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Clostridium perfringens alpha-toxin-induced hemolysis of horse erythrocytes is dependent on Ca^{2+} uptake

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Abstract

Clostridium perfringens alpha-toxin is able to lyse various erythrocytes. Exposure of horse erythrocytes to alpha-toxin simultaneously induced hot–cold hemolysis and stimulated production of diacylglycerol and phosphorylcholine. When A23187-treated erythrocytes were treated with the toxin, these events were dependent on the concentration of extracellular Ca^{2+}. Incubation with the toxin of BAPTA-AM-treated horse erythrocytes caused no hemolysis or production of phosphorylcholine, but that of the BAPTA-treated erythrocytes did. When Quin 2-AM-treated erythrocytes were incubated with the toxin in the presence of $^{45}Ca^{2+}$, the cells accumulated $^{45}Ca^{2+}$ in a dose- and a time-dependent manner. These results suggest that the toxin-induced hemolysis and hydrolysis of phosphatidylcholine are closely related to the presence of Ca^{2+} in the cells. Flunarizine, a T-type Ca^{2+} channel blocker, and tetrandrine, an L- and T-type Ca^{2+} channel blocker, inhibited the toxin-induced hemolysis and Ca^{2+} uptake. However, L-type Ca^{2+} channel blockers, nifedipine, verpamil and diltiazem, an N-type blocker, ω-conotoxin SVIB, P-type blockers, ω-agatoxin TK and ω-agatoxin IVA, and a Q-type blocker, ω-conotoxin MVII C, had no such inhibitory effect. The observation suggests that Ca^{2+} taken up through T-type Ca^{2+} channels activated by the toxin plays an important role in hemolysis induced by the toxin.

Keywords: *Clostridium perfringens*; Alpha-toxin; Hemolysis; Ca^{2+}; Ca^{2+}channel blocker

1. Introduction

Clostridium perfringens alpha-toxin, which possesses hemolytic, lethal, dermonecrotic and cytotoxic activities and in addition phospholipase and sphingomyelinase activities, is thought to play an important role in gas gangrene [1–5]. We have reported that the toxin causes hot–cold hemolysis of rabbit erythrocytes, when given in small amounts in the presence of a low concentration of Ca^{2+} [6]. Horse erythrocytes were reported to be more stable than rabbit erythrocytes [7]. However, horse erythrocytes showed typical hot–cold hemolysis, when incubated with high concentration of the toxin. Hence, the toxin-induced hemolysis is an interesting model for the study of the destructive action of the toxin on cells. Phosphatidylcholine and sphingomyelin in rabbit erythrocytes account for about 34% and 20% of total phospholipids, respectively, and in horse erythrocytes, about 42% and 14%, indicating that the phospholipid contents of rabbit erythrocytes resemble those of horse erythrocytes [8]. On the other hand, we showed that the toxin-induced hemolysis of rabbit erythrocytes is closely related to activation of phospholipid metabolism [6,9]. In addition, we have reported that the toxin-induced contraction of rat ileum and aorta also depends on phospholipid metabolism activated by the toxin [10,11]. Despite these advances, little is known regarding the relationship between the toxin-induced hemolysis and phospholipid metabolism in erythrocytes of animals except rabbits.

Ca^{2+} is known to be necessary for hemolysis induced by the toxin of these erythrocytes [12]. Ikezawa et al. [12] postulated that the toxin combined with Ca^{2+}–phospholipid(s) complex caused hydrolysis of substrate phospholipids in erythrocyte membrane. We reported that the presence of Ca^{2+} allowed the toxin to attach to the target erythrocyte membrane [13], suggesting that Ca^{2+} is essential for the binding of the toxin to erythrocyte membrane. We also previously reported that alpha-toxin induces superoxide

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anion production in rabbit neutrophils through the activation of phospholipid metabolism in the cell membrane [14]. It is known that phospholipid metabolism of the membrane in many cells is closely related to an elevation of intracellular Ca^{2+} [15,16]. Therefore, it is important to know whether or not the toxin induces uptake of Ca^{2+} into erythrocytes, and intracellular Ca^{2+} is important for the hemolytic activity of the toxin.

To clarify the relation between the hemolytic activity of the toxin and intracellular Ca^{2+}, we examined that between the toxin-induced phospholipid metabolism and intracellular Ca^{2+} and the mechanism of uptake of Ca^{2+} in horse erythrocytes.

2. Materials and methods

2.1. Materials

Horse erythrocytes were purchased from Nippon Bio-Test Lab. Inc. (Tokyo, Japan). A23187, *sn*-1,2-diacylglycerol kinase, flunarizine, ionomycin and tetrandrine were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). BAPTA (*O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid), BAPTA-AM(*O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester), *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, Quin 2 (8-amino-2-[(2-amino-5-methylphenoxy)-methyl]-6-methoxyquinoline-*N*,*N*,*N*′,*N*′-tetraacetic acid) and Quin 2-AM (8-amino-2-[(2-amino-5-methylphenoxy)-methyl]-6-methoxyquinoline-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester) were from Dojindo Laboratories Co. (Kumamoto, Japan). 1-Stearoyl-2-arachidonoyl-*sn*-glycerol was purchased from BIOMOL Res. Lab., Inc. (Plymouth Meeting, PA). 5(6)-Carboxyfluorescein diacetate (CF), choline oxidase from *Arthrobacter globiformis*, diltiazem, nifedipine, alkaline phosphatase (from bovine intestinal mucosa), phosphatidylcholine, phosphorylcholine chloride and verapamil were from Sigma Chemical Co. (St. Louis, MO). ω-Agatoxin IVA, ω-agatoxin TK, ω-conotoxin MVIIC and ω-conotoxin SVIB were obtained from Peptide Institute, Inc. (Osaka, Japan). 4-Aminoantipyrine, ascorbate oxidase from *Cucurbita* sp. and peroxidase from horseradish were obtained from Wako Pure Chemical Industries (Osaka, Japan). [γ-^{32}P]ATP (167 TBq/mmol) was purchased from ICN Biochemicals, Inc. (Irvine, CA). ^{45}Ca (0.85 GBq/mg) was supplied by Amersham Pharmacia Biotech KK (Tokyo, Japan). All other agents were of analytical grade.

