***Clostridium perfringens* α -toxin induces rabbit neutrophil adhesion**

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***Clostridium perfringens* α -toxin, which is one of the main agents involved in the development of gas gangrene, stimulates O_2^- production in neutrophils. Exposure of rabbit neutrophils to the α -toxin induced firm adhesion of the cells to fibrinogen and fibronectin. Incubation of rabbit neutrophils and neutrophil lysates with α -toxin led to the production of diacylglycerol (DG) and L- α -phosphatidic acid (PA), respectively. The toxin-induced DG and PA formation preceded the toxin-induced adhesion of the neutrophils to fibrinogen and fibronectin, and the production of O_2^- . Pertussis toxin inhibited the α -toxin-induced formation of PA, the adhesion of the neutrophils to fibrinogen and O_2^- production. GTP γ S stimulated the events induced by the α -toxin, whereas GDP β S inhibited them. The α -toxin stimulated phosphorylation of a protein with a molecular mass of about 40 kDa. In addition, treatment of the cells with 1-oleoyl-2-acetyl-sn-glycerol (OAG) and phorbol-12,13-dibutyrate (PDBu) stimulated cell adhesion, production of O_2^- and phosphorylation of the 40 kDa protein, but had no effect on the formation of PA. The events induced by the presence of OAG and PDBu were not inhibited by pertussis toxin. Protein kinase C inhibitors, H-7, staurosporine and chelerythrine, blocked α -toxin-induced adhesion, production of O_2^- and phosphorylation of the 40 kDa protein. These observations suggested that α -toxin-stimulated adhesion to the matrix and O_2^- production were due to the formation of DG, through activation of phospholipid metabolism by a pertussis-toxin-sensitive GTP-binding protein, followed by activation of protein kinase C by DG.**

Keywords: fibrinogen, gas gangrene

INTRODUCTION

Clostridium perfringens α -toxin, which possesses lethal, haemolytic, dermonecrotic and phospholipase C (PLC) activities (Titball, 1993, 1997a, b, 1999; Sakurai, 1995), has been reported to be a major pathogenic factor in the development of gas gangrene caused by the micro-organism (Awad *et al.*, 1995; Sakurai, 1995; Titball, 1997b; Bryant *et al.*, 2000a, b). Neutrophils are known to respond to invasion by micro-organisms and to kill them through the generation of reactive oxygen metabolites. Several studies have reported that the α -toxin of *C. perfringens* (Patriarca *et al.*, 1970; Stevens *et al.*, 1987;

König *et al.*, 1997; Hofman *et al.*, 2000), and the PLC of *Bacillus cereus* (Styrt *et al.*, 1989), activate neutrophils, measured as an increase in O_2^- formation and enhancement of chemiluminescence induced by opsonized zymosan. However, histopathology has demonstrated an absence of leukocytes within the area of necrosis associated with *C. perfringens* growth and the presence of these cells at the borders of the region showing necrosis (Robb-Smith, 1945; Stevens *et al.*, 1997). Stevens *et al.* (1987) reported that the toxin did not alter the viability, chemotactic responsiveness or morphology of human leukocytes. Bryant & Stevens (1996) reported that exposure to α -toxin induced an up-regulation of the expression of intercellular adherence molecule (ICAM-1), E-selectin and P-selectin expression, and that exposure to θ -toxin induced ICAM-1 and the production of platelet-aggregating factor by endothelial cells. However, Ellemor *et al.* (1999) reported that an infection of

Abbreviations: DG, diacylglycerol; fMLP, formyl-Met-Leu-Pro; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo(1,2-a)pyrazin-3-one; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PA, L- α -phosphatidic acid; PDBu, phorbol-12,13-dibutyrate; PLC, phospholipase C.

C. perfringens resulted in no increase in ICAM-1. Therefore, little is known about the cause of the paucity of leukocytes within the area of necrosis caused by the micro-organism.

We have previously reported that α -toxin induces the contraction of isolated rat aorta (Fujii *et al.*, 1986) and ileum (Sakurai *et al.*, 1990), and that this contraction is related to the turnover of phosphatidylinositol. Notably, the toxin-induced contraction of isolated aorta was found to be closely linked to the stimulation of thromboxane A_2 synthesis (Fujii & Sakurai, 1989). We have also reported that incubation of rabbit erythrocyte membranes with the toxin results in a biphasic production of PA and that the rapid formation of PA, induced by the toxin, is due to activation of endogenous PLC regulated by GTP-binding protein, whereas the late formation is dependent on the activation of endogenous phospholipase D (Sakurai *et al.*, 1993, 1994; Ochi *et al.*, 1996). Therefore, to test if α -toxin affects the adhesion of rabbit neutrophils to tissues, we compared the effect of the toxin on the adhesion of neutrophils to matrixes. Moreover, we present evidence of a relationship between α -toxin-induced adhesion and phospholipid metabolism in rabbit neutrophils.

