

Evidence for Coupling of *Clostridium perfringens* Alpha-Toxin-Induced Hemolysis to Stimulated Phosphatidic Acid Formation in Rabbit Erythrocytes

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When rabbit erythrocytes were exposed to low concentrations of *Clostridium perfringens* alpha-toxin, hot-cold hemolysis was observed. The toxin induced production of phosphatidic acid (PA) in a dose-dependent manner when incubated with erythrocytes at 37°C. When erythrocyte membranes were incubated with the toxin and [γ -³²P]ATP in the presence or absence of ethanol, [³²P]PA formation was maximal within 30 s, then sharply decreased, and began again after 5 min of incubation. Ethanol had no effect on the early appearance (at ~5 min) of PA formation induced by the toxin but significantly inhibited formation of PA over 10 min of incubation. Treatment of erythrocyte membranes with alpha-toxin resulted in the biphasic formation of 1,2-diacylglycerol and PA as well as an increase of inositol-1,4,5-trisphosphate (IP₃) and decrease of phosphatidylinositol-4,5-bisphosphate (PIP₂) within 30 s. Neomycin inhibited the toxin-induced increase in turbidity of egg yolk suspensions but did not inhibit the toxin-induced hemolysis of intact erythrocytes. On the other hand, neomycin inhibited the toxin-induced hemolysis of saponin-treated erythrocytes. In addition, neomycin inhibited PA formation induced by the toxin in erythrocyte membranes. IP₃ was released by incubation of PIP₂ with erythrocyte membranes but not by incubation of PIP₂ with the toxin. The toxin stimulated the membrane-induced release of IP₃ from PIP₂. These data suggest that the toxin-induced hemolysis is dependent on the action of phospholipase C in erythrocyte membranes.

Clostridium perfringens produces alpha-toxin (phospholipase C; EC 3.1.4.3), which hydrolyzes phosphatidylcholine to phosphorylcholine and 1,2-diacylglycerol (25). The toxin has been found to split other phospholipids (sphingomyelin and lysophosphatidylcholine) as well (21, 23). The toxin is known to be hemolytic (19, 24, 44), dermonecrotic (49), and lethal (49). The hemolytic activity of alpha-toxin is observed by incubating the toxin with sheep erythrocytes at 37°C and then chilling the preparation to below 10°C (the phenomenon of hot-cold hemolysis) (47). Meduski and Hochstein (27) reported that hot-cold hemolysis is the response of erythrocytes to agents which alter or remove -N⁺(CH₃)₃ groups of the membrane lipids. Smyth et al. (45) reported that treatment of erythrocyte ghosts with the toxin caused the appearance of droplets which remained associated with membranes treated with the toxin. It was reported that bovine and ovine erythrocytes, which have a high sphingomyelin content (30), also display the hot-cold hemolysis resulting from the action of *Staphylococcus aureus* beta-toxin, which has a sphingomyelinase activity (19). Therefore, it had been thought that these toxins bring about hot-cold hemolysis of sensitive erythrocytes by direct degradation of membrane phospholipids (19).

We have reported that low concentrations of the highly purified toxin (<1.0 µg/ml) potentiate norepinephrine-evoked contraction (41), elevate blood pressure (42), cause contraction of isolated rat aorta (9) and ileum (39), and activate metabolism of the phosphatidylinositol and arachidonic acid cascade (10). On the other hand, Sabban et al. (37) reported that the toxin in the presence of EDTA lysed chicken erythrocytes but did not hydrolyze phospholipids in

the erythrocytes. Furthermore, Sakurai et al. (40) have suggested that the phospholipase C activity of the toxin is not essential for hemolysis induced by the toxin. In addition, Sato et al. (43) reported that the toxin's phospholipase C activity is not identical with its lethal activity. In the present work, to understand the mechanism of alpha-toxin-induced hemolysis, we investigated the biochemical events caused by low concentrations of highly purified toxin in rabbit erythrocytes.

MATERIALS AND METHODS

Chemicals. Saponin (Gypsophila), phospholipids, bovine serum albumin, phenylmethanesulfonyl fluoride (PMSF), 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC), neomycin, propranolol, and phospholipase D (from peanut) were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium deoxycholate was obtained from Difco Laboratories, Detroit, Mich. Dowex 1 × 8 anion-exchange resin was purchased from Dow Chemical Co., Midland, Mich. ³²P_i (carrier free in water) was purchased from the Japan Atomic Energy Research Institute. [γ -³²P]ATP (4,500 Ci/mmol) was purchased from ICN Biochemicals, Inc., Irvine, Calif. Phosphatidyl[2-³H]inositol-4,5-bisphosphate ([³H]PIP₂; 1.0 Ci/mmol) was supplied by Amersham-Japan Co., Tokyo, Japan. [1-³H]ethanol (15 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc., St. Louis, Mo. All other chemicals were of analytical grade.

Purification of alpha-toxin. Alpha-toxin was purified from the culture supernatant of *C. perfringens* type A (NCTC 8237) as described by Fujii et al. (9).

