Volume 219, number 1, 40–44 FEB 04859 July 1987

Botulinum C_2 toxin potentiates activation of the neutrophil oxidase

Further evidence of a role for actin polymerization

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Received 26 March 1987; revised version received 5 May 1987

Botulinum C_2 toxin was employed as a specific inhibitor of actin polymerization in rat neutrophils to determine its role in oxidase activation. This toxin was shown to inhibit actin polymerization and the microfilament-dependent function, phagocytosis. Oxidase activation in response to the chemotactic peptide, f-Met-Leu-Phe (FMLP) was enhanced approx. 3-fold. The enhancement by C_2 toxin did not occur in cells pretreated with cytochalasin B. C_2 toxin had no significant effect on the FMLP-induced intracellular Ca^{2+} rise. These data are consistent with an inhibitory role for actin polymerization in oxidase activation.

Neutrophil; Enzyme activation; Oxidase; Actin polymerization; intracellular Ca^{2+}; (Fura-2)

1. INTRODUCTION

The activation of the neutrophil oxidase represents an important event in the killing of phagocytosed micro-organisms [1]. However, the oxidase can also be triggered by soluble agents, both experimental, such as the calcium ionophore, A23187 and phorbol myristyl acetate (PMA), and physiologically relevant, such as the chemotactic peptide, formylmethionylleucylphenylalanine (FMLP) [2]. It is clear that activation can occur by at least two mechanisms, one dependent on and the other independent of a rise in intracellular Ca^{2+} [3]. Indirect evidence also suggests that actin polymerization may influence the extent of activation when stimulation is via the Ca^{2+}-dependent route [4]. This evidence, however, mainly rests on the use of cytochalasin B and related cytochalasins. Since these agents may exert other effects in the cell, it is important to test the role of actin polymerization by the use of other inhibitors. Botulinum C_2 toxin is such an inhibitor which catalyses intracellular ADP-ribosylation of non-muscle actin thus preventing actin polymerization [5,6]. Furthermore, ADP-ribosylation is specific for non-muscle actin, no other cytoplasmic proteins acting as substrate. The aim of the present work was to use C_2 toxin to test the hypothesis that actin polymerization modifies activation of the neutrophil oxidase.

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2. MATERIALS AND METHODS

Rat neutrophils were prepared from caseinate-induced peritoneal exudate as in [7]. Cells were suspended in Hepes-buffered Krebs medium containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 1.3 mM $CaCl_2$, 25 mM Hepes, pH adjusted to 7.4 with NaOH. Oxidase activation was monitored (i) by detection of reactive oxygen metabolite production using luminol-dependent chemiluminescence as described [7] and (ii) by measurement of oxygen-consumption rate using a Clark-type oxygen elec-

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trode as in [8]. Measurement of G-actin and total actin was by an assay of DNase inhibition [4] and myeloperoxidase secretion was measured using guiacol as indicator [2]. Phagocytosis of unopsonised fluorescein-labelled latex particles (d = 1 μm, PolySciences, Northampton, England) was determined after incubation of cells (10^7/ml) with beads (10^9/ml) for 30 min at 37°C. The suspension was diluted by a factor of 10^2, viewed by fluorescence microscopy and beads associated with cells counted. Botulinum C_2 toxin was prepared as described in [9]. Component 2 was activated by trypsinization before use. Fura-2 acetoxy methyl ester (Molecular Probes, OR) was loaded into the cells to give a final intracellular concentration of approx. 150 μM. Dual-wavelength excitation monitoring was achieved using a Spex Fluorolog and combination system as described by Tsein et al. [10]. The fluorescence ratio of signals at 340 and 380 nm was transformed into free Ca^{2+} from a calibration curve.