2.2. Purification of alpha-toxin

Purification of recombinant alpha-toxin was performed as described in detail previously [13] and was followed by testing for homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunological techniques [13].

2.3. Preparation of horse erythrocytes

Horse erythrocytes were suspended in 0.02 M Tris–HCl buffer (pH 7.5) containing 0.9% NaCl (TBS), and centrifuged at $1100 \times g$ for 3 min. The sedimented erythrocytes were then washed three times in the same buffer. The number of erythrocytes was determined with a cell counter (Celltac; Nihon Kohden Co., Tokyo, Japan).

2.4. Treatment of horse erythrocytes with A23187, ionomycin, BAPTA or BAPTA-AM

Horse erythrocytes were incubated with 0.5 μM A23187, 0.1 μM ionomycin (dissolved in ethanol; 1.0 mM stock solution), various concentrations of BAPTA or BAPTA-AM (dissolved in dimethyl sulfoxide; 50 mM stock solution) and various concentrations of $CaCl_2$ in TBS at 37 °C for 80 min, and then the erythrocytes were washed with TBS. The erythrocytes were resuspended in TBS and the cell count was adjusted to 6.0×10^{11} cells per milliliter.

2.5. Determination of hemolytic activity

Washed treated horse erythrocytes (12×10^{10} cells per milliliter) were incubated with various concentrations of alpha-toxin (0.1–2.0 μg/ml) in TBS at 37 °C for 20 min, and then chilled at 4 °C. Unlysed cells were removed by centrifugation at $1100 \times g$ for 3 min. The extent of lysis was determined by spectrophotometrically measuring the hemoglobin released at A_{550}. All assay mixtures were supplemented with Ca^{2+} to 0.3 mM. Hemolysis was expressed as a percentage of the hemoglobin released from 0.1 ml of erythrocytes suspended in 0.4 ml of 0.4% NaCl.

2.6. Determination of diacylglycerol

Erythrocyte suspensions (12×10^{10} cells per milliliter) were incubated with various concentrations of alpha-toxin (0.1–2.0 μg/ml) in a total volume of 0.5 ml of TBS containing 0.3 mM $CaCl_2$ at 37 °C for 30 min. The reaction was terminated by the addition of 1.8 ml of chloroform–methanol (1:2, vol/vol). The lipids were extracted by the method of Bligh and Dyer [17] except that 0.2 M KCl–5.0 mM EDTA was used instead of water [18]. The final organic phase was dried under a stream of N_2 and used for analysis of the mass amount of diacylglycerol. The diacylglycerol content of the crude lipid fraction was quantified as previously described [6].

2.7. Determination of phosphorylcholine

Erythrocyte suspensions (12×10^{10} cells per milliliter) were incubated with alpha-toxin and various concentrations of Ca^{2+} channel blocker in a total volume of 0.5 ml of TBS containing 0.3 mM $CaCl_2$ at 37 °C for 30 min. The reaction was terminated by the addition of 1.8 ml of chloroform–

methanol (1: 2, vol/vol), and the phases were separated by the addition of 0.49 ml of chloroform and 0.49 ml of 1.0 M KCl. The final water phase was divided into two tubes and evaporated with a Centrifugal Concentrator CC-181 (Tomy Seiko Co., Tokyo, Japan), and incubated in a total volume of 0.5 ml of 0.05 M Tris–HCl buffer (pH 9.0) containing 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ in the presence (phosphatase-treated sample) or absence (non-treated sample) of 10 units of alkaline phosphatase per milliliter at 37 °C for 60 min. To the reaction mixture was then added 0.24 mM 4-aminoantipyrine, 3.9 units of ascorbate oxidase per milliliter, 0.77 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, 2.0 units of choline oxidase per ml and 4.2 units of peroxidase per ml (final volume; 1.0 ml), and incubation continued at 37 °C for 5 min. The A_{600} of the reaction mixture was measured. Known amounts of phosphorylcholine were assayed as described above, and a standard curve was made. The amount of phosphorylcholine in the original sample was determined by subtracting the value for the phosphatase-treated sample from that for the non-treated sample.

2.8. Preparation of liposomes and assay of CF release from the liposomes

Liposomes containing CF were prepared and the amount of CF released was determined by the method of Nagahama et al. [19].

