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Role of Phospholipid Metabolism and G Protein in the Action Induced by *Clostridium Perfringens* Alpha-Toxin

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Abstract

Alpha-toxin (370 residues) of *Clostridium perfringens* is the key virulence determinant in gas gangrene and has also been implicated in the pathogenesis of sudden death syndrome in young animals. Alpha-toxin possesses phospholipase C (PLC), sphingomyelinase (SMase) and biological activities causing hemolysis and lethality. The structure of the toxin reveals two domains: the N-terminal domain containing the catalytic active site and the C-terminal domain involving the binding to membranes. Recent research data showed that alpha-toxin-induced biological activities are responsible for the activation of phospholipid metabolism via a pertussis toxin (PT)-sensitive GTP-binding protein, Gi. In this review, we summary the role of phospholipid metabolism and G protein in the biological activities induced by alpha-toxin. Discussed are activations of the arachidonic acid cascade (Section 1), the phospholipid metabolism (Section 2), the sphingomeylin metabolism (Section 3) and TrkA signaling (Section 4) induced by alpha-toxin.

Keywords: *C. perfringens* alpha-toxin; Phospholipase C; Sphingomyelinase; Diacylglycerol; Ceramide

Abbreviations: AA: Arachidonic Acid; CF: Caboxyfluorescein; DAG: Diacylglycerol; PA: Phosphatidic Acid; PC: Phosphatidyl Choline; PI: Phosphatidyl Inositol; PLC: Phospholipase C; PT: Pertusis-Toxin; S1P: Sphingosine 1-Phosphate; SMase: Sphingomyelinase; *T*m: Phase Transition temperature; TXA_2: Thromboxane A_2

Introduction

Clostridium perfringens produces alpha-toxin, which is an important virulence factor in gas gangrene [1-3]. Alpha-toxin is hemolytic, dermonecrotic, and lethal. Furthermore, it has phospholipase C (PLC) and sphingomyelinase (SMase) activities [1-3]. The toxin has been shown to damage the membranes of various mammalian cells [1-3] as well as artificial membranes [4]. The structure of alpha-toxin shows two domains, the N-domain (residues 1-250) contains the catalytic active site and the C-domain (residues 251-370) responsible for the binding to membranes (Figure 1) [5]. The gene encoding alpha-toxin [6], *Bacillus cereus* PLC (BCPLC) [7], and PLCs from *C. bifermentans* [8] and *Listeria monocytogenes* [9] have been isolated and their nucleotide sequences were determined. The results show that the deduced amino acid sequences of alpha-toxin and these enzymes exhibit significant homology up to approximately 250 residues from the N-terminus. From these findings, alpha-toxin was found to belong to the PLC family. The C-domain is similar to the C2 domain of intracellular eukaryotic proteins involved in vesicular transport and signal transduction [10,11].

We reported that alpha-toxin has two tightly bound zinc metals and an exchangeable divalent cation: residues His-68, -126 and -136 bind an exchangeable divalent cation required for binding to membranes, His-148 and Glu-152 bind one zinc ion essential for the active site of the toxin, and His-11 and Asp-130 tightly bind the other zinc ion required for maintenance of the structure [12,13]. We also reported that Tyr-57 and –65 plays a role in the penetration of the toxin into the bilayer of membranes and access of the catalytic site to sphingomyelin in membranes, but do not participate in the enzymatic activity (Figure 1) [14].

Alpha-toxin induced the leakage of caboxyfluorescein (CF) from liposomes composed of cholesterol and phosphatidylcholine (PC) containing unsaturated fatty acyl residues or shorter chains of saturated fatty acyl residues (12 and 14 carbon atoms), and the toxin-induced release of CF decreased as the chain of the acylresidues of PC increased in length [4]. Therefore, it is possible that the membrane-damaging effect of alpha-toxin on liposomes is related to the phase transition temperature (*T*m) of PC in PC-cholesterol liposomes [4]. We prepared various PCs (C18:0/C18:1) with an unsaturated bond in the *sn*-2 acyl chain. Differential scanning calorimetry showed that *T*m was minimal when the triple bond was positioned at C (9) in the *sn*-2 acyl chain. Our result shows that the binding to liposomes of the toxin and the alpha-toxin-induced release of CF from liposomes increased with a decrease in the *T*m of the PC in liposomes, suggesting that an increase in membrane fluidity promotes the binding of the toxin to liposomes

