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Regulation of *Clostridium perfringens* Alpha-Toxin-Activated Phospholipase C in Rabbit Erythrocyte Membranes

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The rapid phosphatidic acid (PA) formation induced by *Clostridium perfringens* alpha-toxin was stimulated by AlF_4^- in rabbit erythrocyte membranes. GTP[γS] [guanosine 5′-*O*-(3-thiotriphosphate)] stimulated the rapid 1,2-diacylglycerol formation and inositol 1,4,5-trisphosphate release induced by the toxin. On the other hand, treatment of erythrocyte lysates with phorbol 12-myristate 13-acetate (PMA) resulted in inhibition of toxin-induced PA production, and long-term PMA or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) treatment of the lysates led to stimulation of PA formation. Furthermore, treatment of erythrocytes with the toxin caused an increase of protein kinase C activity in membrane fractions. The results suggest that toxin-induced PA formation is mediated by endogenous phospholipase C regulated through GTP-binding protein and protein kinase C in rabbit erythrocytes.

Clostridium perfringens alpha-toxin, which possesses hemolytic (16), lethal, dermonecrotic, and cytotoxic activities (37) among others, is thought to be an important agent in gas gangrene. The toxin also has phospholipase C (PLC) activity, which hydrolyzes phosphatidylcholine to phosphorylcholine and 1,2-diacylglycerol (DG) (11). It had been reported that various biological activities are due to degradation of biological membranes by PLC activity of the toxin (18). However, we have reported that a small amount of the purified toxin causes contraction of isolated rat ileum (29) and aorta (5) tissue and that the toxin-induced contraction of isolated aorta tissue is related to thromboxane A_2 production via activation of phosphatidylinositide metabolism (6). Moreover, it has been reported that biological activities of alpha-toxin are not essentially consistent with PLC activity of the toxin (28, 30, 32). The toxin is known to show hot-cold hemolysis for sheep erythrocytes (34). We have reported that the toxin shows hot-cold hemolysis for rabbit erythrocytes, when incubated with a small amount of the purified toxin in the presence of a low concentration of Ca^{2+} (31). In addition, we showed that the toxin activates endogenous PLC and phospholipase D in rabbit erythrocytes so that phosphatidic acid (PA) is produced by phosphorylation of DG formed from phosphatidylinositol 4,5-bisphosphate (PIP_2) by endogenous PLC and DG via PA produced by phospholipase D (31). On the other hand, it has been reported that chemoattractant (1, 35) induces PA formation by activation of PLC and phospholipase D regulated through a pertussis toxin-sensitive GTP-binding protein in leukocytes, suggesting that one of the signal transductions may be dependent on PA formation through GTP-binding protein. Endogenous PLC also is reported to be blocked by activation of protein kinase C in various cells (22, 23). In the present work, we investigated the regulation of endogenous PLC activated by the toxin.

Alpha-toxin was purified from culture supernatant of *C. perfringens* type A (NCTC 8237), as described by Fujii et al. (5). The purified preparation used in this study showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified toxin contained hemolytic, lethal, dermonecrotic, and PLC activities. Rabbit erythrocytes and erythrocyte membranes were prepared as described elsewhere (30), and the erythrocyte concentration was adjusted to 6×10^{11} cells per ml. Measurement of PA was carried out as previously described (31). Erythrocyte membranes in 0.02 M Tris-HCl buffer (pH 7.5) were incubated in the presence of [γ-^{32}P]ATP (10 μCi/ml) at 37°C with alpha-toxin or agents. After the reaction was terminated by the addition of 0.5 ml of ice-cold 0.02 M Tris-HCl buffer (pH 7.5) and 3.6 ml of chloroform-methanol-concentrated HCl (50:100:1, vol/vol), phospholipids were extracted and concentrated. Phospholipids were separated by thin-layer chromatography employing chloroform–methanol–4N NH_4OH (45:35:10, vol/vol) (7). After the autoradiography, the localized region on the plate corresponding to a standard amount of PA was scraped and counted in a liquid scintillation counter (Aloka Co., Tokyo, Japan). The presence of PA in extracts was confirmed by two-dimensional thin-layer chromatography as described elsewhere (8). ADP-ribosylation was performed in a 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 5 mM dithiothreitol, 10 mM thymidine, 200 μM ATP, 0.025% SDS, 10 μg of activated pertussis toxin (preactivated by incubation with 20 mM dithiothreitol–0.125% SDS at 30°C for 30 min) per ml, 5 μM [adenylate-^{32}P]NAD (20,000 cpm/pmol), and erythrocyte membrane (210 μg) in a total volume of 300 μl (10). The reaction was started by the addition of activated pertussis toxin, and the incubation was carried out at 30°C for 30 min. Pertussis toxin-treated membranes were mixed with ice-cold 7.5% trichloroacetic acid (1 ml) plus bovine serum albumin (15 μg) at 4°C for 30 min. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and then washed twice by centrifugation in 1 ml of ice-cold 7.5% trichloroacetic acid. ADP-ribosylated proteins were analyzed by SDS-PAGE according to Laemmli (12) and by subsequent autoradiography. Rabbit erythrocytes (6×10^{11} cells per ml) were centrifuged at 1,650 × *g* for 5 min, and the pellet was lysed in an equal volume of 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–NaOH buffer (pH 7.0). The lysates were used, as protein kinase C is in both membrane and cytosol (19). The lysates were preincubated with various concentrations of phorbol 12-myristate 13-acetate (PMA) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) at 37°C for 30 min. The