3. RESULTS

3.1. *Effect of C_2 toxin on phagocytosis and exocytosis*

Pre-treatment of rat neutrophils with C_2 toxin (6.65 μg/ml, component 1; 13.3 μg/ml, component 2) inhibited phagocytosis of latex beads and enhanced the secretion of myeloperoxidase stimulated by FMLP (1 μM) (table 1). Pre-treatment with C_2 toxin component 2 alone did not effect subsequent secretion stimulated by FMLP. It was also noted that C_2 toxin triggered slight secretion in the absence of other stimuli, approx. 15% of total myeloperoxidase released compared with approx. 40% in the presence of C_2 toxin and FMLP. This small secretory response could not account for the enhancement of secretion observed with FMLP. Since cytochalasin B also inhibited phagocytosis and enhanced FMLP-triggered secretion of myeloperoxidase, these results were consistent with the effect of C_2 toxin being mediated by inhibition of actin polymerization.

Table 1

Effect of C_2 toxin on phagocytosis, myeloperoxidase secretion and actin polymerization

C_2 toxin	Phagocytosis	Secretion	Actin polymerization
−	22.5 ± 2.0	5.7 ± 1.3	78 ± 6
+	4.0 ± 2.5	37.5 ± 2.5	52 ± 4

The effect of C_2 toxin on (i) phagocytosis of fluorescein-labelled latex beads, after incubation at 37°C for 30 min with beads (10^8/ml); (ii) secretion of myeloperoxidase, 5 min after stimulation with FMLP (1 μM); and (c) polymerization of actin 5 s after stimulation with FMLP (1 μM). Data represent means ± SE for (i) n = 50, (ii) n = 3, (iii) n = 3, and the units are (i) beads/cell, (ii) % of total myeloperoxidase released and (iii) % total actin in polymerized (F-actin) form

3.2. *Effect of C_2 toxin on neutrophil actin polymerization*

Resting rat neutrophils (non-adherent) contained approx. 6 pg actin/cell, of which approx. 50% was in the polymerized form (F-actin). C_2 toxin had no effect on the extent of actin polymerization in these cells. Upon stimulation with FMLP (1 μM) there was a rapid increase in actin polymerization, reaching a peak of approx. 80% polymerization within 5 s. This stimulated increase in actin polymerization was prevented by C_2 toxin (table 1).

3.3. *Effect of C_2 toxin on oxidase activation*

Having established that C_2 toxin inhibited FMLP-induced actin polymerization, this agent was used to test whether actin polymerization played a role in activation of the oxidase. Fig.1 shows a typical experiment. The luminol-dependent chemiluminescent response to FMLP was unaffected by pre-treatment with component 2 of C_2 toxin. However, after treatment with the toxin (components 1,2), the response was enhanced approx. 7-fold. In a series of experiments, the mean enhancement was 7.39 ± 0.56-fold (n = 4, mean ± SE). The enhancement was also observed when oxidase activity was monitored by measurement of the oxygen-consumption rate (fig.1), suggesting the enhancement was not merely an artefact of the luminol assay system. However, since the enhancement of oxygen consumption was less (approx. 3 ± 0.51-fold, n = 3), part of the enhancement of luminol chemiluminescence may be attributed to the presence of secreted peroxidase. Pre-treatment of the cells with cytochalasin B (5 μg/ml) which also enhanced oxidase activa-

