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.

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The Journal of Cell Biology, Volume 109, September 1989 1133-1140

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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)-l-phallacidin and rhodamine-phalloidin were obtained from Molecular Probes, Inc. (Junction City, OR). The N-formyl peptide receptor blocker t-butoxycarbonyl-phe-leu-leu-phe was obtained from Veda Biotechnologies, Inc. (Tuscon, 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 para-oxphenylacetic acid assay as described by Hyslop and 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., 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-phallacidin or rhodamine-phalloidin, and 1 mg/ml lysophosphatidylcholine. The fluorescence intensity of F-actin was measured in a computer (FACS IV, Becton Dickinson & Co., Sunnyvale, CA) as described (21).

Figure 1. Comparison of the 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, respectively. Data representative of a single experiment performed in duplicate and repeated twice in this study (and previously, see reference 12) are shown.

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-phallacidin 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

1. Abbreviation used in this paper: NBD, 7-nitrobenz-2-oxa-1,3-diazole.
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. 3B). 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 dissociation...
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 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 shows 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 (); cells treated with component I (○), component II (Δ), and components I and II (×). In B: control cells (——); cells treated with component I (– –), component II (– –), and components I and II (– –).

The data are consistent with two receptor forms: one form that dissociated with a half-time of ~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 quenched the extracellular ligand while the intracellular ligand was transiently protected. Thus, a calculation of rapidly quenched vs. the protected fluorescence allows an estimate of the extent of internalization (5). Fig. 7 A shows a dot plot of a neutrophil population labeled for 105 s with 10 nM N-formyl peptide. The mean fluorescence channel number...
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^-5 M tBoc-phe-leu-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-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
modifies actin at the same site as botulinum C2 toxin (25, 26), behaves like a capping protein. Provided that ADP-ribosylated neutrophil actin has capping protein function, a small amount of modified actin could be sufficient for blocking the rapid polymerization during activation of neutrophils by capping all polymerization nuclei.

Yuli and Snyderman (30) proposed that the transient right angle light scatter response reflects a cell shape change like membrane ruffling. Sklar et al. (21) showed that this response parallels the actin polymerization in N-formyl peptide–stimulated neutrophils.

Thus, these latter findings argue against an essential role for actin in the affinity change in elutriated neutrophils.

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-sedimented 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 a 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.

J. Norgauer is a recipient of a fellowship from the Boehringer Ingelheim Fonds, Stuttgart, FRG. This work was supported by National Institutes of Health grants AI-19032, AI-17354, and GM-37696.

Received for publication 6 February 1989 and in revised form 8 May 1989.

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