Preparation of rabbit erythrocytes and membranes from erythrocytes. Rabbit blood was withdrawn from the ear veins of New Zealand White rabbits. Acid citrate-dextrose was

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present in each sample as an anticoagulant. Blood samples were centrifuged at $500 \times g$ for 15 min, and the plasma and leukocytes were carefully removed by aspiration. The sedimented erythrocytes were then washed five times in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (135 mM NaCl, 11.9 mM NaHCO₃, 0.36 mM NaH₂PO₄ · H₂O, 5 mM dextrose, 1.5 mM KCl, 0.98 mM MgCl₂ · 6H₂O, 4.6 mM HEPES [pH 7.2]). The number of erythrocytes was determined with a cell counter (Celltac; Nihon Kohden Co., Tokyo, Japan). The erythrocyte concentration was adjusted to 6×10^{11} cells per ml.

Erythrocyte membranes were prepared by washing the membranes a given number of times with 10 volumes of specified hemolytic solution. The washed cells were resuspended in 10 volumes of a specified hypotonic solution and recovered by centrifugation at 4°C. This procedure was repeated for a specified number of times. The hypotonic solution used for this hemolysis and washing procedure was hypotonic HEPES buffer (11.9 mM NaHCO₃, 0.36 mM NaH₂PO₄ · H₂O, 5 mM dextrose, 1.5 mM KCl, 0.98 mM MgCl₂ · 6H₂O, 4.6 mM HEPES [pH 7.2]) or 0.02 M Tris-HCl buffer (pH 7.5).

Measurement of hemolysis induced by alpha-toxin. Alpha-toxin was mixed with 0.1 ml of washed rabbit erythrocytes (6×10^{11} cells per ml) suspended in HEPES buffer (pH 7.2) containing 0.3 mM CaCl₂ and then mixed with the same buffer to give a total volume of 0.5 ml. After the mixtures were incubated at 37°C or at 37°C followed by chilling at 4°C, unlysed cells were pelleted by centrifuged at $1,650 \times g$ for 5 min. The A_{540} of the resulting supernatants was measured to determine the release of hemoglobin. Hemolysis was expressed as a percentage of the A_{540} of the supernatant from 0.1 ml of erythrocytes suspended in 0.4 ml of distilled water.

Determination of phospholipase C activity. Phospholipase C activity was assayed from the toxin-induced increase in turbidity of an egg yolk solution as described by Sakurai et al. (40). Phospholipase C activity was expressed as a percentage of the enzymatic activity of 0.1 µg of alpha-toxin.

Preparation of rabbit erythrocytes treated with saponin. For permeabilization of erythrocytes, rabbit erythrocytes were incubated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.9% NaCl, 1.4 µg of saponin per ml, 1 mM MgCl₂, and 0.3 mM CaCl₂ at 37°C for 10 min, washed by centrifugation ($500 \times g$, 15 min), and suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.9% NaCl, 1 mM MgCl₂, and 0.3 mM CaCl₂. Under the conditions used, no hemolysis of rabbit erythrocytes was observed.

Preparation of ³²P-labeled erythrocytes. The washed erythrocytes were incubated with ³²P_i (70 µCi/ml of packed cells) in phosphate-free HEPES buffer (pH 7.2) at 37°C for 1 h and then washed by centrifugation ($500 \times g$, 15 min). The labeled erythrocytes were pelleted and then suspended in the buffer.

Phosphate-free HEPES buffer (pH 7.2) was prepared by omitting NaH₂PO₄ · H₂O in HEPES buffer (pH 7.2).

Determination of PA in erythrocytes and erythrocyte membranes. Labeled erythrocytes (6×10^{11} cells per ml) were incubated with the toxin in a final volume of 0.5 ml of HEPES buffer or HEPES buffer containing 2% ethanol or 250 µM propranolol at 37°C. The reactions were stopped by addition of 0.5 ml of ice-cold HEPES buffer (pH 7.2) and 3.6 ml of chloroform-methanol-concentrated HCl (50:100:1, vol/vol/vol), and the phases were separated by the addition of 0.96 ml of chloroform and 0.96 ml of 2 M KCl. The lower organic phase was transferred and concentrated to 0.1 ml under a flow of N₂. The lipids were separated on Silica Gel 60 plates (impregnated with 1% potassium oxalate containing

2 mM EDTA) in the solvent system chloroform-methanol-4 N NH₄OH (45:35:10, vol/vol/vol) (12). After autoradiography, radioactive spots on the plate corresponding to standard material of phosphatidic acid (PA) were scraped into scintillation vials, to which 5 ml of scintillation cocktail was added. Radioactivity was counted in a liquid scintillation counter (Aloka Co., Tokyo, Japan).

Erythrocyte membranes (7.2 mg of protein per ml) in 0.02 M Tris-HCl buffer (pH 7.5) were incubated with the toxin in the presence of 10 µCi of [γ -³²P]ATP per ml at 37°C for 20 min. After incubation, 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/vol). Phospholipids were extracted and concentrated as described above and separated by thin-layer chromatography using chloroform-methanol-4 N NH₄OH (45:35:10, vol/vol/vol). After autoradiography, the localized region on the plate corresponding to the PA standard material was scraped and counted in a liquid scintillation counter.

PA in extracts was confirmed by two-dimensional thin-layer chromatography as follows (17). The plate was first developed with chloroform-methanol-concentrated ammonia-distilled water (120:80:10:5, vol/vol). After drying in the presence of nitrogen gas, the plate was reoriented and developed in the second dimension with chloroform-acetone-methanol-acetic acid-distilled water (100:40:30:20:12, vol/vol). After drying, lipids were stained with iodine vapor and radioactive spots were identified by comigration with standard lipids.