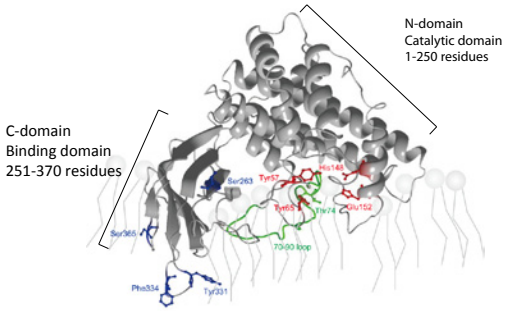

Figure 1: Structure of alpha-toxin.

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[15]. It appears that an increase in membrane fluidity results in the toxin being inserted into the bilayer of the membrane. Accordingly, it is concluded that the membrane-damaging action of alpha-toxin is closely related to the membrane fluidity of liposomes.

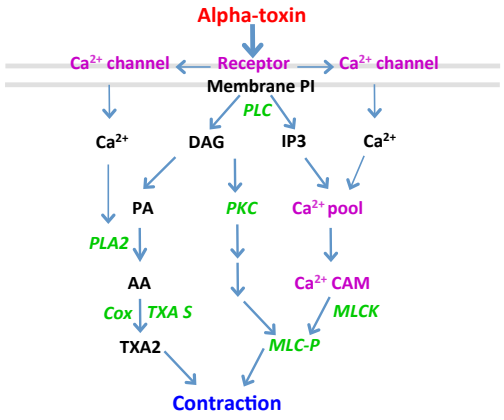
We have reported that the alpha-toxin-induced contraction of isolated rat aorta and ileum [16-18], and hemolysis of rabbit erythrocytes [19-21] are dependent on the activation of glycerophospholipid metabolism. We have also demonstrated that the alpha-toxin-induced hemolysis of sheep erythrocytes is dependent on the activation of the sphingomyelin metabolic system through GTP-binding proteins, especially the formation of sphingosine 1-phosphate [22,23].Therefore, the toxin-induced activation of phospholipid metabolism through G protein is closely involved in the biological activities. In the present review, we demonstrate the relationship between phospholipid metabolism via G protein and the biological activities induced by alpha-toxin.

Activation of the arachidonic acid cascade by alpha-toxin

Alpha-toxin potentiated contractions of the isolated rat vas deferens elicited by noradrenaline [24], and caused a rise in blood pressure with a decrease in blood flow [25]. The toxin caused contractions of the isolated rat aorta in a dose-dependent manner [16]. The response to repeated doses of the toxin was found to be tachyphylactic. The toxin-induced contractions were not inhibited by an alpha-adrenoreceptor blocking agent (phentolamine), a histamine receptor blocking agent (chlorpheniramine), or a muscarinic receptor blocking agent (atropine). The response to the toxin therefore seems to be the result of a direct effect on the aorta. Alpha-toxin-induced contractions were inhibited by Ca channel blockers such as verapamil and nifedipine [16]. Furthermore, the toxin-induced contractionswere not observed in Ca-free medium. The data indicate that the direct action of the toxin is mainly due to an increase in Ca permeability across the smooth muscle cell membranes [16]. Labelling by ^{32}P phosphate of phosphatidylinositol (PI) and phosphatidic acid (PA) in fragments of the aorta was enhanced by alpha-toxin [16]. Therefore, Ca influx caused by the toxin is thought to be associated with the stimulation of phospholipid metabolism. On the other hand, it is possible that the increased turnover of phospholipids caused by the toxin stimulates the breakdown of PI into diacylglycerol (DAG) and inositolphosphates. Alpha-toxin-induced contractions were significantly inhibited by cyclo-oxygenase inhibitors such as indomethacin and aspirin [17]. Furthermore, the toxin was found to stimulate release of arachidonic acid (AA), indicating that stimulation of AA release relates to activation of phospholipid metabolism by the toxin. The release of AA caused by the toxin was greater in the presence than absence of indomethacin, suggesting that AA is mainly metabolized to cyclo-oxygenase products, indicating the toxin-induced contraction to be associated with cyclo-oxygenase product(s) metabolized from AA [17]. The toxin-evoked contraction and AA release were unaffected by the phospholipase A_2 inhibitor quinacrine. Therefore, the release of AA seems to be due to the hydrolysis of diacylglycerol by diacylglycerol lipase. The thromboxane synthetase inhibitor OKY-046 and thromboxane A_2 (TXA_2) antagonist ONO-3708 were found to inhibit contractions of the aorta produced by the toxin. OKY-046 and indomethacin blocked the production of TXA_2 caused by the toxin. TXA_2 is produced enzymatically by endothelial cells of rabbit, bovine and human umbilical blood vessels [17]. The toxin did not induce contractions after treatment of the isolated aorta with collagenase or rubbing of the tissue to remove endothelial cells from the intimal surface of the tissue [17]. Thus, the toxin-induced contractions of the isolated rat aorta associated with the production of TXA_2 required an intact endothelium. Furthermore, TMB-8 (intracellular Ca^{2+} antagonist), trifluoperazine, W-7(calmodulin inhibitor) and H-7 (PKC inhibitor) significantly blocked the toxin-induced contractions [16]. From these observations, in endothelial cells of the rat aorta, alpha-toxin stimulates phosphotidylinositol metabolism and arachidonic acid release, leading to the production of TXA_2 and the phosphorylation of myosin light chain which then elicits contractions of the adjacent aorta smooth muscle (Figure 2).