METHODS

Purification of wild-type α -toxin and the H148G variant.

Recombinant plasmids of pHY300PLK harbouring the structural genes of wild-type α -toxin or H148G (Nagahama *et al.*, 1995) were introduced into *Bacillus subtilis* ISW1214 by transformation. Transformants were grown in Luria-Bertani broth containing 50 μ g ampicillin ml^{-1} to an OD_{600} of 0.8–0.85, with continuous aeration. The culture was centrifuged (18000 g, 20 min) and ammonium sulfate (472 g l^{-1}) was added to the culture supernatant fluid. The ammonium sulfate fraction obtained was used as the starting material for purifying the toxins. Purification of wild-type toxin and the H148G variant was performed as described previously (Nagahama *et al.*, 1995), and was followed by homogeneity-testing with SDS-PAGE and immunological techniques (Nagahama *et al.*, 1995). The PLC activity of the wild-type α -toxin was determined photometrically (A_{405}) with *p*-nitrophenylphosphorylcholine (Sigma) as the substrate (Kurioka & Matsuda, 1976). One unit of PLC activity was defined as the amount of enzyme necessary to liberate 1 μ mol *p*-nitrophenol min^{-1} from *p*-nitrophenylphosphorylcholine at 37 °C. In this case, one unit corresponds to about 9 μ g of wild-type α -toxin. H148G variant toxin bound to sheep erythrocyte membranes and phosphatidylcholine-cholesterol liposomes, but did not contain PLC (*p*-nitrophenylphosphorylcholine hydrolytic activity), sphingomyelinase, or haemolytic and lethal activity, as reported previously (Nagahama *et al.*, 1995).

Preparation of rabbit neutrophils and cell lysates. Rabbit blood was withdrawn from the ear arteriae of New Zealand White rabbits. Acid citrate-glucose (0.085 M sodium citrate, 0.065 M citric acid, 2% glucose) was present in each sample as an anticoagulant. Blood samples were centrifuged at 380 g for 5 min, and the supernatant containing the leukocytes was collected and mixed with 4.5% dextran (Amersham) in 0.9% NaCl. The supernatant (the plasma layer containing the leukocytes) was recovered after 30 min sedimentation and then centrifuged at 380 g for 5 min. Contaminated erythro-

cytes contained within the pellet were removed by haemolysis with hypotonic ammonium chloride solution (0.155 M NH_4Cl , 0.119 M $NaHCO_3$, 0.1 mM EDTA). Following centrifugation (380 g, 5 min), the white cell pellet was washed and resuspended in Hanks' balanced salt solution without divalent cations (HBSS; 137 mM NaCl, 5.36 mM KCl, 0.337 mM Na_2HPO_4 , 0.441 mM KH_2PO_4 , 4.17 mM $NaHCO_3$, 5.55 mM glucose, pH 7.4). The suspended cells were overlaid with lymphoprep (New England Nuclear) and centrifuged at 280 g for 20 min. The neutrophil pellet was washed and resuspended to a concentration of 1×10^8 cells ml^{-1} in HBSS. The neutrophils were routinely of high purity (> 90%) and viability (> 95%). Rabbit neutrophil lysates were prepared by sonication; the resulting sonicates were termed the lysate.

Measurement of active oxygen in rabbit neutrophils.

The generation of active oxygen was evaluated by the MCLA [2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-(1,2-a)pyrazin-3-one]-dependent chemiluminescence method (Nishida *et al.*, 1989; Nakano, 1990). MCLA reacts with O_2^- or 1O_2 (singlet oxygen) to emit light, via the dioxetanone analogue. Superoxide dismutase (a scavenger of O_2^-), or NaN_3 (a quencher of 1O_2) can be used for differentiating between O_2^- and 1O_2 -dependent luminescence. Thus, MCLA is an excellent chemiluminescence probe for the detection of O_2^- in terms of its specificity and selectivity. Rabbit neutrophils (1.5×10^6 cells ml^{-1}) were incubated with α -toxin at 37 °C in a final volume of 0.2 ml HBSS containing 0.3 mM $CaCl_2$ and 1.25 μ M MCLA (Tokyo Kasei Kogyo). The maximal chemiluminescence intensity was determined by measuring its peak height (c.p.m.) using a chemiluminescence reader (luminescencer-JNR; Atto, Japan). Xanthine oxidase (XO, Sigma) was assayed by the reduction of ferrocytochrome *c* (Sigma), in terms of the conversion of hypoxanthine (Wako Pure Chemical Industries) to urate. Briefly, the reaction mixture contained, in a final volume of 0.2 ml HBSS, 50 μ M hypoxanthine and 40 μ M XO. Adding the XO at 37 °C started the reaction and the change in absorbance at 550–540 nm was monitored by a spectrophotometer. In this case, one unit of XO corresponded to the generation of 0.248 nmol O_2^- min^{-1} and a MCLA-dependent maximal chemiluminescence intensity of 744000 c.p.m.