Determination of 1,2-diacylglycerol in erythrocyte membranes. Erythrocyte membranes were incubated with alpha-toxin at 37°C. The incubation was terminated by addition of chloroform-methanol (1:2, vol/vol). The lipids were extracted by the method of Bligh and Dyer (3) except that 0.2 M KCl-5 mM EDTA was used instead of water (29). The final organic phase was dried under a stream of N₂ and used for analysis of the mass amount of 1,2-diacylglycerol. 1,2-Diacylglycerol content in crude lipid fractions was measured by the conversion of 1,2-diacylglycerol into [³²P]PA by *Escherichia coli* 1,2-diacylglycerol kinase in the presence of [γ -³²P]ATP, using the method of Preiss et al. (34) with the 1,2-diacylglycerol assay kit (Amersham-Japan Co.).

Determination of IP₃ in erythrocyte membranes. Erythrocyte membranes were incubated with alpha-toxin at 37°C, and the reaction was terminated by the addition of ice-cold 10% perchloric acid. The samples were kept on ice for 20 min and then centrifuged at $2,000 \times g$ for 15 min at 4°C. The pH of the supernatant was adjusted to 7.5 with 10 N KOH. The solution was kept on ice for 30 min and centrifuged at $2,000 \times g$ for 15 min at 4°C. Inositol-1,4,5-trisphosphate (IP₃) in the supernatant was determined with the IP₃ assay kit (Amersham-Japan Co.) (33).

Determination of PIP₂ in erythrocyte membranes. Erythrocyte membranes (7.2 mg of protein per ml) were incubated with 10 µCi of [γ -³²P]ATP per ml at 37°C for 60 min. The membranes were washed with 0.02 M Tris-HCl buffer (pH 7.5) by centrifugation at $20,000 \times g$ for 10 min. The membrane preparations were used as the labeled membranes and resuspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM MgCl₂, 0.3 mM CaCl₂, and 1 mM ATP. The labeled membranes were incubated with alpha-toxin in the buffer at 37°C. The reaction was terminated by addition of 3.6 ml of chloroform-methanol-concentrated HCl (50:100:1, vol/vol/vol) followed by the addition of 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.2). Phospholipids were extracted and separated as described above. After autoradiography, PIP₂ was iden-

tified by using unlabeled standards that were visualized by exposure to iodine. The amount of radioactivity in the PIP₂ was determined by liquid scintillation counting.

Measurement of PIP₂ hydrolysis activity. PIP₂-specific phospholipase C activity in rabbit erythrocyte membranes was determined by a slight modification of the method described by Homma et al. (16). Phospholipase C activity was assayed with 500 μ M [³H]PIP₂ (400 cpm/nmol) in 50 μ l of 50 mM morpholineethanesulfonic acid (MES)-NaOH buffer (pH 6.5) containing 50 μ M phosphatidylethanolamine, 2.4 mM sodium deoxycholate, 200 μ M KCl, and 1 mg of bovine serum albumin per ml. Free calcium ion concentration in the buffer was adjusted in 10 μ M, using buffer containing 2 mM EGTA and 1.4 mM CaCl₂. The reaction was started by addition of alpha-toxin and/or rabbit erythrocyte membranes to the solution and was run in a final volume of 50 μ l at 37°C. The reaction was stopped by addition of 0.1 ml of 5 mM EGTA and 0.1 ml of chloroform followed by 0.2 ml of chloroform-methanol-concentrated HCl (10:20:1, vol/vol/vol). A 0.2-ml aliquot of the upper aqueous phase was then neutralized before being applied to anion-exchange columns (Dowex 1 \times 8, formate form). [³H]IP₃ was separated and quantitated by the procedure described by Berridge et al. (1). A 2-ml portion of each fraction was mixed with 2 ml of distilled water, and its radioactivity was determined by scintillation counting after addition of 6 ml of scintillation fluid.

Determination of phosphatidylethanol in erythrocyte membranes. Washed erythrocyte membranes were suspended (7.2 mg of protein per ml) in 20 mM HEPES-NaOH buffer (pH 7.0) containing 1 mM MgCl₂ and preincubated with 50 μ Ci of [³H]ethanol at 37°C for 30 min, and the toxin (5 ng) was added into the suspension (0.5 ml). After 30 min of incubation, the reaction was stopped by addition of 0.5 ml of ice-cold 20 mM HEPES-NaOH buffer (pH 7.0) and 3.6 ml of chloroform-methanol (1:2, vol/vol), and a standard sample of phosphatidylethanol (10 μ g) was added. The phases were separated by the addition of 0.96 ml of chloroform and 0.96 ml of 2 M KCl. The lower chloroform phase was dried and spotted on Silica Gel 60 plates. The plates were developed in a solvent system consisting of chloroform-methanol-acetic acid-distilled water (75:45:3:1, vol/vol) (11). After drying, the plates were sprayed with En³Hance (New England Nuclear, Boston, Mass.) and subjected to autoradiography. The labeled phosphatidylethanol was identified by comparison with a nonlabeled standard made visible by iodine staining. The area corresponding to phosphatidylethanol was scraped into a vial and counted with a liquid scintillation counter.

The phosphatidylethanol standard was prepared by the phospholipase D-mediated transphosphatidylation of egg phosphatidylcholine (4 mg) in the presence of 2% ethanol as described elsewhere (11).

