2 toxin. Recently, Wegener and Aktories (28) have shown that G-actin ADP-ribosylated by Clostridium perfringens iota toxin, which
The primary point of contention is whether cytoskeletal association is obligatory to form or maintain the high-affinity state. For example, a change in the Triton extractability in cells treated with cytochalasin could reflect changes in the nature of the extract. Thus, a failure to coisolate high-affinity receptors could reflect a change in the extract as well as a blockade of the formation of high-affinity receptors. That receptors remain high affinity after their transient association with cytoskeleton (9) indicates that continued association is not obligatory to maintain the high affinity.
The conclusions of Painter et al. (16) are problematical. In contrast to the previously published observation in broken cells (19), Painter et al. (16) detected rapidly dissociating ligand–receptor complexes in control cell membranes in the absence of guanine nucleotide. The extent of rapidly dissociating ligand–receptor complexes is enhanced by dihydrocytochalasin B alone or in combination with formyl peptide. While such results cannot be dismissed, they point to a fundamental difference in the application of the fluorescence detection systems for receptor analyses in our two laboratories.

Receptor Number and Internalization

Cytoskeletal elements are suggested to play roles in the up-regulation of the N-formyl peptide receptor. Bender et al. (3) and Jesaitis and al. (10) showed an enhanced expression of N-formyl peptide receptors after cytochalasin treatment. In these studies, the enhanced receptor numbers were correlated with the enhanced oxygen radical production in elutriated neutrophils (10). We performed real-time flow cytometric N-formyl peptide binding studies with ligand concentrations appropriate to cell response and acquired fluorescence profiles every 30 s for 5 min on gelatin-sedimentated and elutriated neutrophils. In our studies with botulinum C2 toxin, the total amount and the rate of the oxygen radical production compared with control neutrophils were enhanced, as reported previously (12). We did not observe, however, any significant influence of botulinum C2 toxin on the N-formyl peptide ligand–receptor binding kinetics. Analogous results were obtained in cytochalasin B–treated neutrophils (data not shown).

One important difference between the results obtained in the present studies by using botulinum C2 toxin and cytochalasin B and previous results with cytochalasin (3, 10) might be the method used for the preparation of the neutrophils. Several groups (6, 27) have shown that lipopolysaccharide enhances the formyl peptide receptor number in a time- and concentration-dependent manner in cells prepared in lipopolysaccharide-free media by a mechanism called priming by Goldman et al. (6). In this study, neutrophils were prepared by using a gelatin-sedimentation step in which prior exposure to lipopolysaccharide appears to cause maximal receptor up-regulation. Current studies in our laboratory, which are performed with neutrophils isolated with lipopolysaccharide-free media, support the view that there is similar degree of up-regulation of N-formyl peptide receptors with lipopolysaccharide without degranulation or under degranulating conditions with cytochalasin B and botulinum C2 toxin (Norgauer, J., K. Aktories, and L. A. Sklar, manuscript in preparation).

Finally, it has been shown that cytochalasin treatment blocks the uptake of the N-formyl peptide receptor (10). Here we demonstrate by real-time cytometric methods that endocytosis of the N-formyl peptide ligand–receptor complex still occurs even in the absence of F-actin. All of the cells, either completely or partially depleted of F-actin, were capable of internalization. However, the rate of endocytosis was influenced by botulinum C2 toxin. The estimated half-time of the endocytosis of botulinum C2 toxin–treated neutrophils was ~8 min compared with 4 min in control cells. These findings suggest that a microfilament network is not essential for endocytosis, but modulates its velocity. Some indication that the processing may be altered is suggested in Fig. 8 A.

Here, the quenching of the intracellular ligand in toxin-treated cells occurs at a comparable, but not identical, rate after the extracellular pH is changed. How the fate and processing of the receptor depends upon the network remains to be determined.

In conclusion, botulinum C2 toxin inhibited the polymerization of actin, permitted degranulation, and delayed the endocytosis of the N-formyl peptide ligand–receptor complex. However, in neutrophil preparations examined in this study, there was no obligatory enhancement of N-formyl peptide receptor with the release of enzymes. Alterations in ligand receptor dynamics could not be correlated to the enhanced oxygen radical production in botulinum C2 toxin–treated cells. Moreover, depletion of F-actin did not influence the conversion of the affinity states in primed neutrophils. Neither was actin essential for the endocytosis of the N-formyl peptide ligand–receptor complex.

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