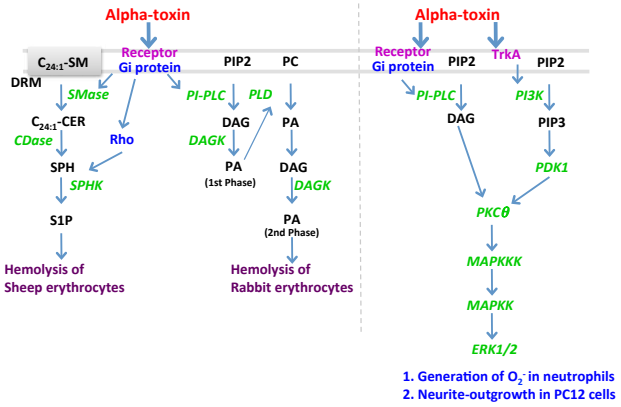
Activation of phosholipid metabolism through GTP-binding protein induced by alpha-toxin in rabbit erythrocytes

Alpha-toxin induces hot-cold hemolysis of rabbit erythrocytes. The toxin induced production of PA in a dose-dependent manner when incubated with erythrocytes at 37°C. We have reported that incubation of rabbit erythrocyte membranes with the toxin results in biphasic production of PA [19] (Figure 3). When erythrocyte membranes are incubated with the toxin in the presence of [$\gamma^{32}P$]ATP at 37°C, the formation of [^{32}P] phosphatidic acid (PA) is biphasic, the first phase lasting 30s and the second phase, c. 20 min [19]. 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 (IP_3) and decrease of phosphatidylinositol-4,5-bisphosphate (PIP_2) within 30 s.The formation of PA in the first and secondphases was stimulated by AlF_4^- and/or GTP [γS] [19]. The former was inhibited by phorbol ester and stimulated by protein kinase C inhibitor; however, these agents had no effect on thesecond phase [20]. It therefore seems that thetoxin-induced formation of PA in the first phase is stimulated by endogenous PLC which is activated by G-protein and is inhibited by protein kinase C, and the formation of PA inthe second phase is stimulated by endogenous phospholipase D which is activated by G-protein, but is not controlled by protein kinaseC [20]. Treatment of rabbit erythrocytes simultaneously with neomycin resulted in inhibition of the toxin-induced formation of PA and hemolysis. Furthermore, GTP [γS] stimulated the toxin-induced formation of PA and hemolysis, and GDP [βS] inhibited them in a dose-dependent manner [21]. Accordingly, it seems likely that the toxin-induced formation of PA is tightly linked to hemolysis elicited by the toxin. Enzymatic activities of alpha-toxin are essential for hemolysis [21]. We have reported that the PLC activity of the toxin plays a role in



Abbreviations: PI: Phosphatidylinositol; PLC: Phospholipase C; DAG: Diacylglycerol, IP3: Inositol Trisphosphate; PA: Phosphatidic Acid; PKC: Protein Kinase C; AA: Arachidonic Acid; CAM: Calmodulin; PLA2: Phospholipase A_2; COX: Cyclooxygenase; TXA S: Thromboxane A_2 Synthetase; TXA2: Thromboxane A_2; MLCK: Myosin Light Chain Kinase; MLC-P: Phosphorylated MLC

Figure 2: Mechanism of aortic contraction induced by alpha-toxin.