Measurement of active oxygen in permeabilized rabbit neutrophils.

Rabbit neutrophils (2.0×10^6 cells ml^{-1}) were permeabilized in HBSS containing 10 μ M cytochalasin B (Nacalai Tesque) and 2 μ M streptolysin O (Sigma) (pre-activated by incubation with 0.45 mM dithiothreitol at 37 °C for 5 min) at 37 °C for 5 min (at this concentration of streptolysin O, $0.9 \pm 0.2\%$ of the total lactate dehydrogenase activity was released from cells during a 30 min incubation) (Bhakdi & Tranum-Jensen, 1988). The permeabilized neutrophils (1.5×10^6 cells ml^{-1}) were incubated with α -toxin at 37 °C in HBSS containing 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM glutathione (oxidized form) and 1.25 μ M MCLA in the absence or presence of a guanine nucleotide analogue (GTP γ S or GDP β S, Roche). The chemiluminescence intensity was determined by a chemiluminescence reader.

Measurement of rabbit neutrophil adhesion.

The adhesion of neutrophils to extracellular matrixes was evaluated in 24-well tissue culture plates coated with ligand. Prior to the addition of neutrophils, the plates were incubated with 500 μ l per well of 50 μ g human fibrinogen ml^{-1} (Calbiochem-Novabiochem), 50 μ g bovine type I collagen ml^{-1} (Sigma) or 50 μ g bovine fibronectin ml^{-1} (Nacalai Tesque) for 2 h at 37 °C. The wells were washed once with HBSS, blocked with 1% BSA in HBSS for 1 h at 37 °C, washed twice with HBSS containing 0.1%

Fig. 1. Effect of α -toxin on neutrophil adhesion to fibrinogen and superoxide generation. (a) Rabbit neutrophils ($1.2 \times 10^6 \text{ ml}^{-1}$) were incubated with various concentrations of α -toxin in fibrinogen- ($\square$), fibronectin- ($\triangle$) or type I collagen- ($\circ$) coated wells at 37°C for 20 min. Values are means $\pm$ SE for five or six experiments. (b) Rabbit neutrophils ($1.2 \times 10^6 \text{ ml}^{-1}$) were incubated with various concentrations of fMLP in fibrinogen-coated wells at 37°C for 15 min. Adherent cells were determined as described in Methods. Values are means $\pm$ SE for five or six experiments. (c) Rabbit neutrophils ($1.5 \times 10^6 \text{ ml}^{-1}$) were incubated without ($\square$) or with various concentrations of α -toxin (∇ , $1 \mu\text{g ml}^{-1}$; $\blacktriangle$, $2.5 \mu\text{g ml}^{-1}$; $\triangle$, $5 \mu\text{g ml}^{-1}$; $\circ$, $10 \mu\text{g ml}^{-1}$; $\bullet$, $20 \mu\text{g ml}^{-1}$) at 37°C for different periods and the chemiluminescence was measured by a chemiluminescence reader. The results from one of five experiments yielding similar results are shown. (d) Rabbit neutrophils ($1.5 \times 10^6 \text{ ml}^{-1}$) were incubated without ($\square$) or with $10 \mu\text{g ml}^{-1}$ wild-type α -toxin ($\circ$) or $100 \mu\text{g ml}^{-1}$ H148G ($\triangle$) at 37°C for various periods and the chemiluminescence was measured by a chemiluminescence reader. A typical result from one of five experiments is shown.

Tween 20 and then once with HBSS. Rabbit neutrophils ($1.2 \times 10^6 \text{ cells ml}^{-1}$) were then added to individual wells. After incubation with α -toxin in the presence of 0.3 mM CaCl_2 for 30 min at 37°C , non-adherent cells were gently washed twice with warm PBS ($1.06 \text{ M KH}_2\text{PO}_4$, $2.97 \text{ mM Na}_2\text{HPO}_4 \cdot 7 \text{ H}_2\text{O}$, 154 mM NaCl) containing 1 mM CaCl_2 . Adherent neutrophils were then stained with $250 \mu\text{l}$ 0.25% rose bengal (Wako Pure Chemical Industries) solution for 10 min at room temperature. The staining solution was aspirated off and each well was washed twice with PBS and then $250 \mu\text{l}$ ethanol/PBS ($1:1$, v/v) solution was added. After incubation at 37°C for 30 min, to allow the cell-retained stain to be completely dissolved, the A_{557} of each well was determined with a microplate spectrophotometer (SPECTRAMax 340PC, Molecular Devices), using wells containing medium alone as a control. Cell adhesion to wells coated with BSA was not detected, even when neutrophils were treated with the toxin in the presence of Ca^{2+} .