Determination of protein concentration. Protein concentration was determined by the method of Lowry et al. (22), using bovine serum albumin as a standard.

Statistical analysis. In all cases, mean values and standard errors (SE) were determined. Statistical analysis was performed by using Student's *t* test; a *P* value of 0.05 or less was considered statistically significant.

RESULTS

Effect of temperature on hemolysis induced by alpha-toxin. The effect of temperature on the toxin-induced hemolysis of rabbit erythrocytes was investigated (Fig. 1). To do so, the toxin was incubated with rabbit erythrocytes in HEPES

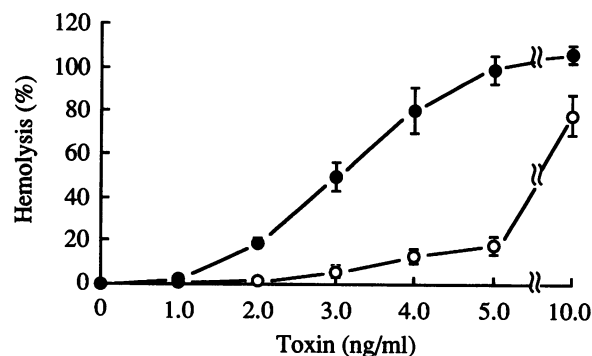


FIG. 1. Effect of temperature on alpha-toxin-induced hemolysis of rabbit erythrocytes. The toxin was mixed with 0.1 ml of erythrocytes suspended in HEPES buffer, and then the mixture was incubated at 37°C for 30 min (○) or at 37°C for 30 min followed by chilling at 4°C for 10 min (●). Percent hemolysis was determined as described in Materials and Methods. Values represent means \pm SE for five to six experiments.

buffer (pH 7.2) containing 0.3 mM CaCl₂ at 37°C for 30 min, and then the preparation was chilled at 4°C for 10 min. Alpha-toxin in the range of 1.0 to 5.0 ng/ml caused a dose-dependent increase in hemolysis. Toxin concentrations above 5.0 ng/ml caused maximal hemolysis under the conditions used. Little hemolysis was evident at 37°C until the mixture had been cooled at 4°C when the mixture was incubated with 3.0 ng of the toxin per ml. On the other hand, incubation of erythrocytes with 5.0 and 10 ng of the toxin per ml at 37°C resulted in approximately 15 and 80% hemolysis, respectively. The data demonstrate that low concentrations of the toxin produce a typical hot-cold hemolysis of rabbit erythrocytes. Furthermore, when rabbit erythrocytes (6×10^{11} cell per ml) were incubated with the toxin (5 ng) in 0.02 M Tris-buffered saline at 37°C for various times and then chilled at 4°C for 10 min, hot-cold hemolysis was dependent on the duration of incubation of the toxin with erythrocytes at 37°C (data not shown).

Effect of alpha-toxin on formation of PA. Fujii et al. reported that treatment of rat isolated aorta with alpha-toxin caused stimulation of PA, phosphatidylinositol, arachidonic acid, and thromboxane A₂ production in the tissue (9, 10). Therefore, the formation of [³²P]PA was investigated after incubation of labeled erythrocytes with the toxin at 37°C for 20 min. The radioactivity of PA increased progressively with toxin dose (Fig. 2), suggesting that the toxin stimulates PA formation in erythrocytes. On the other hand, chilling at 4°C for 10 min after incubation at 37°C did not cause additional formation of [³²P]PA.

It appears that PA is generally produced from phosphorylation of 1,2-diacylglycerol or hydrolysis of phospholipids by phospholipase D. Furthermore, it has been reported that PA-produced phospholipase D is converted to 1,2-diacylglycerol by PA phosphatase. Therefore, we determined contents of [³²P]PA in erythrocyte membranes treated with the toxin in the presence of [γ -³²P]ATP at 37°C. As shown in Fig. 3, the toxin dose dependently stimulated formation of [³²P]PA, and formation reached a maximum at 10 ng of the toxin per ml. The time course for formation of [³²P]PA in toxin-treated erythrocyte membranes was investigated (Fig. 4). PA was formed rapidly, reaching a maximum within 30 s, and then decreased rapidly. Over 5 min of incubation, [³²P]PA formation began again and reached a maximum after

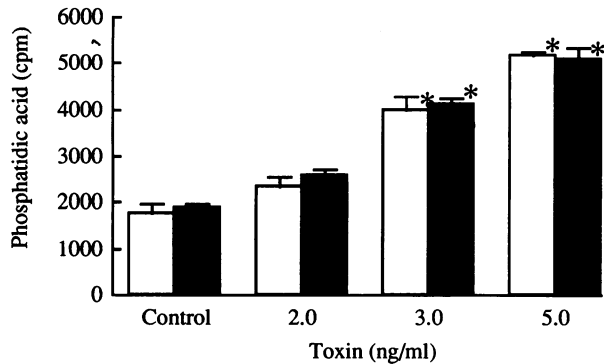


FIG. 2. Effect of incubation temperature on production of PA by alpha-toxin in rabbit erythrocytes. ^{32}P -labeled erythrocytes (6×10^{11} cells) were incubated without the toxin (control) or with various concentrations of the toxin in 0.5 ml of HEPES buffer (pH 7.2) at 37°C for 30 min (\square) or at 37°C for 30 min followed by chilling at 4°C for 10 min (\blacksquare). ^{32}P incorporation into PA was determined as described in Materials and Methods. Values represent means \pm SE for five to six experiments. *, $P < 0.05$ compared with the control value.