Abbreviations: SM: Sphingomyelin; PIP2: Phosphatidylinositol 4,5-Bisphosphate; PI-PLC: Phosphatidylinositol Phospholipase C; DAG: Diacylglycerol, PC: Phosphatidyl Choline; SMase: Sphingomyelinase; PLD: Phospholipase D; PA: Phosphatidic Acid; PKCθ: Protein Kinase C θ; PI3K: Phosphatidylinositol 3-Kinase; PIP3: Phosphatidylinositol 3,4,5-Trisphosphate; CDase: Ceramidase; CER: Ceramide; SPH: Sphingosine; S1P: Sphingosine 1-Phosphate; DAGK: DAG kinase; PDK1: Phosphatidylinositide Dependent Kinase 1; DRM: Detergent-Resistant Membrane; TrkA: Neurotrophic Tyrosine Kinase Receptor Type1; MAPKK: Mitogen-Activated Protein Kinase Kinase; Erk1/2: Extracellular Signal-Regulated Kinase ½

Figure 3: Relationship between phospholipid metabolism and biological activities of alpha-toxin.

the activation of endogenous PIP_2-specific PLC in rabbit erythrocytes, which stimulates the glycerophospholipid system in the membrane, and that activation of the system leads to hemolysis of the erythrocytes [21]. The mechanism of the formation of PA is thought to be as shown in Figure 3. First, hydrolysis of membrane phospholipids by PLC activity of the toxin causes activation of G-protein. Second, activation of endogenous PI-PLC and PLD via G-protein activated by the toxin results in stimulation of PA formation, which leads to hemolysis of rabbit erythrocytes. These results demonstrate that the toxin-induced hemolysis is due to activation of phospholipid metabolism systems through GTP-binding proteins.

Activation of sphingomyelin metabolism through GTP-binding protein induced by alpha-toxin in rabbit erythrocytes