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TABLE 1. Effect of AlF_4^- on alpha-toxin-induced PA formation[a]

Treatment	PA (cpm) formed at indicated time (s)		
	0	30	60
None	1,100 ± 70	1,330 ± 80	1,310 ± 40
AlF_4^-	1,090 ± 40	2,700 ± 20	1,240 ± 30
Alpha-toxin	1,420 ± 70	3,360 ± 40	2,390 ± 20
Alpha-toxin + AlF_4^-	1,200 ± 50	11,670 ± 290[b]	6,020 ± 270[b]

[a] Erythrocyte membranes (720 μg) were incubated with or without alpha-toxin (5 ng) and/or AlF_4^- (750 μM) in the presence of [γ-^{32}P]ATP at 37°C for 30 or 60 s. The quantity of PA was determined as described in the text. Values are means ± SE for five to six experiments.

[b] Significantly different from PA formation induced by the toxin alone or AlF_4^- alone ($P < 0.01$).

mixture was used for the assay of PA. Rabbit erythrocytes (6×10^{11} cells per ml) treated with PMA (0.1 μM) at 37°C for 10 h were washed with 0.02 M HEPES–NaOH buffer (pH 7.0) containing 150 mM NaCl and subsequently lysed as described above (long-term treatment with PMA). Rabbit erythrocytes (6×10^{11} cells per ml) were incubated with the toxin (3 ng) in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.9% NaCl at 37°C for 60 s and centrifuged at 1,650 × *g* for 5 min, and the pellets were lysed in an equal volume of 0.02 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 50 μg of phenylmethylsulfonyl fluoride per ml, 10 mM benzamidine, and 0.3% β-mercaptoethanol. The lysate was centrifuged at 100,000 × *g* for 60 min. The precipitate was assayed for protein kinase C activity with the Amersham PKC enzyme assay kit (36). Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard. All mean values are shown with their calculated standard errors (SE). Statistical analysis was performed with Student's *t* test; a *P* value of 0.05 or less was considered statistically significant.

We have reported that the rapid formation of PA induced by the toxin is due to endogenous PLC (31). Bigay et al. (2) and Paris and Pouyssegur (25) reported that AlF_4^- activates GTP-binding protein coupled to endogenous PLC. For these reasons, the effect of AlF_4^- on the rapid PA formation induced by the toxin was investigated (Table 1). When erythrocyte membranes were incubated with AlF_4^- in the presence of [γ-^{32}P]ATP, the first appearance of labeled PA was within 30 s and was followed by a second rise (after about 20 min) in labeled PA production (data not shown), indicating that rapid PA formation also is induced by AlF_4^- alone. It thus appears that rabbit erythrocytes have GTP-binding protein activated by AlF_4^- coupled to PLC. When erythrocyte membranes were incubated with the toxin and AlF_4^-, PA formation markedly increased, compared with levels of PA formation induced by the toxin or AlF_4^- alone. We have reported that the toxin caused biphasic production of DG in membranes and that the time course of the toxin-induced DG formation was coincident with that of the toxin-induced PA formation (31). Therefore, the effect of GTP[γS] [guanosine 5′-*O*-(3-thiotriphosphate)] on the rapid DG formation induced by the toxin was investigated. GTP[γS] markedly increased the effect of the toxin on DG formation with near-maximal effects observed with 0.2 μM GTP[γS], as shown in Fig. 1A. ATP, ADP, GDP, and GMP (each at 200 μM) were ineffective on the formation of DG (data not shown). We also have reported that the toxin stimulates inositol 1,4,5-triphosphate (IP_3) release from erythrocyte membranes within 30 s after incubation (31). Therefore, to investigate whether GTP[γS] stimulates the effect of the