2.9. Measurement of uptake of $^{45}Ca^{2+}$ into horse erythrocytes

Horse erythrocytes were incubated with 5.0 μM Quin 2-AM at 37 °C for 60 min. They were then washed with TBS containing 5.0 mg/ml of BSA (bovine serum albumin) to remove extracellular Quin 2-AM. The erythrocytes were resuspended in at 6.0×10^{10} cells/ml and equilibrated with 300 μM $^{45}Ca^{2+}$ (specific activity; 0.8 kBq/ml) at 37 °C for 30 min before the addition of alpha-toxin. At the desired times after addition of the toxin, cell suspensions were centrifuged to pellet the erythrocytes. Cell pellets were washed first in TBS containing 5.0 mM EGTA and then three times in TBS before lysis in deionized water. The lysed erythrocytes were decolorized with H_2O_2 before liquid scintillation analysis of $^{45}Ca^{2+}$ content. Except using the medium containing 10 mM pyruvate or 10 mM pyruvate and 10 mM glucose, the control experiments were carried out by the same procedure.

2.10. Measurement of Ca^{2+} influx into resealed hose erythrocyte ghosts containing Fura 2

Horse erythrocytes (6.0×10^{10} cells per milliliter) were lysed in 35 volumes of the ice-cold sodium phosphate buffer (pH 7.5) containing 7.0 mM KCl and 0.1 mM EGTA. The membranes were collected by centrifugation at 40,000 × *g* for 2 min at 4 °C. The membranes suspended at ice-cold sodium phosphate buffer (pH 7.5) containing 7.0 mM KCl and 0.1 mM EGTA were incubated in 30 μM Fura 2-AM at 4 °C for 15 min. To restore isotonicity, a small volume of the mixture of KCl, $MgCl_2$, and dithiothreitol (DTT) was added to the membrane suspension, giving a final concentration of 100 mM KCl, 1.0 mM $MgCl_2$, and 1.0 mM DTT. The ghost suspension was incubated at 37 °C for 30 min to allow the ghosts to reseal, and then the resealed erythrocyte ghosts were washed with TBS and resuspended in TBS. Resealed erythrocyte ghosts containing Fura 2 were incubated with various concentrations of alpha-toxin (0.1–2.0 μg/ml) in the presence of 0.3 mM $CaCl_2$ at 37 °C for 15 min. The fluorescence of Fura 2 was measured by fluorescence spectrophotometer with excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm [20]. Changes in Fura 2 fluorescence intensity were monitored from ratio for excitation at 340 and 380 nm (F340/380). Fluorescence was expressed as a percentage of F340/380 induced by addition of 0.5% Triton X-100 instead of alpha-toxin.

2.11. Others

Measurement of cellular ATP levels was performed using the bioluminescence ATP kit (Toyo Ink., Co., Tokyo, Japan) with Iuminometer (Promega, Madison, WI). The protein concentration was determined by the method of Lowry et al. [21], using BSA as a standard. In all cases, mean and standard error (S.E.) values were determined. Statistical analysis was performed by using Student's *t* test; a *P* value of 0.05 or less was considered statistically significant.

3. Results

3.1. Effect of alpha-toxin on hemolysis and hydrolysis of phosphatidylcholine

Horse erythrocytes were incubated with alpha-toxin in TBS at 37 °C for 30 min and then chilled at 4 °C for 10 min. The toxin at concentrations of 0.3–2.0 μg /ml caused a dose-dependent increase in hemolysis, and production of diacylglycerol and phosphorylcholine (Fig. 1). Toxin concentrations above 1.0 μg/ml caused maximal lysis of the cells and production of diacylglycerol and phosphorylcholine under the conditions (Fig. 1). However, incubation with 10 μg /ml of the toxin at 37 °C for 30 min resulted in no hemolysis (6.0 ± 1.0 %), but induced the formation of diacylglycerol (18 ± 1.0 μM) and phosphorylcholine (40 ± 2.0 μM).

3.2. Effect of Ca^{2+} influx on the toxin-induced hemolysis and phosphatidylcholine hydrolysis in horse erythrocytes

It is known that Ca^{2+} plays a role in hemolysis induced by the toxin; Ca^{2+} has been reported to be essential for the binding of the toxin to biological membranes [12,13]. In the