Alpha-toxin induces hemolysis of rabbit erythrocytes through the activation of glycerophospholipid metabolism [19-21]. Sheep erythrocytes contain large amounts of sphingomyelin (SM) but not PC. Alpha-toxin simultaneously induced hemolysis and a reduction in the levels of SM in the membrane and an increase in ceramide and sphingosine 1-phosphate (S1P) [22]. It seemed that the toxin-induced hemolysis of sheep erythrocytes is closely related to the hydrolysis of SM in the membrane by the SMase activity of alpha-toxin. Moreover, the level of phosphorylcholine markedly increased in the cells treated with the toxin, compared with that of ceramide. PC was not detected in sheep erythrocytes. These results show that the phosphorylcholine released is mostly derived from SM. The levels of phosphorylcholine released by treatment with the toxin were significantly higher than those of ceramide, suggesting that ceramide is rapidly metabolized to sphingosine. However, a markedly lower level of sphingosine, compared with S1P, was detected, although S1P increased with an increase in the dose of the toxin. It therefore is likely that sphingosine is rapidly metabolized to S1P in the cells treated with the toxin [22]. N-Oleoylethanolamine, a ceramidase inhibitor, inhibited the toxin-induced hemolysis and caused ceramide to accumulate in the toxin-treated cells. The data show that the agent specifically suppresses ceramidase to block hemolysis. Furthermore, DL-threo-dihydrosphingosine and B-5354c, isolated from a novel marine bacterium, both sphingosine kinase inhibitors, blocked the toxin-induced hemolysis and production of S1P and caused sphingosine to accumulate. S1P potentiated the toxin-induced hemolysis of saponin-permeabilized erythrocytes but had no effect on that of intact cells suggesting that intracellular S1P is important for the toxin-induced hemolysis and that the hemolytic effect is not dependent on the action of S1P outside the cells. These observations indicate that S1P plays an intracellular role in the toxin-induced hemolysis, not an extracellular one, suggesting that it functions as a second messenger in the process. S1P itself caused no hemolysis of saponin-permeabilized cells, suggesting that hemolysis may be induced by a combination of S1P and other events elicited by the toxin in the cells [22] (Figure 3). GTP[γS] stimulated alpha-toxin-induced hemolysis, hydrolysis of SM, and formation of S1P in sheep erythrocytes. It therefore appears that the activation of GTP-binding proteins is required in the toxin-activated SM metabolic system. Preincubation of lysated sheep erythrocytes with pertussis toxin blocked the alpha-toxin-induced formation of ceramide from SM, suggesting that alpha-toxin activates endogenous SMase through a pertussis toxin-sensitive G_i type GTP-binding protein. In addition, incubation of *C. botulinum* C3 exoenzyme-treated lysates of sheep erythrocytes with alpha-toxin caused an accumulation of sphingosine and inhibition of the formation of S1P, indicating that sphingosine kinase is controlled by Rho (small molecular GTP-binding protein) in the cells. Therefore, it appears that Rho activated directly or indirectly by alpha-toxin stimulates sphingosine kinase. These observations suggest that the alpha-toxin-induced hemolysis of sheep erythrocytes is dependent on the activation of the SM metabolic system through GTP-binding proteins, especially the formation of S1P [23].The level of $C_{24:1}$-ceramide was highest among the ceramides with an unsaturated bond in the fatty acyl chain in the detergent-resistant membranes (DRMs). The toxin specifically bound to DRMs, resulting in the hydrolysis of N-nervonoic sphingomyelin ($C_{24:1}$-SM) in DRMs [23] (Figure 3). Treatment of the cells with pertussis toxin (PT) inhibited the alpha-toxin-induced formation of $C_{24:1}$-ceramide from $C_{24:1}$-SM in DRMs and hemolysis, indicating that endogenous sphingomyelinase, which hydrolyzes $C_{24:1}$-SM to $C_{24:1}$-ceramide is controlled by a PT-sensitive GTP-binding protein in membranes. In summary, alpha-toxin hydrolyzes the unsaturated SM, especially $C_{24:1}$-SM, by activating endogenous SMase through Gi in the DRMs of sheep erythrocyte membranes, indicating that alpha-toxin activates endogenous SMase specific for $C_{24:1}$-SM. $C_{24:1}$-ceramides are rapidly metabolized to sphingosine in the cells treated with the toxin, implying that the toxin activates ceramidases which selectively recognize unsaturated ceramides as a substrate [23]. These results show that the toxin-induced metabolism of $C_{24:1}$-SM to S1P in DRMs plays an important role in the toxin-induced hemolysis of sheep erythrocytes (Figure 3).

Activation of TrkA signaling through GTP-binding protein induced by alpha-toxin

Alpha-toxin stimulated adhesion to the matrix and the generation of O_2^- in rabbit neutrophils due to the formation of DAG through activation ofendogenous PLC by a PT-sensitive GTP-binding protein [26,27]. Treatment of the cells with the toxin resulted in tyrosine phosphorylation of TrkA (nerve grown factor high-affinity receptor) [27]. Anti-TrkA antibody inhibited the productionof O_2^- and binding of the toxin to the TrkA. It has been reported that the phosphatidylinositol 3-kinase (PI3K) signaling pathway has an important role in several