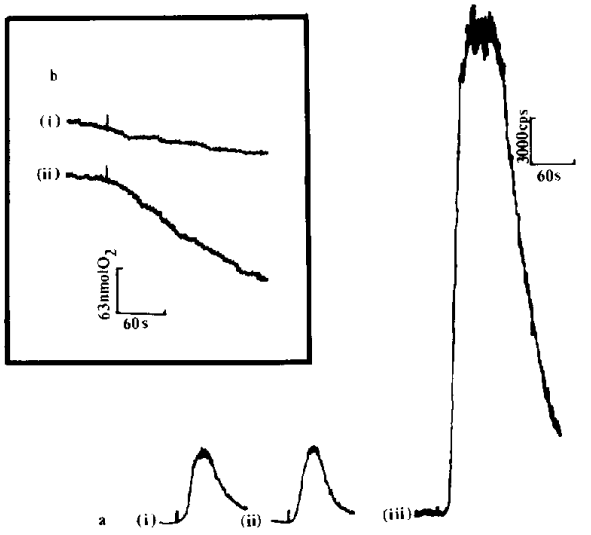

Fig.1. Effect of botulinum C_2 toxin on oxidase activation by FMLP. (a) Activation of oxidase monitored by luminol-dependent chemiluminescence, from: (i) untreated cells; (ii) cells treated with toxin component 2 (13.3 μg/ml); (iii) cells treated with both toxin components 1 (6.65 μg/ml) and 2 (13.3 μg/ml). At the mark indicated on the trace the cells were stimulated with FMLP (1 μM). The calibration bar shows luminescence counts per second (cps) recorded. (b) Consumption of oxygen by: (i) untreated cells; (ii) cells treated with components 1 (6.65 μg/ml) and 2 (13.3 μg/ml) of the toxin. At the mark the cells were stimulated with 1 μM FMLP.

tion totally inhibited FMLP-induced actin polymerization [4]. No further enhancement of the oxidase response by C_2 toxin was possible under these conditions (fig.2), suggesting that both agents acted at a common site. As with cytochalasin B, no enhancement was observed with stimulation by PMA (fig.2A).

3.4. *Effect of C_2 toxin on resting oxidase activity*

Addition of C_2 toxin to neutrophils resulted in a weak activation of the oxidase system representing a peak response approx. 5% of that observed after C_2 toxin with FMLP. This response was not observed with either component alone, both components 1 and 2 being essential for the response (fig.2B). The response parallels the secretion of myeloperoxidase. The response was not abolished by t-Boc-Met-Leu-Phe, an antagonist of the formylated peptide reception, at concentrations up to 10 mM, although some inhibition was observed at high concentrations. In these conditions actin polymerization was unaffected.

3.5. *The effect of C_2 toxin on intracellular Ca^{2+}*

The possibility that the concentrations of C_2 toxin used here resulted in perturbation of the cell membrane and increased Ca^{2+} permeability was tested by measurement of intracellular Ca^{2+} using fura-2 as indicator. Under the conditions of the experiment, the fura-2 signal rose slowly over the 12 min incubation with C_2 toxin, reaching a plateau level corresponding to 195 ± 9 nM. This compared with the concentration in untreated cells of 114 ± 13 nM. Stimulation of cells with FMLP (1 μM) resulted in a transient intracellular Ca^{2+} elevation to 987 ± 7 nM in untreated cells compared with a fura-2 signal corresponding to 1148 ± 56 nM for C_2 toxin-treated cells (fig.3). The time course of the fura-2 signal was also altered after C_2 toxin treatment. Since approx. 8% of the cell-associated fura-2 was within secretory granules in those cells, the quantitative and qualitative differences in the fura-2 signal after C_2 toxin may have resulted from secretion of granular fura-2 into extracellular medium containing saturating concentrations of Ca^{2+} (1.3 mM).

4. DISCUSSION

Studies of the role of actin polymerization and microfilament function often rely upon the use of cytochalasins. However, it is well documented that these agents can exert other effects on cells including inhibition of glucose uptake [11] and thymidine incorporation [12]. The use of these agents has therefore been criticised. An alternative agent for preventing actin polymerization, such as botulinum C_2 toxin, therefore is important in testing conclusions reached by the use of cytochalasins [6]. The toxin consists of two components [13], component 2 which binds to the cell membrane, and component 1 which is transported into the cytoplasm where it catalyses ADP-ribosylation of G-actin [5,6]. The ribosylation is specific for non-muscle actin, muscle actin and other proteins not being ribosylated [6]. Since ADP-ribosylated actin is unable to participate in actin polymerization, C_2 toxin represents a useful