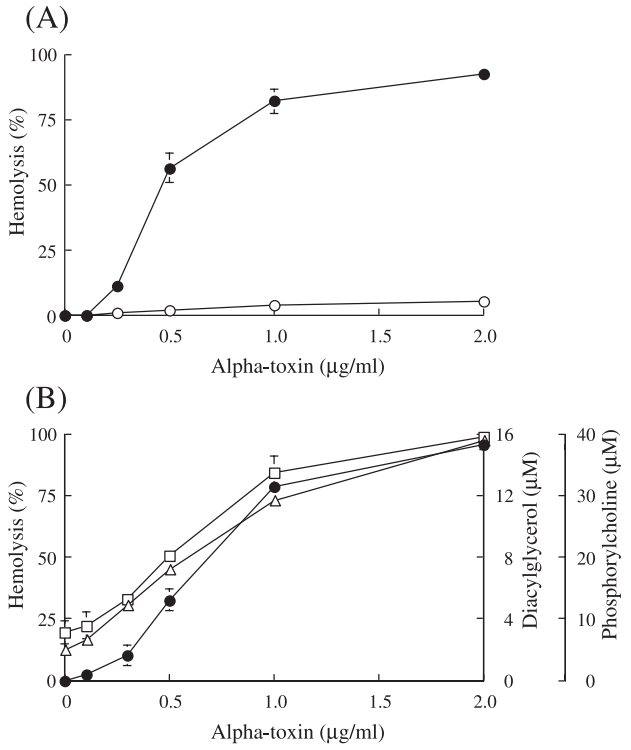

Fig. 1. Effect of alpha-toxin on hemolysis, diacylglycerol and phosphorylcholine formation of horse erythrocytes. (A) Horse erythrocytes were incubated with various concentrations of alpha-toxin in 0.5 ml of TBS containing 0.3 mM $CaCl_2$ at 37 °C for 30 min (○) or at 37 °C for 30 min followed by chilling at 4 °C for 10 min (●). Hemolysis was assessed as described in Materials and methods. (B) Horse erythrocytes were incubated with various concentrations of alpha-toxin in 0.5 ml of TBS containing 0.3 mM $CaCl_2$ at 37 °C for 30 min. After the incubation, hot–cold hemolysis (●), diacylglycerol (△), and phosphorylcholine (□) formation were determined as described in Materials and methods. Values are means ± S.E. S.E. for five to six experiments.

present study, we investigated the effect of intracellular Ca^{2+} on the toxin-induced events. When horse erythrocytes treated with A23187 (0.5 μM), a Ca^{2+} ionophore, were incubated with 0.3 μg/ml of the toxin, a subhemolytic dose, in the presence of various concentrations of Ca^{2+} at 37 °C for 30 min, the toxin-induced hemolysis and phosphorylcholine formation increased with an increase in the dose of Ca^{2+} (0.3–2.0 mM) (Table 1). However, the incubation of untreated erythrocytes with 0.3 μg/ml of the toxin had no effect on the cells (Table 1). The same result was obtained when ionomycin (Ca^{2+} ionophore) was used instead of A23187 (data not shown).

To evaluate whether the toxin induces Ca^{2+} influx into the cells, the cells incubated with BAPTA, an impermeable Ca^{2+} chelating agent, or BAPTA-AM, a permeable Ca^{2+} chelating agent, were incubated with 1.0 μg/ml of the toxin in the presence of 0.3 mM. BAPTA-AM inhibited the toxin-induced hemolysis and production of phosphorylcholine in a dose-dependent manner, but BAPTA did not, as shown in Fig. 2. We examined Ca^{2+} influx induced by alpha-toxin in erythrocytes, which were loaded with Fura 2-AM, cell-permeant and Ca^{2+}-sensitive fluorophore with an affinity for Ca^{2+}. However, we were not able to precisely measure a fluorescence of the fluorophore because of high fluorescent background. It is thought that hemoglobin in erythrocyte disturbs measurement of fluorescence of the fluorophore. Accordingly, we examined the Ca^{2+} influx by the toxin in resealed erythrocyte ghosts containing Fura 2. The toxin (0.5–1.0 μg/ml) induced an increase of Fura 2 fluorescence in the ghosts in a dose-dependent manner, as shown Fig. 3. In the absence of the toxin, no increase of the fluorescence was observed in the resealed ghosts under the condition. Next, to obtain a more quantitative evaluation of the capacity of the toxin to promote Ca^{2+} influx into the cells, $^{45}Ca^{2+}$ was used to monitor the influx. Horse erythrocytes were incubated with 1.0 μg /ml of the toxin in the presence of $^{45}Ca^{2+}$ at 37 °C for 30 min, washed and assayed for radioactivity. Little radioactivity was detected in the cells (data not shown). From these results, it was speculated that the influxed Ca^{2+} is sharply drawn out of the cells. Therefore, the cells were treated with Quin 2-AM, a permeable Ca^{2+} chelating agent,

Table 1
Effect of Ca^{2+} on alpha-toxin-induced hemolysis, and diacylglycerol and phosphorylcholine formation in untreated or A23187-treated horse erythrocytes

$CaCl_2$ (mM)	Hemolysis[a] (%)		Diacylglycerol[b] (μM)		Phosphorylcholine[b] (μM)	
	− A23187	+ A23187	− A23187	+ A23187	− A23187	+ A23187
0	10 ± 0.3	10 ± 0.2	4 ± 0.1	4 ± 0.2	12 ± 0.8	14 ± 1.1
0.3	12 ± 0.2	37 ± 3.1**	3 ± 0.1	5 ± 0.2*	13 ± 1.1	16 ± 1.0*
0.5	11 ± 0.4	43 ± 3.6**	3 ± 0.1	8 ± 2.1**	12 ± 0.7	28 ± 2.8**
1.0	12 ± 0.6	62 ± 4.0**	5 ± 0.2	12 ± 1.9**	14 ± 0.9	32 ± 2.3**
2.0	13 ± 0.2	76 ± 2.2**	4 ± 0.2	14 ± 3.4**	12 ± 1.3	34 ± 2.1**

Horse erythrocytes were incubated with (" + A23187" in the table) or without (" − A23187" in the table) 0.5 μM A23187 in the presence of various concentrations of $CaCl_2$ (0–2.0 mM) at 37 °C for 80 min, and then the erythrocytes were washed with TBS.
Values represent means ± S.E. for four to five experiments.

* $P < 0.05$, compared with the value for intact erythrocytes.

** $P < 0.01$, compared with the value for intact erythrocytes.

[a] Alpha-toxin (0.3 μg/ml) and 0.3 mM $CaCl_2$ were mixed with untreated or 0.5 μM A23187-treated erythrocytes, and the mixture was incubated at 37 °C for 30 min and then chilled at 4 °C for 10 min. Hemolysis was evaluated as described in Materials and methods.