effector functions including the generation of O_2^- [28]. PI3K is known to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is recognized by a pleckstrin homology domain identified as a specialized lipid-binding module [29]. Several studies have reported that 3-phosphoinositide-dependent proteinkinase 1 (PDK1) requires PIP_3 as its activator for effective catalytic activity [29]. The toxin induced phosphorylation of PDK1. K252a, an inhibitor of the TrkA receptor and LY294002, an inhibitor of PI3K, reduced the toxin-induced production of O_2^- and phosphorylation of PDK1, but not the formation of DAG. The result shows that the toxin-induced activation of PI3K occurs upstream of the phosphorylation of PDK1, which is an important step in the toxin-induced generation of O_2^- [27]. It is likely that the toxin-induced phosphorylation of PDK1 is a process independent of the toxin-induced formation of DAG. Alpha-toxin induced phosphorylation of PKCθ and PKCζ/λ, and the generation of O_2^- induced by the toxin was inhibited by rottlerin and calphostin C, an inhibitor of PKCθ. We reported that the formation of DAG induced by alpha-toxin in rabbit neutrophils plays an important role in the generation of O_2^- [26]. K252a and LY294002 inhibited the toxin-induced phosphorylation of protein kinase Cθ (PKCθ). It therefore appears that the toxin-induced generation of O_2^- is dependent on the activation of PKCθ, through binding of PKCθ phosphorylated by PDK1 to DAG [27]. U73122, a PLC inhibitor, and pertussis toxin inhibited the toxin-induced generation of O_2^- and formationof DAG, but not the phosphorylation of PDK1. These observations show that the toxin independently induces production of DAG throughactivation of endogenous PLC and phosphorylation of PDK1 via the TrkA receptor signaling pathway and that these events synergistically activate PKCθ in stimulating an increase in O_2^- [27]. In addition, several studies have reported that the activation of PKC by various stimuli results in the generation of O_2^- via the activation of mitogen-activated protein kinase (MAPK) systems [30-32]. The toxin causes phosphorylation of ERK1/2, but not p38 and SAPK/JNK, implying that the process is dependent on a MAPK system containing MEK1/2 and MAPK/ERK1/2, but not systems containing p38 and SAPK/JNK. We showed the participation of MAPK-associated signaling events via activation of PKCθ in the toxin-induced generation of O_2^-. In conclusion, we demonstrated that alpha-toxin induces formation of DAG through the activation of endogenous PLC by a PT-sensitive GTP-binding protein and phosphorylation of PDK1 via stimulation of the TrkA receptor, so that DAG and PDK1 synergistically activate PKCθ, and that the activation of PKCθ stimulates generation of O_2^- through MAPK-associated signaling events in rabbit neutrophils [27] (Figure 3). To clarify the mechanism responsible for the toxin-induced activation of TrkA, the effect of alpha-toxin on TrkA in PC12 cells, a model for studying the differentiation of neuronal cells in response to TrkA [33], was investigated. The toxin induced neurite-outgrowth and phosphorylation of TrkA in the cells in a dose-dependent manner [34]. TrkA inhibitor K252a and shRNA for TrkA inhibited the toxin-induced neurite-outgrowth, and phosphorylation of TrkA and ERK1/2. Furthermore, the binding of the toxin to PC12 cells transfected with TrkA-specific shRNA vectors decreased, compared with that to the intact cells, suggesting that the binding of the toxin to TrkA and the activation of TrkA are required for neuritogenesis. Nerve growth factor (NGF), which binds to TrkA, is reported to be required for the differentiation and survival of nerve cells [35]. Several studies have reported that neurite-outgrowth induced by NGF is dependent on the activation of MAPK systems [36-38]. Alpha-toxin caused the phosphorylation of ERK1/2, but not that of p38 and SAPK/JNK, and PD98059, an inhibitor of Erk1/2 cascade, attenuated the neurite-outgrowth and the phosphorylation of ERK1/2 induced by alpha-toxin. K252a inhibited the phosphorylation of ERK1/2. These results showed that the toxin-induced neurite-outgrowth is dependent on activation of the ERK1/2 pathway via TrkA [34]. The wild-type toxin induced the formation of DAG, and neurite-outgrowth, but H148G, a variant toxin which binds to cell membranes and has lost the enzymatic activity, did not. Therefore, it appears that the phospholipid metabolism caused by the enzymatic activity of alpha-toxin plays an important role in the activation of TrkA-ERK1/2 signal transduction and neurite-outgrowth. We demonstrated that the phosphorylation of TrkA through the phospholipid metabolism induced by the toxin synergistically plays a key role in neurite-outgrowth [34] (Figure 3).

Conclusion

C. perfringens alpha-toxin is an important agent of gas gangrene. Alpha-toxin induces the hemolysis of rabbit erythrocytes and the generation of superoxide anion in rabbit neutrophils through the activation of endogenous PLC via a PT-sensitive GTP-binding protein, Gi. On the other hand, the toxin also induces the hemolysis of sheep erythrocytes through the activation of endogenous SMase via Gi and Rho. The enzymatic activity of the toxin is essential for activation of phospholipid metabolism via G protein.

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