Measurement of diacylglycerol (DG) and L- α -phosphatidic acid (PA) formation induced by α -toxin in rabbit neutrophils. For DG measurements, rabbit neutrophils ($1.5 \times 10^7 \text{ cells ml}^{-1}$) were incubated with α -toxin in a final volume of 0.08 ml HBSS containing 0.3 mM CaCl_2 and 1 mM MgCl_2 at 37°C . After the reaction was terminated, by the addition of 0.3 ml chloroform/methanol ($1:2$, v/v), DG was extracted and quantified as previously described (Sakurai *et al.*, 1993). In brief, DG in the extracted lipids was converted into [^{32}P]PA by *Escherichia coli* DG kinase in the presence of [γ - ^{32}P]ATP. [^{32}P]PA was

separated by TLC and measured in a liquid scintillation counter. The amount of DG converted to [^{32}P]PA was calculated based upon the specific activity of [γ - ^{32}P]ATP and the sample volume. A standard curve for the conversion of DG to PA was constructed using known quantities of 1-stearoyl-2-arachidonyl-*sn*-glycerol (Biomol Research Laboratories), and quantitated from the specific activity. A known amount of 1-stearoyl-2-arachidonyl-*sn*-glycerol was run with each assay to quantify the conversion.

For PA measurements, rabbit neutrophil lysates ($1.5 \times 10^7 \text{ cells ml}^{-1}$) were incubated with α -toxin in a final volume of 0.5 ml HBSS containing 0.3 mM CaCl_2 , 1 mM MgCl_2 and $10 \mu\text{Ci ml}^{-1}$ (370 kBq ml^{-1}) [γ - ^{32}P]ATP (ICN Biochemicals) in the presence or absence of a guanine nucleotide analogue (GTP γ S or GDP β S) at 37°C . After the reaction was terminated by the addition of 0.5 ml ice-cold HBSS and 3.6 ml chloroform/methanol/concentrated HCl ($50:100:1$, by vol.), [^{32}P]PA was extracted and quantified as previously described (Sakurai *et al.*, 1993). Briefly, the extracted [^{32}P]PA was separated by TLC and the radioactivity measured in a liquid scintillation counter.

Detection of protein phosphorylation induced by α -toxin in rabbit neutrophils. Rabbit neutrophil lysates ($1.5 \times 10^7 \text{ cells ml}^{-1}$) were incubated with α -toxin at 37°C for 5 min in HBSS containing 0.3 mM CaCl_2 , 1 mM MgCl_2 , $0.1 \text{ mM Na}_3\text{VO}_4$ and $10 \mu\text{Ci}$ [γ - ^{32}P]ATP ml^{-1} . After incubation, the reaction was terminated by the addition of 0.5 ml ice-cold 7.5%

trichloroacetic acid and kept on ice for 30 min. The precipitate was collected by centrifugation at 10000 *g* for 20 min and then washed twice by centrifugation in 1 ml ice-cold 7.5% trichloroacetic acid. Phosphorylated proteins were analysed by SDS-PAGE according to Laemmli (1970) and by subsequent autoradiography.

Determination of protein concentration. Protein concentration was determined by the Lowry method, using BSA as a standard.

Statistical analysis. All mean values are shown with their calculated standard error (SE). Student's *t*-test was used to determine the significance of differences between controls and experimental groups; a *P* value of 0.05 or less was considered statistically significant.

RESULTS

Effect of α -toxin on rabbit neutrophils

To investigate the effect of α -toxin on neutrophil adhesion to fibrinogen and on the chemiluminescence responses of rabbit neutrophils, neutrophils were incubated with various concentrations of the toxin at 37 °C for 30 min. As shown in Fig. 1(a, c), toxin added in the range 2.5–20 $\mu\text{g ml}^{-1}$ induced adhesion of the cells to fibrinogen and chemiluminescence responses in a dose-dependent manner. The adhesion started 1–5 min after incubation when the cells were incubated with 20 $\mu\text{g } \alpha$ -toxin ml^{-1} , and was maximal after 20 min of incubation (data not shown). Formyl-Met-Leu-Pro (fMLP), which was used as a positive control, increased cell adhesion to fibrinogen in a dose-dependent manner (Fig. 1b). The toxin-induced chemiluminescence responses began about 1.5 min after the incubation of the cells with toxin concentrations less than 20 $\mu\text{g ml}^{-1}$, and maximal responses were observed about 4, 5 and 7 min after incubation with 20, 10 and 2.5 $\mu\text{g } \alpha$ -toxin ml^{-1} , respectively (Fig. 1c). Toxin-induced chemiluminescence was completely inhibited by 0.5 μM superoxide dismutase, whereas it was not inhibited significantly by 0.5 mM N_3^- ($^1\text{O}_2$ quencher), 20 μg catalase ml^{-1} (H_2O_2 scavenger), or 50 mM mannitol ($\text{HO}^\bullet$ scavenger) (data not shown). Treatment of rabbit neutrophils with less than 50 $\mu\text{g } \alpha$ -toxin ml^{-1} caused no release of lactate dehydrogenase from the cells. Therefore, it appears that the toxin does not harm the cells, as reported by Stevens *et al.* (1987). Toxin pretreated with 1 mM EDTA, and the H148G variant toxin, which does not possess PLC or haemolytic activity (Nagahama *et al.*, 1995), did not induce cell adhesion to fibrinogen or a chemiluminescence response (data not shown). We tested the ability of the α -toxin-treated rabbit neutrophils to adhere to other extracellular matrix ligands, such as type I collagen and fibronectin. The toxin induced cell adhesion to these ligands in a dose-dependent manner (Fig. 1a). The adhesion of neutrophils to fibronectin was comparable with that to fibrinogen, but the toxin-induced adhesion to type I collagen was about 30% of that to fibrinogen. It is likely that the toxin-induced adhesion of rabbit neutrophils is matrix-specific.