[b] Alpha-toxin (0.3 μg/ml) and 0.3 mM $CaCl_2$ were mixed with untreated or 0.5 μM A23187-treated erythrocytes, and the mixture was incubated at 37 °C for 30 min. The mixture was assayed for diacylglycerol and phosphorylcholine formation.

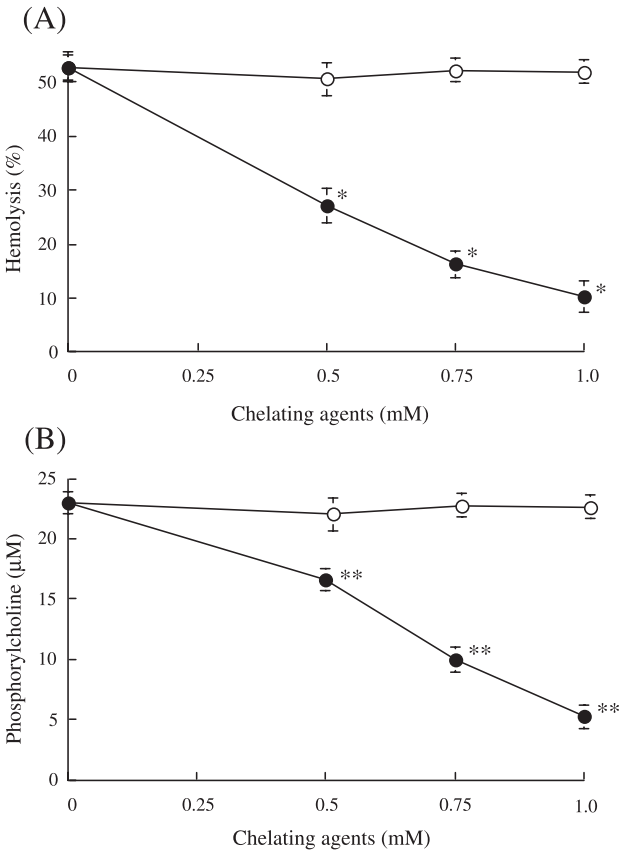

Fig. 2. Hemolysis and phosphorylcholine formation induced by alpha-toxin in horse erythrocytes treated with BAPTA or BAPTA-AM. Horse erythrocytes were incubated with BAPTA (○) or BAPTA-AM (●) at 37 °C for 80 min. (A) The washed erythrocytes were incubated with 1.0 μg/ml of alpha-toxin and 0.3 mM $CaCl_2$ at 37 °C for 30 min and then chilled at 4 °C. Hemolysis was determined as described in Materials and methods. (B) The washed erythrocytes were incubated with 1.0 μg/ml alpha-toxin and 0.3 mM $CaCl_2$ at 37 °C for 30 min, and phosphorylcholine content was determined as described in Materials and methods. Values are means ± S.E. for five to six experiments. *$P<0.01$, compared with hemolysis induced by alpha-toxin of horse erythrocytes in the absence of BAPTA-AM. **$P<0.05$, compared with phosphorylcholine formation induced by alpha-toxin in horse erythrocytes in the absence of BAPTA-AM.

to retard the rapid expulsion of the Ca^{2+}. The Quin 2-loaded erythrocytes were incubated with the toxin in the presence of $^{45}Ca^{2+}$ at 37 °C. As shown in Fig. 4, the toxin (0.25–1.0 μg/ml) induced an increase in Ca^{2+} influx into the Quin 2-loaded cells in a dose- and a time-dependent manner. We used AM compound (Quin 2-AM) for a trapping of influxed extracellular Ca^{2+}. It is known that this compound produces formaldehyde, which interferes with glycolysis, in erythrocytes [22] and that the glycolitic blockade causes ATP depletion in the cell [23], but pyruvate is able to prevent the blockade [24]. We investigated if alpha-toxin-induced $^{45}Ca^{2+}$ uptake is responsible for ATP depletion caused by formaldehyde produced by a hydrolysis of the Quin 2-AM. Addition of 10 mM pyruvate and/or 10 mM glucose resulted in no effect on the toxin-induced $^{45}Ca^{2+}$ uptake into Quin 2-loaded erythrocytes (data not shown). Furthermore, horse

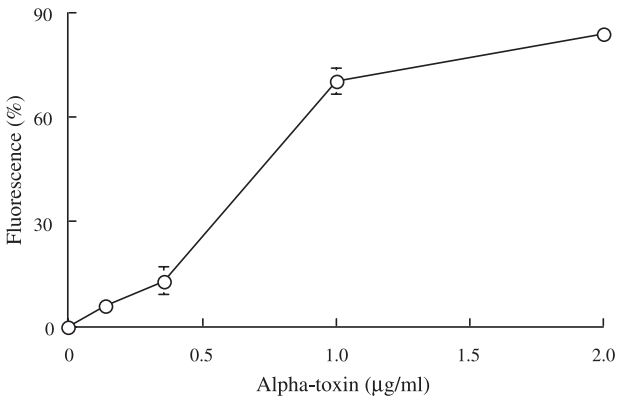

Fig. 3. Effect of alpha-toxin on Ca^{2+} uptake into resealed horse erythrocyte ghosts containing Fura 2. Resealed horse erythrocyte ghosts containing Fura 2 were incubated with various concentrations of alpha-toxin in the presence of 0.3 mM $CaCl_2$ at 37 °C for 15 min. The fluorescence of Fura 2 was measured as described in Materials and methods. Fluorescence was expressed as a percentage of F340/380 induced by addition of 0.5% Triton X-100 instead of alpha-toxin. Values are means ± S.E. for five to six experiments.