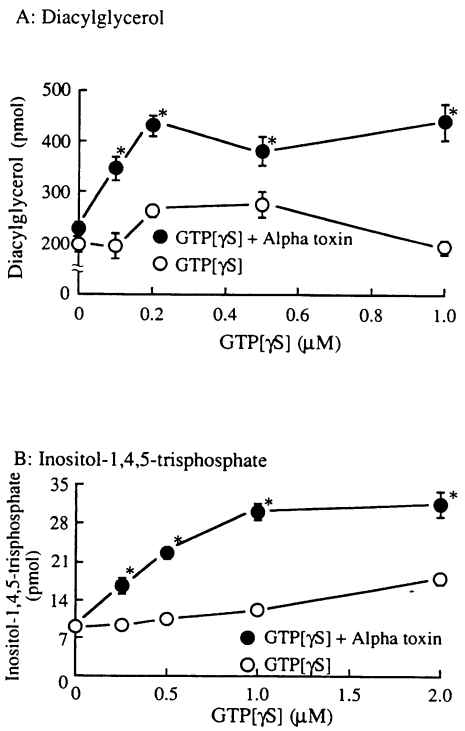

FIG. 1. Effect of GTP[γS] on alpha-toxin-induced DG and IP_3 production. Erythrocyte membranes (720 μg) were incubated with GTP[γS] in the presence or absence of the toxin (5 ng) in 0.02 M Tris-HCl buffer (pH 7.5) at 37°C for 30 s. DG (A) and IP_3 (B) masses were determined as described previously (24, 26, 29). Values are means ± SE for four to five experiments. *, $P < 0.01$ compared with GTP[γS] alone.

toxin on IP_3 release from membranes, erythrocyte membranes were incubated with the toxin in the presence or absence of GTP[γS] (Fig. 1B). GTP[γS] significantly increased the effect of the toxin on IP_3 release from membranes, with near-maximal effects observed with 1.0 μM GTP[γS]. Pertussis toxin has been reported to ADP-ribosylate GTP-binding protein so that PLC is blocked (3, 20). The effect of pertussis toxin on the rapid PA formation induced by alpha-toxin was investigated. Table 2 shows that prior exposure of erythrocyte membranes to pertussis toxin resulted in almost total abolition of alpha-toxin-induced PA formation. Heated pertussis toxin did not block alpha-toxin-induced PA formation. We determined whether or

TABLE 2. Effect of pertussis toxin on alpha-toxin-induced PA formation[a]

Alpha-toxin treatment	PA (cpm) with indicated pertussis toxin treatment		
	Control	Pertussis toxin	Heated pertussis toxin
None	1,290 ± 70	1,600 ± 150	1,430 ± 110
Alpha-toxin	4,600 ± 140	1,560 ± 170[b]	3,030 ± 200

[a] Erythrocyte membranes (720 μg) treated with pertussis toxin or heated pertussis toxin (10 μg) or not treated with pertussis toxin (control) at 37°C for 30 min were incubated with or without alpha-toxin (5 ng) in the presence of [γ-^{32}P]ATP at 37°C for 30 s. The quantity of PA was determined as described in the text. Values are means ± SE for five to six experiments.

[b] Significantly different from PA formation with alpha-toxin (control) ($P < 0.05$).

not proteins in erythrocyte membranes are ADP-ribosylated by pertussis toxin in the presence of [^{32}P]NAD (data not shown). Exposure of erythrocyte membranes to pertussis toxin resulted in labeling of a protein band corresponding to approximately 41 kDa. On the other hand, when erythrocyte membranes were incubated with heated pertussis toxin in the presence of [^{32}P]NAD, no labeling of proteins was observed.

Phorbol esters, such as PMA, are reported to bind to (9) and directly activate protein kinase C (31). It is known that long-term PMA treatment results in down-regulation of protein kinase C (4). Moreover, several workers have reported negative regulation of PLC by protein kinase C in various cells (4, 22, 23). For these reasons, to determine the involvement of protein kinase C in endogenous PLC regulation, the lysates of erythrocytes were incubated with the toxin in the presence or absence of PMA for 30 s. Toxin-induced PA formation was strongly inhibited by 5 nM and more concentrated PMA, as shown in Fig. 2A. The result suggests that activation of protein kinase C may cause depression of endogenous PLC activity in erythrocyte membranes. Therefore, to down-regulate protein kinase C, erythrocytes were treated with 0.1 μM PMA for 10 h. The lysates of the treated erythrocytes were incubated with the toxin at 37°C for 30 s. PA formation in response to the toxin was significantly stimulated in the long-term PMA-treated lysates, compared with the control (Fig. 2B). Furthermore, to confirm the effect of protein kinase C on toxin-induced PA formation, the lysates of intact erythrocytes were incubated with the toxin in the presence or absence of H-7 (protein kinase C inhibitor). Figure 2C indicates that H-7 (more than 10 μM) significantly stimulated toxin-induced PA formation, compared with the control. The effect of H-7 treatment on PA formation was consistent with that of long-term PMA treatment. These data indicate that the rapid formation of PA induced by the toxin is inversely proportional to protein kinase C activity. When erythrocytes were incubated with the toxin at 37°C for 60 s, the activity of protein kinase C in erythrocyte membrane fractions increased by 149% ± 2% (mean ± SE) compared with the activity in untreated membranes.