20 min of incubation. Recently, PA has been reported to be derived from the action of phospholipase C and/or phospholipase D on phospholipids (14, 18). Furthermore, phospholipase D is reported to catalyze a transphosphatidylation reaction in which the phosphatidyl moiety of the phospholipid substrate is transferred to appropriate nucleophiles such as ethanol. Therefore, to understand the effects of these enzymes on the formation of PA, we determined the levels of PA in erythrocyte membranes treated with the toxin in the absence or presence of 2.0% ethanol. Figure 4 shows that the addition of ethanol resulted in no effect on PA formation within 5 min of incubation but led to a significant decrease in PA formation over 10 min of incubation compared with PA formation in the absence of ethanol. When erythrocyte membranes (720 μg) were incubated with the toxin (5 ng) in the presence of [^3H]ethanol (50 μCi) at 37°C for 30 min, radioactivity (12,427 dpm) was significantly incorporated into phosphatidylethanol, which confirmed the activation of phospholipase D by the toxin. On the other hand, incubation of membranes with [^3H]ethanol in the absence of the toxin

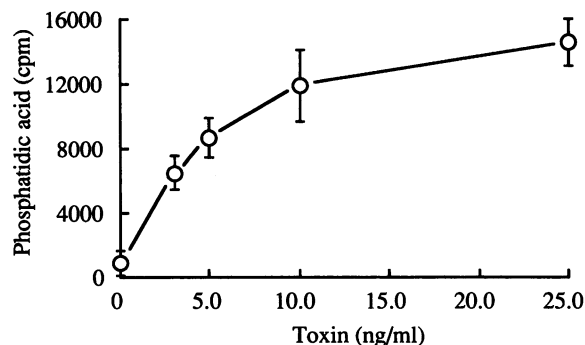


FIG. 3. Dose effect of alpha-toxin on PA production in rabbit erythrocyte membranes. Erythrocyte membranes were incubated with various concentrations of the toxin and [γ - ^{32}P]ATP in 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.5) at 37°C for 30 min. ^{32}P incorporation into PA was determined as described in Materials and Methods. Values represent means \pm SE for five to six experiments.

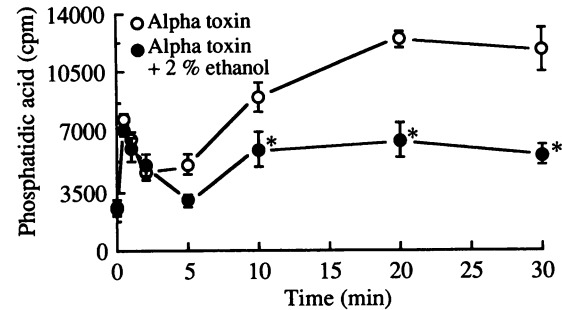


FIG. 4. Time course of PA production induced by alpha-toxin in rabbit erythrocyte membranes. Erythrocyte membranes (720 μg of protein) were incubated with the toxin (5.0 ng) in 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.5) in the absence (\circ) and presence (\bullet) of 2% ethanol at 37°C for various times. PA content was determined as described in Materials and Methods. Values represent means \pm SE for five to six experiments. The effect of ethanol was significant (* indicates $P < 0.05$).

resulted in little incorporation of radioactivity (2,792 dpm) into phosphatidylethanol. The effect of propranolol, which inhibits the conversion of PA to 1,2-diacylglycerol, on [^{32}P]PA formation in response to the toxin in the presence of [γ - ^{32}P]ATP was investigated. Treatment with 250 μM propranolol resulted in a significant decrease in PA formation induced by the toxin over 10 min, but not within 5 min, of incubation (data not shown).

Changes in 1,2-diacylglycerol, IP₃, and PIP₂ in erythrocyte membranes in response to alpha-toxin. According to the current paradigm, binding of various agents, such as agonists, hormones, and bacterial toxins, to the receptors on biological membranes leads to the phosphodiesteric cleavage of PIP₂, yielding 1,2-diacylglycerol and IP₃ (2, 15, 31, 38). After exposure of erythrocyte membranes to the toxin, levels of 1,2-diacylglycerol in toxin-treated membranes were determined. As shown in Fig. 5A, 1,2-diacylglycerol was produced rapidly; the level reached a maximum within 15 s and then rapidly decreased. Within 2 min of incubation, 1,2-diacylglycerol production was stimulated again. The effect of the toxin on IP₃ release from erythrocyte membranes was investigated. Figure 5B shows that release of IP₃ reached a maximum within 30 s after exposure of erythrocyte membranes to the toxin and then sharply decreased. After 60 s of incubation, levels of IP₃ were not significantly different from control levels. Levels of [^{32}P]PIP₂ in the labeled membranes treated with the toxin were determined. As shown in Fig. 5C, the level of [^{32}P]PIP₂ decreased significantly 15 to 30 s after exposure of the labeled membranes to the toxin, but after 60 s of the incubation, levels of [^{32}P]PIP₂ in toxin-treated membranes were not significantly different from control levels. These data suggest that the time course for rapid breakdown of PIP₂ is coincident with those of PA, 1,2-diacylglycerol, and IP₃ generation.