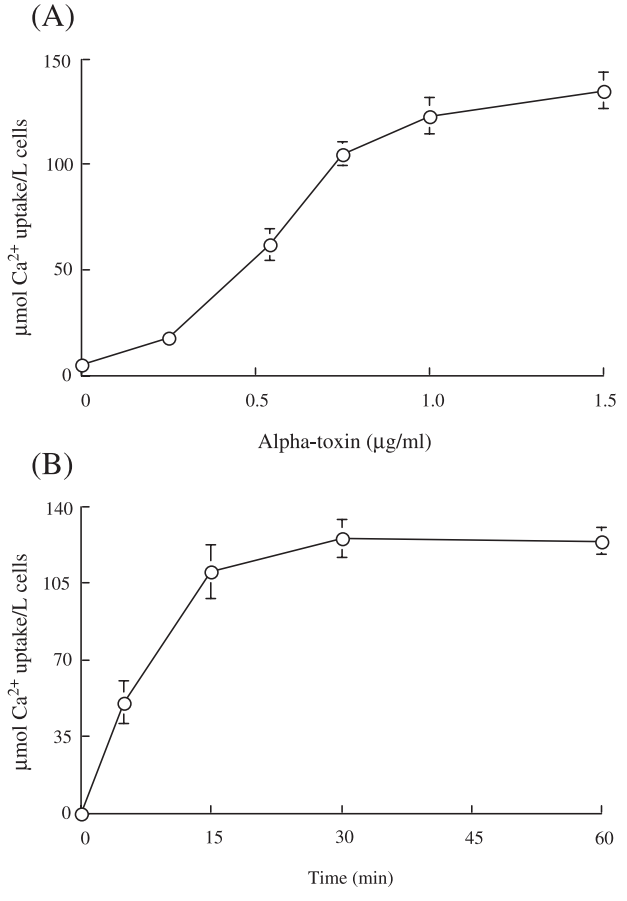

Fig. 4. Dose and time dependency of $^{45}Ca^{2+}$ uptake induced by alpha-toxin into horse erythrocytes. (A) Quin 2-loaded erythrocytes were incubated with various concentrations of alpha-toxin at 37 °C for 30 min. (B) Quin 2-loaded erythrocytes were incubated with 1.0 μg/ml of alpha-toxin for various periods. The erythrocytes were washed and assayed for radioactivity. Values are means ± S.E. for five to six experiments.

erythrocytes (12×10^{10} cells per milliliter) were incubated with or without BAPTA-AM at 37 C for 80 min and ATP was measured in treated and untreated cells. ATP level in untreated cells was $3.0 \pm 0.1 \times 10^{-5}$ mole per 12×10^{10} cells and was not significantly different from that in treated cells (data not shown). It therefore is unlikely that formaldehyde generated by hydrolysis of the acetoxymethyl esters of BAPTA-AM, perhaps Quin 2-AM, depletes ATP in the cells.

3.3. Effect of Ca^{2+} channel blockers on the toxin-induced hemolysis and Ca^{2+} influx

To understand the toxin-stimulated Ca^{2+} uptake pathway, we investigated the effect of Ca^{2+} channel blockers on the toxin-induced Ca^{2+} entry and hemolysis (Table 2). Treatment of the cells with 50 μM of flunarizine resulted in a drastic reduction of the toxin-induced Ca^{2+} influx and hemolysis. In addition, 50 μM of tetrandrine, an L- and T-type Ca^{2+} channel blocker, also reduced by about 30% of the toxin-induced Ca^{2+} entry and hemolysis, compared with the untreated cells. These agents dose-dependently inhibited the toxin-induced Ca^{2+} entry and hemolysis (Fig. 5); however, 100 μM of nifedipine, verapamil and diltiazem, an L-type Ca^{2+} channel blocker, did not inhibit them (Table 2). At 200 nM, ω-conotoxin SVIB, an N-type Ca^{2+}channel blocker, ω-agatoxin TK and ω-agatoxin IVA, P-type Ca^{2+} channel blockers, and ω-conotoxin MVIIC, a Q-type Ca^{2+} channel blocker, also had no effect on the toxin-induced Ca^{2+} entry and hemolysis (Table 2). We reported that phosphatidylcholine hydrolyzed by the toxin in liposomes induces CF leakage from liposomes [19]. As shown in Table 2, the blockers used did not inhibit the toxin-induced CF leakage from liposomes, showing that they had no effect on the hydrolysis of phosphatidylcholine in the bilayer by enzymatic activity of the toxin.

Table 2
Effect of various Ca^{2+} channel blockers on alpha-toxin-induced $^{45}Ca^{2+}$ uptake and CF release from liposomes

Inhibitor	Type	Hemolysis[a] (%)	$^{45}Ca^{2+}$ uptake[b] (cpm)	CF-release[c] (%)
Control (Alpha-toxin alone)	–	95 ± 2	1218 ± 164	95 ± 3
100 μM Nifedipine	L	102 ± 4	1265 ± 121	103 ± 4
100 μM Diltiazem	L	107 ± 5	1223 ± 89	116 ± 7
100 μM Verapamil	L	102 ± 3	1031 ± 112	102 ± 3
200 nM ω-conotoxin SVIB	N	103 ± 3	1268 ± 91	97 ± 4
200 nM ω-Agatoxin TK	P	99 ± 4	1242 ± 103	101 ± 3
200 nM ω-Agatoxin IVA	P	108 ± 3	1216 ± 97	98 ± 2
200 nM ω-conotoxin MVIIC	Q	103 ± 2	1280 ± 110	97 ± 4
50 μM Flunarizine	T	8 ± 1*	150 ± 10*	102 ± 4
50 μM Tetrandrine	L, T	29 ± 2*	540 ± 14*	105 ± 3

Values represent means ± S.E. for four to five experiments.