AlF_4^-, which is known as an activator of GTP-binding protein, caused a significant increase in the rapid [^{32}P]PA formation induced by the toxin, suggesting that the rapid PA formation induced by the toxin is associated with a GTP-binding protein. As shown in Fig. 3, we also have reported that PA is produced from phosphorylation of DG by DG kinase under the experimental conditions (31). The rapid formation of DG induced by the toxin also was stimulated by GTP[γS]. Furthermore, GTP[γS] stimulated the effect of the toxin on IP_3 release from membranes within 30 s. Accordingly, the rapid DG formation is associated with a rapid IP_3 release. It is generally thought that DG and IP_3 are simultaneously generated from hydrolysis of PIP_2 by PLC (21). We have reported that the toxin does not hydrolyze PIP_2 but stimulates hydrolysis of PIP_2 by endogenous PLC in membranes (31). Therefore, GTP[γS] seems to be associated with activation of endogenous PLC activated by the toxin, suggesting that endogenous PLC activated by the toxin is regulated through GTP-binding protein. In addition, the toxin-induced PA formation was strongly inhibited by pretreatment of erythrocyte membranes with pertussis toxin. Pertussis toxin ADP-ribosylated a protein of about 41 kDa in erythrocyte membranes, when incubated with membranes. Therefore, the process of PA formation induced by alpha-toxin appears to be associated with a protein ADP-ribosylated by pertussis toxin. Several workers have reported that pretreatment of various cells or membrane fractions with pertussis toxin results in blockage of PA formation induced by agonists or agents and that such PA formation is regulated by

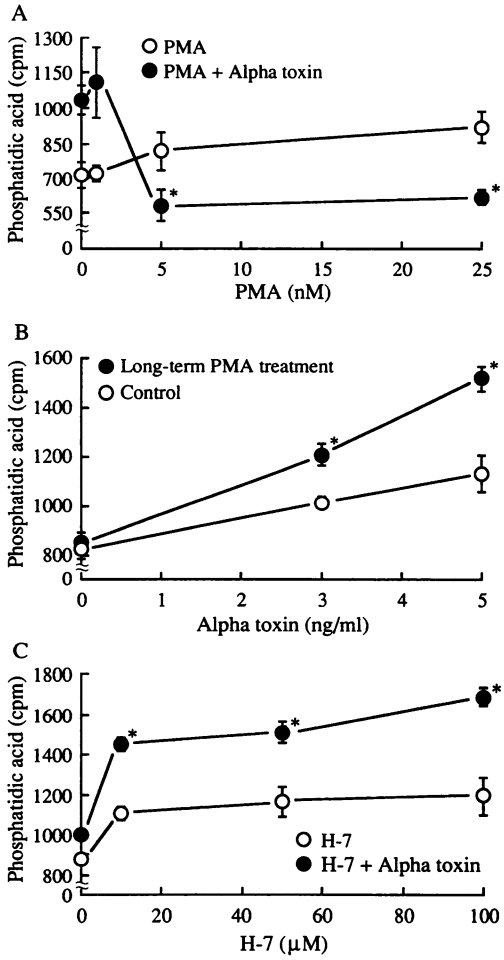

FIG. 2. Effects of PMA or H-7 on alpha-toxin-induced phosphatidic acid formation. The lysates of erythrocytes were treated with PMA (A) or H-7 (C) as described in the text. PMA- or H-7-pretreated lysates (720 μg) were incubated with the toxin (5 ng) in the presence of [γ-^{32}P]ATP at 37°C for 30 s. (B) Long-term PMA-pretreated lysates (720 μg) were incubated with various concentrations of the toxin in the presence of [γ-^{32}P]ATP at 37°C for 30 s. The quantity of PA was determined as described in the text. Values are means ± SE for four to five experiments. *, $P < 0.05$ compared with the control.