We determined whether the toxin or erythrocyte membranes contain PIP₂-specific phospholipase C activity, which degrades PIP₂ to IP₃ and 1,2-diacylglycerol. To do so, [^3H]PIP₂ was incubated with the toxin and/or erythrocyte membranes, and the [^3H]IP₃ content was measured (Table 1). Incubation of [^3H]PIP₂ with the toxin caused little release of [^3H]IP₃, but incubation with erythrocyte membranes resulted in a marked release of [^3H]IP₃. On the other hand, when [^3H]PIP₂ was incubated with the toxin and erythrocyte membranes, [^3H]IP₃ release increased significantly com-

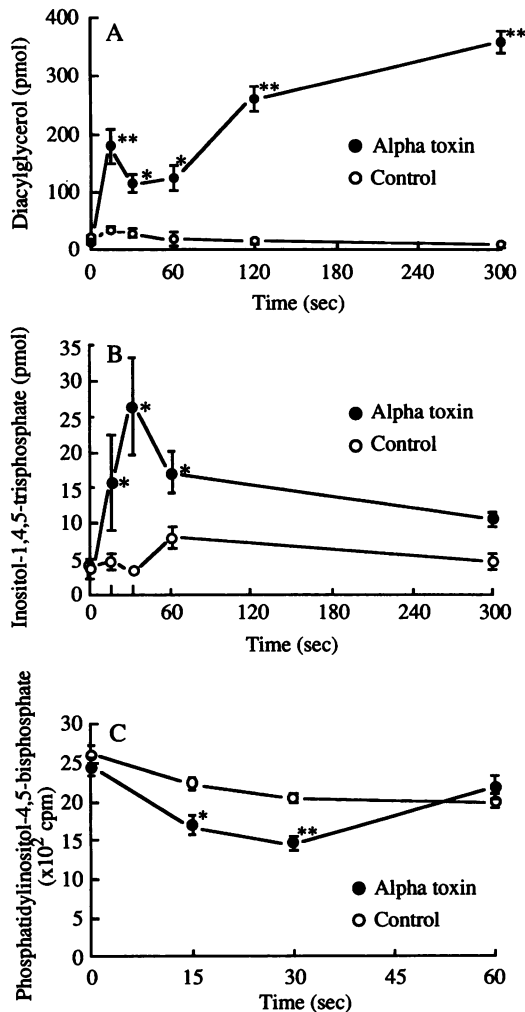


FIG. 5. Time course of rapid changes in 1,2-diaclycerol, IP₃, and PIP₂ in response to alpha-toxin. Erythrocyte membranes (720 µg of protein) were incubated without (control; ○) or with (●) the toxin (5.0 ng) in 0.02 M Tris-HCl buffer (pH 7.5) at 37°C for various times. Levels of 1,2-diaclycerol (A) and IP₃ (B) were determined as described in Materials and Methods. The labeled membranes (720 µg of protein) were incubated without (control; ○) or with (●) the toxin (5.0 ng) in 0.02 M Tris-HCl buffer (pH 7.5) at 37°C for various times. PIP₂ content in membranes (C) was determined as described in Materials and Methods. Values represent means ± SE for five to six experiments. The effect of alpha-toxin was significant (* indicates *P* < 0.05; ** indicates *P* < 0.01).

pared with the release of [³H]IP₃ upon incubation of [³H]PIP₂ with erythrocyte membranes alone.

Effects of phospholipase C inhibitors on alpha-toxin-induced hemolysis and PA production. It has been reported that neomycin (7, 46) and NCDC (48) are effective inhibitors of phosphatidylinositol phosphodiesterase in erythrocyte membranes and platelets, respectively. When the toxin was incubated with egg yolk suspensions in the presence of phospholipase C blockers, each of the blockers inhibited the increase in turbidity of the egg yolk solution induced by the toxin in a dose-dependent manner (Table 2). Treatment with 1.0 mM NCDC or 3.0 mM neomycin resulted in more than 90% inhibition. However, PMSF, which inhibits phospholipase C of human platelets (48), had no effect on the activity of toxin under the experimental conditions used.

TABLE 1. Effects of alpha-toxin on degradation of PIP₂ to IP₃^a

Addition		IP ₃ (dpm)
Alpha-toxin	Erythrocyte membranes	
-	-	0
-	+	443 ± 35
+	-	1 ± 1
+	+	687 ± 28 ^b

^a The toxin (5.0 ng) and/or erythrocyte membranes (96 µg of protein) were incubated with labeled PIP₂ in 0.02 M Tris-HCl buffer (pH 7.5) at 37°C for 10 min. IP₃ was determined as described in Materials and Methods.

^b *P* < 0.01 compared with the value for erythrocyte membranes alone.