*$P < 0.01$, compared with the value induced by the toxin alone (control).

[a] Horse erythrocytes were incubated with 1 μg/ml of alpha-toxin and various Ca^{2+} channel blockers at 37 °C for 30 min and then 4 °C for 10 min. Hemolysis was evaluated as described in Materials and methods.

[b] Quin 2-loaded horse erythrocytes were suspended in TBS containing $^{45}Ca^{2+}$. After 30 min equilibration, the erythrocytes were incubated with 1 μg/ml of alpha-toxin and various Ca^{2+} channel blockers at 37 °C for 30 min, then washed and assayed for radioactivity.

[c] CF-loaded phosphatidylcholine–cholesterol liposomes were incubated with 1 μg/ml of alpha-toxin in the presence of various Ca^{2+} channel blockers at 37 °C for 30 min. The toxin-induced CF-release was determined by measuring the fluorescence intensity.

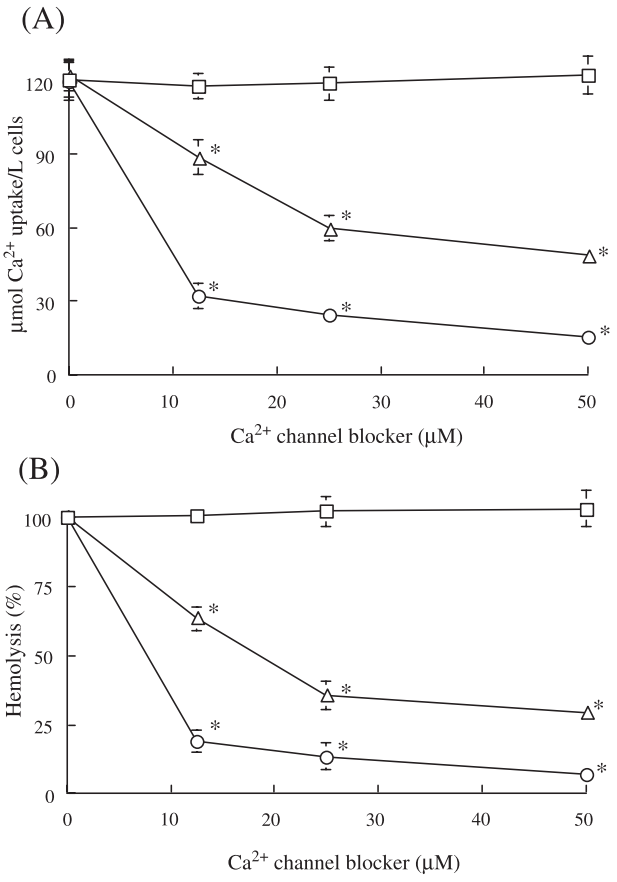

Fig. 5. Effect of various Ca^{2+} channel blockers on alpha-toxin-induced $^{45}Ca^{2+}$ uptake and hemolysis in horse erythrocytes. (A) Quin 2-loaded horse erythrocytes were incubated with 1.0 μg/ml alpha-toxin in TBS and $^{45}Ca^{2+}$ in the absence or presence of various concentrations of Ca^{2+} channel blocker (□, Nifedipine; △, Tetrandrine; ○, Flunarizine) at 37 °C for 30 min, then the erythrocytes were washed and assayed for radioactivity as described in Materials and methods. (B) Horse erythrocytes were incubated with 1.0 μg/ml alpha-toxin in TBS in the absence or presence of various concentrations of Ca^{2+} channel blocker (□, Nifedipine; △, Tetrandrine; ○, Flunarizine). Hemolysis was determined as described in Materials and methods. Values are means ± S.E. for five to six experiments. *$P < 0.05$, compared with the value induced by the toxin alone.

4. Discussion

The X-ray crystallographic structure of alpha-toxin has been solved [25]. Alpha-toxin is composed of two domains:

the N-terminal domain containing an active site for phospholipase C, and the C-terminal domain analogous to the eukaryotic Ca^{2+} and membrane-binding C2 domains [25–27]. It has been reported that the enzymatic activity of the N-domain is potentiated by a smaller C-terminal domain [26,28]. Ca^{2+} is known to be essential for the binding of the toxin to erythrocytes [13]. From crystallographic analysis of the toxin, Naylor et al. [27] showed that the C-terminal domain of the toxin contains three Ca^{2+} binding sites which are close together, in an approximately straight line, along the putative membrane-binding surface. They speculated that the binding of Ca^{2+} to the C-terminal domain become the cause which forms the active form of the toxin [27]. Therefore, these findings permit us to speculate that the toxin binds to the target cell membrane through Ca^{2+} bound to the C-terminal domain.