pertussis toxin-sensitive GTP-binding protein (3, 20). It therefore is likely that endogenous PLC activated by the toxin is under regulation by pertussis toxin-sensitive GTP-binding protein. On the other hand, binding of stimulant to receptors is thought to cause activation of GTP-binding protein. Although the receptor for alpha-toxin on the surface of rabbit erythrocytes is not characterized yet, these events that arise from the interaction of the toxin with erythrocytes suggest that the specific receptor of the toxin may be located on rabbit erythrocytes. However, it is possible that hydrolysis of membrane phospholipids by PLC activity of the toxin results in activation of endogenous PLC. We have reported that one of the earliest events induced by the toxin is the hydrolysis of membrane PIP_2 by endogenous PLC to IP_3 and DG, as shown in Fig. 3 (31). From these data, it is reasonable to conclude that GTP-binding protein is responsible for the coupling of alpha-toxin receptor occupancy or hydrolysis of phospholipids by the toxin to endogenous PLC activation (Fig. 3). Therefore, it is speculated

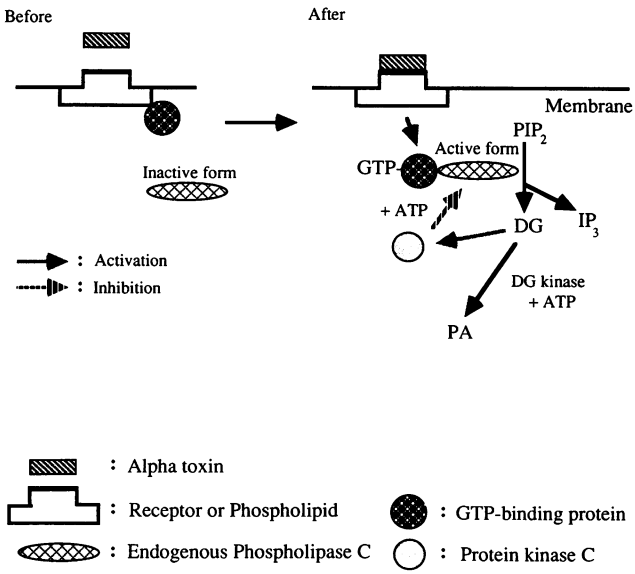

FIG. 3. A possible model of alpha-toxin action.

that activation of PLC via GTP-binding protein activated by the toxin results in activation of phosphatidylinositol metabolism in tissues (Fig. 3).

It has been reported that protein kinase C inhibits phosphoinositide hydrolysis in response to receptors which are coupled to PLC activation via GTP-binding protein (19, 27). In addition, Lawrence et al. reported that rabbit erythrocytes have protein kinase C (13). Therefore, we determined the relationship between toxin-induced PA formation and protein kinase C. PMA, which activates protein kinase C, inhibited toxin-induced PA formation in the lysates of erythrocytes, suggesting that activation of protein kinase C may inhibit endogenous PLC activated by the toxin. Brown et al. reported that in Swiss 3T3 cells protein kinase C is down-modulated by prolonged exposure to phorbol ester (4). Long-term PMA treatment of the erythrocytes led to stimulation of toxin-induced PA formation. Furthermore, H-7, which inhibits protein kinase C, enhanced toxin-induced PA formation in the lysates. It therefore appears that deletion or inhibition of protein kinase C causes activation of endogenous PLC induced by the toxin in erythrocytes. Moreover, treatment of erythrocytes with the toxin resulted in a significant increase in protein kinase C activity in membranes. From these data, it is likely that endogenous PLC activated by the toxin also is regulated by protein kinase C. An inhibitory effect of this type on cells of the following types has been reported: thrombin-stimulated platelets (17), norepinephrine-stimulated DDT_1 MT-2 cells (14), and bradykinin-stimulated human keratinocytes (33). Hydrolysis of PIP_2 by endogenous PLC activated by the toxin yields two biologically active molecules, DG and IP_3 (Fig. 3). DG is known to activate protein kinase C. Thus, the sudden decrease of PA production induced by the toxin may be due to inhibition of endogenous PLC by activation of protein kinase C activated by DG (Fig. 3). On the other hand, IP_3 is known to promote release of Ca^{2+} from internal reservoirs in cytosol. The rise in Ca^{2+} is reported to result in contraction of muscle tissues or activation of PLC and phospholipase A_2. It therefore appears that the toxin induces contraction of the aorta and the ileum, activation of phosphatidylinositol metabolism, and arachidonic acid cascade.

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