Fig. 2. Effect of α -toxin on DG formation in neutrophils and PA formation in neutrophil lysates. (a) Rabbit neutrophils ($1.5 \times 10^7 \text{ ml}^{-1}$) or lysates were incubated with various concentrations of α -toxin at 37 °C for 5 min. DG ($\circ$) and PA ($\bullet$) were determined as described in Methods. Values are means $\pm$ SE for five or six experiments. (b) Rabbit neutrophils ($1.5 \times 10^7 \text{ ml}^{-1}$) or lysates were incubated with 10 $\mu\text{g } \alpha$ -toxin ml^{-1} at 37 °C for various periods. DG ($\circ$) and PA ($\bullet$) were determined as described in Methods. Values are means $\pm$ SE for five or six experiments. The lack of error bars indicates that the SE was smaller than the symbol.

Effect of α -toxin on phospholipid metabolism in rabbit neutrophils

We have previously reported that α -toxin induces haemolysis of rabbit erythrocytes through activation of phospholipid metabolism (Sakurai *et al.*, 1993). Therefore, the effect of α -toxin on DG formation in rabbit neutrophils and PA formation in the cell lysates was examined. Fig. 2(a) shows that α -toxin in the range 1–20 $\mu\text{g } \alpha$ -toxin ml^{-1} induced formation of DG and PA in a dose-dependent manner. We then determined the time course of the α -toxin-induced production of DG and PA (Fig. 2b). The formation of DG began within 30 s of the incubation and was maximal after 1 min; formation of PA started 1 min after the incubation and gradually increased until at least 5 min. Therefore, it appears that toxin-induced DG production was followed by PA production under the conditions tested.

Effect of pertussis toxin, GTP γ S and GDP β S on the α -toxin-induced events

We have previously reported that α -toxin-induced haemolysis and PA formation in rabbit erythrocytes is due to activation of endogenous PLC, via pertussis-toxin-

Fig. 3. Effect of pertussis toxin on α -toxin-induced neutrophil adhesion and superoxide generation. (a) Rabbit neutrophils ($1.2 \times 10^6 \text{ ml}^{-1}$) were incubated with various concentrations of pertussis toxin at 37°C for 120 min. Pertussis-toxin-treated neutrophils were incubated in fibrinogen-coated wells in the presence or absence of α -toxin at 37°C for 20 min. Adherent cells were determined as described in the Methods. Values are means $\pm$ SE for five or six experiments. The lack of error bars indicate that the SE was smaller than the horizontal line of the bar. *, $P < 0.05$ compared with adhesion induced by α -toxin. (b) Rabbit neutrophils ($1.5 \times 10^6 \text{ ml}^{-1}$) were preincubated without ($\square$) or with various concentrations of pertussis toxin ($\circ$, 1 $\mu\text{g ml}^{-1}$; $\triangle$, 5 $\mu\text{g ml}^{-1}$; $\blacktriangledown$, 10 $\mu\text{g ml}^{-1}$) at 37°C for 120 min. Pertussis toxin-treated neutrophils were incubated without ($\square$) or with 20 $\mu\text{g ml}^{-1}$ α -toxin ($\bullet$, $\circ$, $\triangle$, $\blacktriangledown$) at 37°C for various periods. The results from one of five experiments yielding similar results are shown.

Fig. 4. Effect of pertussis toxin on α -toxin-induced PA formation in neutrophil lysates. Rabbit neutrophils ($1.5 \times 10^7 \text{ ml}^{-1}$) were incubated with various concentrations of pertussis toxin at 37°C for 120 min. Pertussis-toxin-treated neutrophils were lysed and then incubated with α -toxin and 10 μCi [γ - ^{32}P]ATP at 37°C for 5 min. PA was determined as described in Methods. Values are means $\pm$ SE for five or six experiments. *, $P < 0.05$, compared with PA formation induced by α -toxin.