The effects of these inhibitors on the hemolysis induced by the toxin were investigated (Table 2). These inhibitors had no effect on the toxin-hemolysis of intact erythrocytes. On the other hand, various cells permeabilized with saponin have been used to study the effects of molecules that normally do not penetrate. When saponin-treated erythrocytes were incubated with the toxin in the presence of neomycin, neomycin in a range of 0.5 to 3.0 mM dose dependently inhibited the toxin-induced hemolysis, but NCDC and PMSF did not. The effects of the inhibitors on PA production induced by the toxin in erythrocyte membranes were investigated. As shown in Fig. 6, treatment of erythrocyte membranes with 3.0 mM neomycin resulted in more than 90% inhibition. However, treatment of erythrocyte membranes with 1.0 mM NCDC or 2.0 mM PMSF had no effect on production of PA induced by the toxin. These effects of the blockers on the toxin-induced hemolysis of saponin-treated erythrocytes were consistent with those of the blockers on toxin-induced PA formation.

DISCUSSION

Erythrocytes of most laboratory animals are known to be sensitive to *C. perfringens* alpha-toxin; exceptions are those

TABLE 2. Effects of PMSF, NCDC, and neomycin on egg yolk or hemolytic activity of alpha-toxin^a

Agent	Concn (mM)	Phospholipase C activity (%)	Hemolysis (%)	
			Intact cells	Saponin-treated cells
None (control)		100	100	100
PMSF	1.0	101 ± 5	97 ± 3	96 ± 5
	2.0	99 ± 4	107 ± 6	94 ± 3
NCDC	0.1	84 ± 3	94 ± 2	112 ± 8
	0.25	61 ± 6 ^b	ND ^c	ND
	0.5	7 ± 3 ^d	110 ± 1	88 ± 4
	1.0	3 ± 1 ^d	108 ± 6	93 ± 3
Neomycin	0.1	83 ± 2	96 ± 4	87 ± 10
	0.5	56 ± 3 ^b	105 ± 3	63 ± 8 ^b
	1.0	27 ± 3 ^d	ND	ND
	3.0	9 ± 1 ^d	102 ± 4	25 ± 4 ^d

^a The toxin (100 ng) and various inhibitors were simultaneously added to 1.9 ml of egg yolk solution, and the mixture was incubated at 37°C for 60 min. Phospholipase C activity was determined as described by Sakurai et al. (40). The toxin (5.0 ng) and various inhibitors were simultaneously added to intact erythrocyte suspensions or saponin-treated erythrocyte suspensions, and the mixtures were incubated at 37°C for 30 min and then chilled at 4°C for 10 min. Percent hemolysis was determined as described in Materials and Methods.

^b *P* < 0.05 compared with the control value.

^c ND, not determined.

^d *P* < 0.005 compared with the control value.

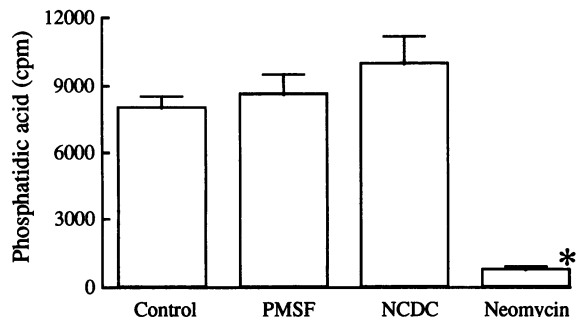


FIG. 6. Effects of PMSF, NCDC, and neomycin on PA production induced by alpha-toxin. The erythrocyte membranes (720 μ g of protein) were incubated with the toxin (5.0 ng) in 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.5) in the absence (control) or presence of 2.0 mM PMSF, 1.0 mM NCDC, or 3.0 mM neomycin at 37°C for 20 min. PA content was determined as described in Materials and Methods. Values represent means \pm SE for five to six experiments. *, $P < 0.05$ compared with the control value.

of horses and goats (20). MacFarlane (24) and Jolivet-Reynaud et al. (20) reported a hot-cold phenomenon for sheep erythrocytes and found that rabbit erythrocytes are more sensitive to the toxin than are sheep erythrocytes, but the phenomenon does not occur with rabbit erythrocytes. A large amount (more than 10 ng) of the toxin caused hemolysis of rabbit erythrocytes at 37°C, as reported by other workers (6, 20). The hot-cold hemolysis of rabbit erythrocytes was observed upon exposure to a small amount of the purified toxin (<5 ng) in the presence of a low concentration (300 μ M) of Ca^{2+} . On the other hand, other investigators reported the hemolysis of rabbit erythrocytes in the presence of a high concentration of Ca^{2+} (20, 24). In addition, Ikezawa et al. reported that 10 to 50 mM Ca^{2+} is optimal for the toxin-induced hot-cold hemolysis of sheep, horse, and rat erythrocytes (19). Therefore, it seems that the hot-cold phenomenon of rabbit erythrocytes is not observed upon exposure to a large amount of the toxin in the presence of a high concentration of Ca^{2+} .

Our results provide the first evidence that alpha-toxin-induced hemolysis is coupled to generation of 1,2-diacylglycerol, IP_3 and PA and degradation of PIP_2 . When erythrocyte membranes were incubated with the toxin, formation of 1,2-diacylglycerol and [γ - ^{32}P]ATP was biphasic, the first phase occurring within 15 to 30 s and the second phase occurring at approximately 20 min. At the same time, levels of IP_3 and PA reached a maximum and levels of PIP_2 were minimal. The time course for 1,2-diacylglycerol and IP_3 generation was coincident with that for breakdown of PIP_2 within 1 min of incubation. Downes and Michell (7) demonstrated that rabbit erythrocytes contain an active PIP_2 -specific phospholipase C, which degrades PIP_2 to 1,2-diacylglycerol and IP_3 . On the other hand, when exogenous 1,2-diacylglycerol was incubated with erythrocyte membranes in the presence of [γ - ^{32}P]ATP, [^{32}P]PA levels increased with an increase of diacylglycerol added in the presence or absence of the toxin (data not shown). It therefore appears that erythrocyte membranes have 1,2-diacylglycerol kinase. Incubation of erythrocyte membranes with the toxin in the presence of [γ - ^{32}P]ATP resulted in a significant increase in labeled PA content. Furthermore, the time course for the toxin-induced PA formation was coincident with that for toxin-induced 1,2-diacylglycerol genera-

tion. These results suggest that PA formation depends on the production of 1,2-diacylglycerol.