We have reported that entry of Ca^{2+} is induced in response to the toxin in rat ileum [10] or aorta [11]. The data presented here show that an influx of Ca^{2+} induced by the toxin is required for the toxin-induced hemolysis of horse erythrocytes and production of diacylglycerol and phosphorylcholine. First, A23187, a Ca^{2+} ionophore, elevated the sensitivity of horse erythrocytes to the toxin and production of diacylglycerol and phosphorylcholine in the presence of Ca^{2+}. In addition, horse erythrocytes treated with BAPTA-AM, a Ca^{2+} chelating agent, were resistant to the action of the toxin, but the cells treated with BAPTA were not. Tiffert and Lew [29] reported that the loading of free chelator (BAPTA) could be accomplished by incubating intact erythrocytes with the acetoxymethyl ester forms (BAPTA-AM) and by using hydrolysis of a membrane-permeant ester. Furthermore, Lew et al. [30] showed that BAPTA non-disruptively loaded into intact erythrocytes uniformly distributes throughout the cytosol and chelates intracellular Ca^{2+} [29]. Thus, these reports support that the chelating of Ca^{2+} in the cytosol of erythrocytes inhibited the toxin-induced hemolysis of horse erythrocytes and production of diacylglycerol and phosphorylcholine. It therefore appears that Ca^{2+} in the cytosol is essential for the toxin-induced events. Second, the toxin induced an increase in intracellular $^{45}Ca^{2+}$ in the cells preloaded with Quin 2-AM. Moreover, it promoted an increase in Fura 2 fluorescence in the resealed cells containing Fura 2. These observations show that the toxin induces Ca^{2+} influx into the cells. However, no Ca^{2+} influx was detected in the cells in the presence or absence of BAPTA and Quin 2, an impermeable Ca^{2+} chelating agent. Lew et al. [31] reported that Ca^{2+} fluxes could not be measured in erythrocytes without loading a Ca^{2+} chelator or artificially increasing the Ca^{2+} pool (e.g. ionophore treatment) because of ATP-dependent Ca^{2+} extrusion in the erythrocyte membranes [31–33]. It therefore appears that the influxed Ca^{2+} was rapidly removed from the cytosol by Ca^{2+}-ATPase in horse erythrocyte membrane. In addition, there are evidences that addition of pyruvate and/or glucose resulted in no effect on the toxin-induced $^{45}Ca^{2+}$ uptake in Quin 2-loaded erythrocytes and that treatment of the cells with BAPTA-AM had no effect on ATP level in the cells. These observations show that the toxin activates Ca^{2+} channel in membrane. Finally, the toxin-induced Ca^{2+} influx and hemolysis were inhibited by flunarizine and tetrandrine (L- and T-type Ca^{2+} channel blockers), but not by L-type Ca^{2+} channel blockers (nifedipine, diltiazem and verapamil) and other Ca^{2+} channel blockers. The pharmacology of T-type Ca^{2+} channels is complicated by many factors because the channels are rarely expressed alone and typically at low densities [34]. There are no compounds that are highly sensitive to a T-type Ca^{2+} channel [34]. Flunarizine was reported to inhibit both L- and T-type Ca^{2+} channels [35]. On the other hand, Akaike et al. [36] reported that flunarizine was most potent at blocking the T-type current in hypothalamic neurons. Therefore, the toxin-induced Ca^{2+} uptake seems to be mediated by T-type Ca^{2+} channels.

Horse erythrocytes contain large amounts of phosphatidylcholine [8]. Incubation of the cells with the toxin resulted in production of diacylglycerol and phosphorylcholine, showing that the toxin induces hydrolysis of phosphatidylcholine in the cells. The hydrolysis was dependent on the toxin-induced Ca^{2+} influx into the cells. Ca^{2+} has been reported to affect phospholipid turnover in human erythrocytes [15,16]. These findings suggest that endogenous PLC is activated by the influx of Ca^{2+} induced by the toxin in horse erythrocytes. We reported that alpha-toxin stimulated Ca^{2+} uptake into isolated rat ileum and activated phospholipid metabolism [10]. Furthermore, we have reported that the toxin activates endogenous PLC and PLD in rabbit erythrocyte membrane, and that the activation is closely associated with hemolysis [6,37]. Alpha-toxin is known to bind to biological membranes, but not to enter the cells. Incubation of the BAPTA-loaded erythrocytes, the flunarizine- or the tetrandrine-treated erythrocytes with the toxin resulted in a significant reduction in formation of phosphorylcholine and hemolysis, suggesting that endogenous PLC activity, which is related to the hemolysis, is dependent on Ca^{2+} influx mediated through a T-type Ca^{2+} channel activated by the toxin.

We can not explain the mechanism of the toxin-induced Ca^{2+} entry. However, H148G (variant alpha-toxin), which binds to the cells, but does not hydrolyze phosphatidylcholine in membrane, did not induce Ca^{2+} entry into the cells (data not shown). It therefore appears that hydrolysis of phosphatidylcholine in membrane by the toxin plays an important role in open of Ca^{2+} channel. The elevation of intracellular Ca^{2+} elicited by the toxin is important for the toxin-induced hemolysis, as mentioned above. On the other hand, A23187 at the concentration of 2.0 mM induced hemolysis in the presence of the sub-hemolytic dose of the toxin, but did not hemolysis in the absence of the toxin (Table 1). The observation suggests that the elevation of intracellular Ca^{2+} alone does not induce lysis of the cells under our experimental condition. From these findings, it appears that the event is due to a combination of elevation of intracellular Ca^{2+} and the action of the toxin.

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