Fig. 5. Effect of GDP- β -S on α -toxin-induced superoxide generation in rabbit neutrophils. Rabbit neutrophils ($1.5 \times 10^6 \text{ ml}^{-1}$) were incubated without ($\square$) or with 10 $\mu\text{g ml}^{-1}$ α -toxin ($\bullet$) in the presence of GDP- β -S ($\circ$, 50 μM ; $\triangle$, 75 μM ; $\blacktriangle$, 100 μM) at 37°C for various periods and the chemiluminescence was measured by a chemiluminescence reader. The results from one of five experiments yielding similar results are shown.

Table 1. Effect of GTP- γ -S or GDP- β -S on α -toxin-induced PA formation in permeabilized neutrophil lysates

Permeabilized rabbit neutrophils ($1.5 \times 10^7 \text{ ml}^{-1}$) were preincubated with various concentrations of GTP- γ -S or GDP- β -S at 37°C for 30 min and then incubated with α -toxin and 10 μCi [γ - ^{32}P]ATP at 37°C for 5 min. The mass of PA was determined as described in Methods. Data are expressed as a percentage of the control (α -toxin alone). Values are means $\pm$ SE for five or six experiments.

Treatment	PA (percentage of control response)
Control (α -toxin alone)	100
GTP- γ -S	
1 μM	173 $\pm$ 4†
5 μM	653 $\pm$ 6†
10 μM	786 $\pm$ 5†
GDP- β -S	
0.02 μM	110 $\pm$ 5
2 μM	40 $\pm$ 4†
4 μM	32 $\pm$ 5†

† $P < 0.05$, compared with PA formation induced by the toxin alone.

sensitive GTP-binding protein, which is activated by the toxin (Sakurai *et al.*, 1993, 1994; Ochi *et al.*, 1996). Thus, we tested whether prior treatment of neutrophils with pertussis toxin affects α -toxin-induced adhesion and chemiluminescence responses. As shown in Fig. 3(a, b), α -toxin-induced adhesion and chemiluminescence responses in neutrophils pretreated with 1 μg pertussis toxin ml^{-1} were about 50% as extensive as those induced by the toxin of untreated neutrophils, and the treatment with 10 μg pertussis toxin ml^{-1} resulted in a complete loss of the responses induced by α -toxin. We then investigated the effect of pertussis toxin on α -toxin-induced PA formation. Fig. 4 shows that pretreatment of

Fig. 6. α -Toxin-induced phosphorylation of 40 kDa protein and adhesion to fibrinogen. (a) Rabbit neutrophil ($1.5 \times 10^7 \text{ ml}^{-1}$) lysates were incubated with various concentrations of α -toxin, $0.5 \mu\text{M}$ PDBu ml^{-1} or $10 \mu\text{g}$ OAG ml^{-1} at 37°C for 5 min. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography as described in Methods. The experiment was repeated with similar results. (b) Rabbit neutrophil ($1.5 \times 10^7 \text{ ml}^{-1}$) lysates were preincubated at 37°C for 15 min with various concentrations of protein kinase C inhibitor (staurosporine or H-7) and then incubated with α -toxin and $10 \mu\text{Ci}$ [γ - ^{32}P]ATP ml^{-1} at 37°C for 5 min. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography as described in Methods. The experiment was repeated with similar results. (c) Rabbit neutrophils ($1.2 \times 10^6 \text{ ml}^{-1}$) were preincubated with various concentrations of protein kinase C inhibitor (H-7 or staurosporine) at 37°C for 15 min and then incubated with α -toxin in fibrinogen-coated wells at 37°C for 20 min. Adherent cells were determined as described in Methods. Values are means $\pm$ SE for five or six experiments. *, $P < 0.05$, compared with adhesion induced by α -toxin. (d) Rabbit neutrophils ($1.5 \times 10^6 \text{ ml}^{-1}$) were incubated without ($\square$) or with $10 \mu\text{g}$ α -toxin ml^{-1} ($\bullet$) in the presence of H-7 ($\circ$, $50 \mu\text{M}$; $\triangle$, $100 \mu\text{M}$) or staurosporine ($\blacktriangle$, 50 nM ; $\blacktriangledown$, 100 nM) at 37°C for various periods and the chemiluminescence was measured. The results from one of five experiments yielding similar results are shown.

neutrophil lysates with pertussis toxin inhibited α -toxin-induced PA formation in a dose-dependent manner, as reported previously. To obtain further evidence for a relationship between the toxin-induced events and the GTP-binding protein, the effect of GDP β S on these toxin-induced events was examined. α -Toxin was incubated with streptolysin-O-treated neutrophils in the presence of GDP β S. The toxin-induced chemiluminescence responses in the presence of 50 and $75 \mu\text{M}$ GDP β S were about 60 and 16% of the responses seen in the absence of GDP β S, respectively (Fig. 5). Furthermore, GTP γ S significantly enhanced the toxin-induced responses (data not shown). When the toxin was incubated with the lysates which had been preincubated in the presence of GTP γ S or GDP β S, the formation of PA increased with increase in the dose of GTP γ S and decreased with increase in the dose of GDP β S (Table 1).