The toxin alone did not hydrolyze exogenous PIP_2 . However, formation of 1,2-diacylglycerol at the first phase seems to be due to PIP_2 hydrolysis through activation of endogenous phospholipase C in membranes by the toxin. Formation of IP_3 and degradation of PIP_2 were not observed at the second phase. It therefore is unlikely that the late appearance of PA is due to diacylglycerol production from PIP_2 by phospholipase C. Martinson et al. (26) reported that activation of phospholipase D in astrocytoma cells results in delayed accumulation of 1,2-diacylglycerol as a result of the relatively slow conversion of PA to 1,2-diacylglycerol by PA phosphatase. It has been reported that phospholipase D activity is implicated in the formation of PA by mammalian systems activated by stimulants and that the enzyme is able to catalyze a transphosphatidyl action (4, 18, 26, 32, 35, 36) in which the phosphatidyl group of phosphatidylcholine and/or other phospholipids is transferred to ethanol, producing phosphatidylethanol (13, 50). In the present work, incubation of erythrocyte membranes with the toxin in the presence of [3H]ethanol caused increased incorporation of radioactivity into phosphatidylethanol, suggesting that the erythrocyte membrane contains phospholipase D. When a small amount of ethanol was added into the mixture of erythrocyte membranes and the toxin, ethanol had no effect on PA formation induced by the toxin at the first phase but caused significant inhibition of formation at the second phase. Moreover, propranolol, which appears to inhibit the conversion of PA to 1,2-diacylglycerol (5, 8, 28), markedly inhibited [^{32}P]PA formation at the second phase upon incubation of membranes with the toxin in the presence of [γ - ^{32}P]ATP. These data show that the early appearance (at ~5 min) of PA is due primarily to phospholipase C and that the late appearance (at ~10 min) of PA is related to phospholipase D. It has been known that the toxin contains phospholipase C but not phospholipase D activity. Therefore, the data suggest that the toxin activates phospholipase D in erythrocyte membranes. However, from these data, we cannot determine whether phospholipase D is directly related to the toxin-induced hemolysis.

Downes and Michell (7) reported that neomycin blocks PIP_2 -specific phospholipase C in human erythrocytes. Walenga et al. (48) reported that NCDC and PMSF inhibit phosphatidylinositol-specific phospholipase C in human platelets. Neomycin and NCDC inhibited increased turbidity in egg yolk emulsions induced by the toxin, which contains phospholipase C activity, but PMSF did not, suggesting that neomycin interacted with the toxin and/or phospholipids so that the inhibitor blocked the toxin-induced increased turbidity in egg yolk emulsions. Neomycin had no effect on the toxin-induced hemolysis of intact erythrocytes. Therefore, the hemolytic activity of toxin seems not to be due to a direct action of the phospholipase C activity of the toxin. On the other hand, neomycin inhibited the toxin-induced hemolysis of saponin-treated erythrocytes in a dose-dependent manner. Various cells permeabilized with saponin have been used to study the effect of molecules that normally do not penetrate. Moreover, it has been reported that neomycin directly interacts with phospholipids. Therefore, neomycin appears to penetrate saponin-treated erythrocytes and inhibit hydrolysis of phospholipids by phospholipase C. In addition, neomycin blocked PA production induced by the toxin in erythrocyte membranes, but NCDC and PMSF did not. The effects of these inhibitors on the toxin-induced hemolysis of saponin-treated erythrocytes were consistent

with the effects of these agents on PA production induced by the toxin. On the other hand, the effect of neomycin on the toxin-induced hemolysis of intact erythrocytes was different from the effect of the agent on the toxin-induced hemolysis of saponin-treated erythrocytes. These data indicate that phospholipids hydrolyzed by phospholipase C are in and/or inside erythrocyte membrane so that the toxin cannot attack them, suggesting that endogenous phospholipase C may be related to hemolysis induced by the toxin under the experimental conditions used. Furthermore, the toxin alone did not hydrolyze PIP₂ but stimulated PIP₂-specific phospholipase C activity in erythrocyte membranes. These data suggest that the toxin may interact with receptors on the erythrocyte membrane and then activate PIP₂-specific phospholipase C in erythrocyte membranes.

We do not exclude the possibility that the initial step induced by the toxin is hydrolysis of phospholipids by the phospholipase C activity of the toxin. However, it does not appear that hemolysis induced by the toxin is due only to degradation of erythrocyte membranes by the phospholipase C activity of the toxin. The results of this study suggest that activation of PIP₂-specific phospholipase C and/or phospholipase D by the toxin in erythrocyte membranes is related to hemolysis induced by the toxin under the our experimental conditions.

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