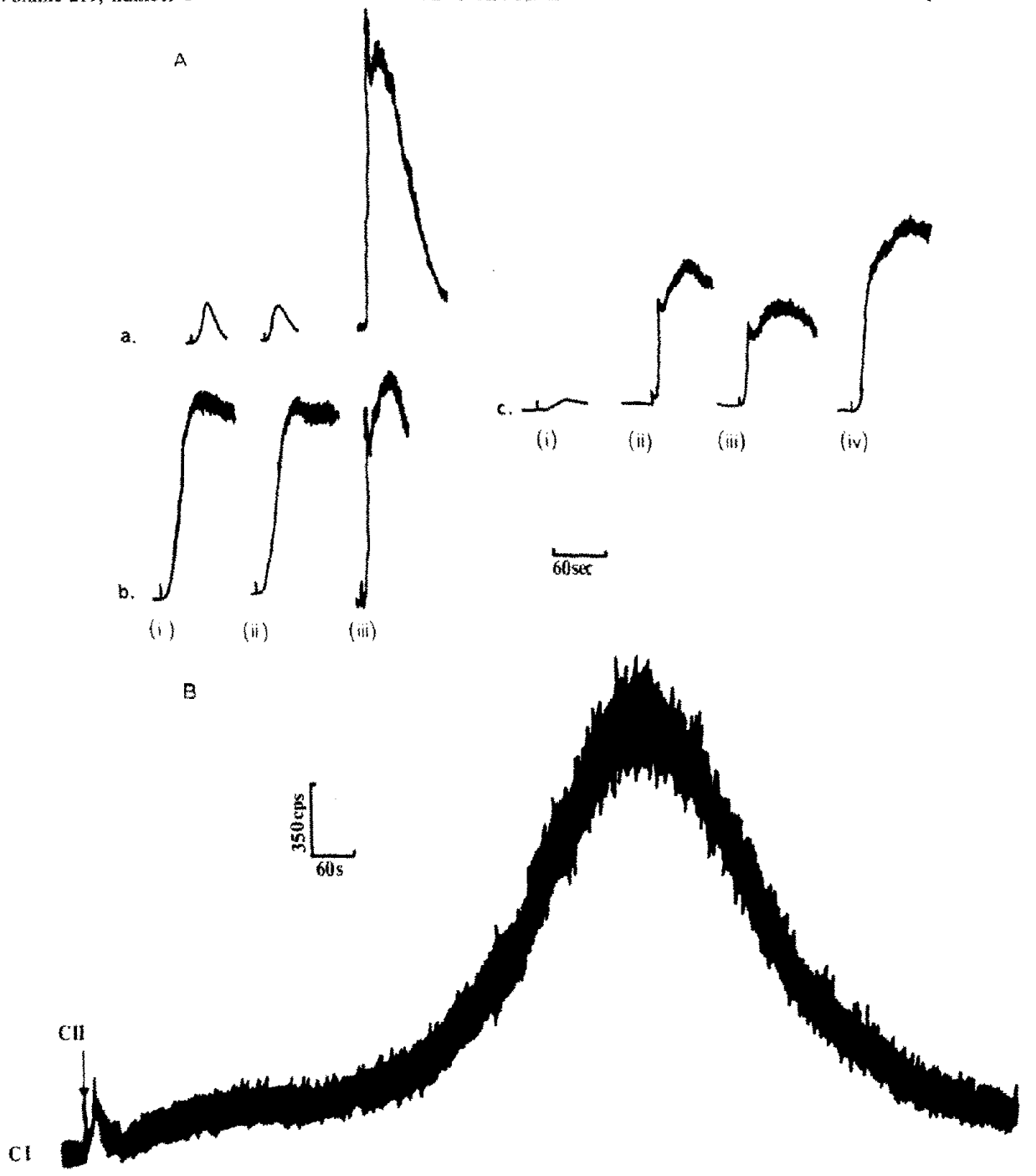

Fig.2. (A) Interaction of botulinum C_2 toxin with PMA and cytochalasin B luminol-dependent chemiluminescence from neutrophils stimulated by (a,c) 1 μM FMLP and (b) PMA at 0.1 μg/ml. In (a,b), traces (i) show the response from untreated cells, (ii) from cells treated with toxin component 2 and (iii) from cells treated with complete toxin. In (c), the trace shows the response from (i) untreated cells, (ii) cells treated with C_2 toxin, (iii) cells treated with C_2 toxin and cytochalasin B (5 μg/ml) and (iv) cells treated with cytochalasin B. The calibration scale is shown as luminescence cps and is different for traces (c) from that for traces (a,b). (B) Effect of C_2 toxin on resting oxidase activity. Luminol-dependent chemiluminescence is shown from neutrophils, pre-treated with toxin component 1 (6.65 μg/ml) which produced no increase in response, when toxin component 2 (13.3 μg/ml) was added at the time indicated by the arrow. Component 2 alone produced no response (see fig.1; and this figure, panel A).

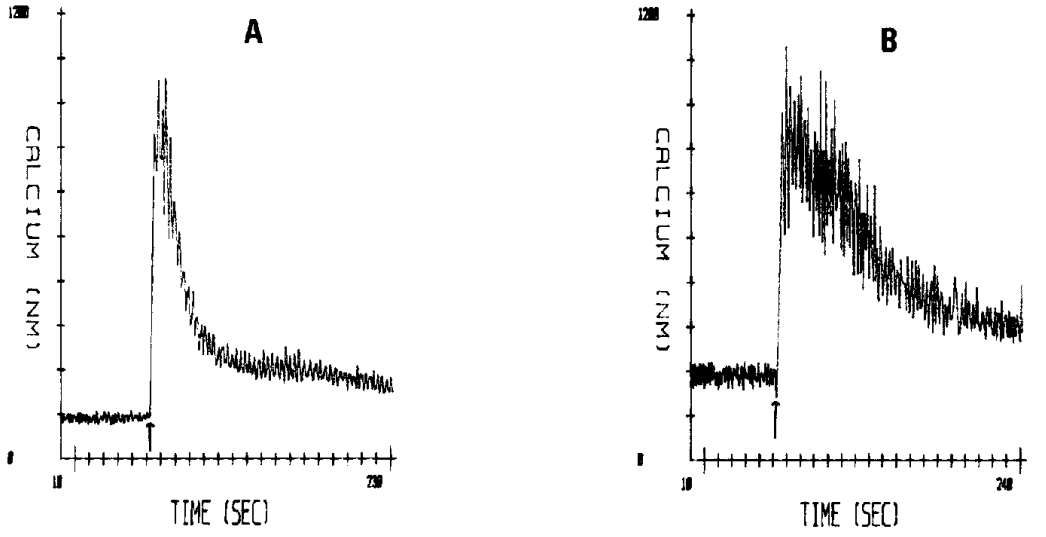

Fig.3. Effect of C_2 toxin on fura-2 signals. The traces show the fura-2 (340 nm/380 nm ratio) signal deconvoluted and expressed as intracellular Ca^{2+} concentration. (A) Untreated cells. (B) C_2 toxin treated. At the arrow FMLP (1 μM) was added.

specific inhibitor of actin polymerization in non-muscle cells.

The present data show the use of this agent in testing whether actin polymerization played a role in neutrophil oxidase activation. The agent inhibited actin polymerization and a microfilament-dependent cellular event, phagocytosis. Oxidase activation in response to FMLP was enhanced. This enhancement was accompanied by the secretion of myeloperoxidase. Enhancement was not observed with PMA as stimulus, nor was enhancement achieved in cells pre-treated with cytochalasin B. Although the toxin had an effect on the fura-2 signal from loaded cells, it was possible that this resulted from secretion of fura-2 which was within granules (see table 1), and that no significant effect on the FMLP-mediated intracellular Ca^{2+} rise occurred. These results are, therefore, consistent with C_2 toxin acting at a site after Ca^{2+} influx. The use of C_2 toxin has therefore supported the hypothesis that actin polymerization exerts an inhibiting influence on FMLP-triggered oxidase activation. The mechanism for this inhibitory influence remains to be determined.

ACKNOWLEDGEMENT

F.A.A.-M. holds a scholarship from the Saudi Ministry of Defence.

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