Action of α -toxin on DG-activated protein kinase C

Hayakawa *et al.* (1986) reported that the production of the superoxide anion in human neutrophils is stimulated through phosphorylation of 44–48 kDa proteins by DG-activated protein kinase C. It also was reported that aggregation of human platelets is induced by phosphorylation of a 40 kDa protein by DG-activated protein

kinase C (de Chaffoy de Courcelles *et al.*, 1985; Nunn & Watson, 1987). We examined whether neutrophil adhesion and chemiluminescence responses were induced on addition of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG, an analogue of DG). Both responses increased linearly with an increase in the dose of OAG and phorbol-12,13-dibutyrate (PDBu), which activate protein kinase C (data not shown). One to twenty micrograms of α -toxin was incubated with neutrophil lysates and [γ - ^{32}P]ATP, and the phosphorylated proteins were analysed by SDS-PAGE. Fig. 6(a) shows that the toxin induced phosphorylation of a 40 kDa protein in neutrophils in a dose-dependent manner. When neutrophil lysates were treated with $10 \mu\text{g}$ toxin ml^{-1} , the 40 kDa protein was strongly phosphorylated, but phosphorylation of 47, 57 and 65 kDa proteins was less than 15% of that of the 40 kDa protein. OAG and PDBu also stimulated the phosphorylation of a 40 kDa protein in the neutrophils. The effect of staurosporine and H-7, which inhibit protein kinase C, on the phosphorylation of the 40 kDa protein by the toxin was also investigated. Fig. 6(b) shows that H-7 (50 and $100 \mu\text{M}$) and staurosporine (50 and 100 nM) inhibited phosphorylation of the 40 kDa protein by the toxin in a dose-dependent manner. Furthermore, H-7 ($> 50 \mu\text{M}$) and staurosporine (20– 100 nM) blocked the toxin-induced adhesion (Fig. 6c)

and chemiluminescence responses in a dose-dependent manner (Fig. 6d). In addition, 25 μ M chelerythrine, a protein kinase C inhibitor, significantly inhibited the toxin-induced events (data not shown). On the other hand, treatment of α -toxin with these inhibitors at 37 °C for 15 min had no effect on the PLC activity of the toxin.

DISCUSSION

Gas gangrene caused by *C. perfringens* is characterized by a rapidly spreading oedema and tissue necrosis, associated with the growth of the micro-organism (Stevens, 2000). Several studies have found aggregates of leukocytes in vessels at the borders of the necrotic tissue and that leukocytes were absent from the necrotic tissue (Stevens *et al.*, 1997; Bryant *et al.*, 2000a). This phenomenon could be a result of the impairment of leukocytes by exotoxins produced by *C. perfringens*, or of the expression of adhesion molecules and cytokines *in vivo*. θ -Toxin is known to be cytotoxic to leukocytes (Stevens *et al.*, 1987; Rossjohn *et al.*, 1999), suggesting that it contributes to the paucity of leukocytes in the necrotic tissue. However, this does not explain why leukocytes accumulate in vessels at the borders of necrotic tissue.

It has been reported that α -toxin induced the expression of E-selectin and ICAM-1 in human umbilical vein endothelial cells (Bryant & Stevens, 1996), as well as the expression of P-selectin *in vivo* (Bunting *et al.*, 1997; Bryant *et al.*, 2000a). It has also been reported that intramuscular injection of the toxin induced growth of intravascular aggregates of activated platelets, fibrin and leukocytes in a manner dependent on the fibrinogen receptor glycoprotein IIb/IIIa (gpIIb/IIIa; Bryant *et al.*, 2000a). However, Ellemor *et al.* (1999) reported that infection with *C. perfringens* did not induce the expression of adhesion molecules, such as ICAM-1, *in vivo*. In addition, it is known that selectin-dependent adhesion of leukocytes does not lead to firm adhesion unless another set of adhesion molecules is engaged (Albelda *et al.*, 1994), and that the firm adhesion of neutrophils requires activation of the β 2 (CD18) integrin family, resulting in binding to one of the intercellular adhesion molecules on the surface of endothelial cells (Ley, 1996). If the exotoxins produced by *C. perfringens* activate cellular functions in neutrophils adhered to biological surfaces, neutrophils which are exposed to the toxin at the borders of the necrotic tissue would bind to adjacent vessels at the borders. In the present study, we demonstrated that treatment of neutrophils with α -toxin enhanced their adhesion to fibrinogen and fibronectin, but resulted in weak adhesion to collagen. It is known that the adhesion of neutrophils to fibrinogen is mediated by a group of β 2 and β 3 integrins (Humphries, 2000). Taking these findings into consideration, the toxin-induced cell adhesion suggests that the toxin induces activation of β 2 integrin(s) on neutrophils and the expression of integrin ligands, such as ICAM-1, on the surface of endothelial cells. Therefore, it is possible that the toxin-induced adhesion of the cells to matrixes

promotes the growth of intravascular aggregates, as reported by Bryant *et al.* (2000a).

It has been reported that α -toxin induced a respiratory burst in guinea pigs (Patriarca *et al.*, 1970) and in human polymorphonuclear leukocytes (PMNLs) (Kaplan *et al.*, 1972; König *et al.*, 1997; Yan & Novak, 1999), potentiated the chemiluminescence response to opsonized zymosan in human PMNLs, and elicited superoxide production in bovine neutrophils (Styrt *et al.*, 1989). α -Toxin also induced adhesion of the cells to matrix ligands, such as fibrinogen and fibronectin, and the production of reactive oxygen intermediates in rabbit neutrophils. Inhibition of MCLA chemiluminescence by superoxide dismutase, but not by N_3^- , indicated that O_2^- elicited MCLA luminescence without the involvement of 1O_2 , i.e. the production of O_2^- in rabbit neutrophils. The results provide evidence of a relationship between intracellular transduction events (e.g. activation of GTP-binding protein, endogenous PLC and protein kinase C) and events such as O_2^- production and adhesion of the cells to these matrix ligands in toxin-activated rabbit neutrophils. The production of DG and PA induced by the toxin followed these biological events.

Pertussis toxin inhibited toxin-induced PA formation, adhesion and O_2^- production in neutrophils and GTP γ S stimulated these events, but GDP β S inhibited them. These observations suggest that the activation of phospholipid metabolism plays an important role in these events, which are induced by the toxin, and is dependent on activation of the pertussis-toxin-sensitive GTP-binding protein. Ohta *et al.* (1985) reported that fMLP induced production of the superoxide anion which was mediated by the pertussis-toxin-sensitive GTP-binding protein present in human neutrophils. In addition, we have reported that α -toxin induced the metabolism of phospholipid and haemolysis through activation of the pertussis toxin-sensitive GTP-binding protein in rabbit erythrocytes (Sakurai *et al.*, 1994; Ochi *et al.*, 1996). The process of toxin-induced neutrophil activation is supported by the results obtained with fMLP and by the haemolysis induced by the toxin.

α -Toxin stimulated the production of DG and PA with phosphorylation of a 40 kDa protein in rabbit neutrophils. However, OAG and PDBu, both protein kinase C activators, activated cell adhesion and O_2^- production, but did not enhance DG and PA production. The results show that the phosphorylation of the 40 kDa protein, induced by these activators, occurred downstream of the toxin-induced phospholipid metabolism. In addition, protein kinase C inhibitors inhibited the toxin-induced O_2^- production, cell adhesion and phosphorylation of the 40 kDa protein. It appears that the toxin-induced adhesion is closely related to the production of DG, a protein kinase activator, through activation of phospholipid metabolism. Several studies have shown that α -toxin can elicit the activation of protein kinase C in rat skeletal muscle (Henriksen *et al.*, 1989) and mouse epidermal HEL-37 cells (Jones & Murray, 1986). Grzeskowiak *et al.* (1985) reported that the neutrophil

responses induced by the toxin, activation of a respiratory burst and secretion of specific granules, were dependent on the formation of DG with the breakdown of phospholipids. They hypothesized the involvement in the neutrophil responses of protein kinase C. Our result was consistent with the hypothesis proposed by Grzeskowiak *et al.* (1985). Yan & Novak (1999) reported that treatment of human neutrophils with tumour necrosis factor α and fMLP, which stimulated adhesion of the cells to fibrinogen, resulted in the phosphorylation of cellular proteins with molecular masses of approximately 115 kDa. Hayakawa *et al.* (1986) reported that treatment of human neutrophils with DG caused the production of the superoxide anion and the phosphorylation of proteins with molecular masses of 44–48 kDa. Fuchs *et al.* (1997) reported that phorbol ester induced phosphorylation of a 40 kDa protein in HL60 cells that had differentiated into neutrophils, and that the protein phosphorylation was closely related to the kinetics of O_2^- production. Therefore, the toxin-induced adhesion and O_2^- production appears to be involved in the phosphorylation of cellular proteins in neutrophils. The phosphorylated 40 kDa protein was reported to be p40phox, a soluble component of NADPH oxidase (Fuchs *et al.*, 1997; Babior, 1999). However, Bianca *et al.* (1999) reported that antigen–antibody reactions of antibodies against the components of NADPH oxidase were species-specific and identified human and mouse NADPH oxidase components. Rabbit NADPH oxidase components, however, have not yet been isolated. Therefore, in the present study, the phosphorylated 40 kDa protein in rabbit neutrophils treated with the toxin was difficult to identify.

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