J. Biochem. **136**, 569–574 (2004)
DOI: 10.1093/jb/mvh161

Clostridium perfringens Alpha-Toxin: Characterization and Mode of Action

Jun Sakurai*, Masahiro Nagahama and Masataka Oda

Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514

Received July 16, 2004; accepted August 25, 2004

***Clostridium perfringens* type A strains that produce alpha-toxin cause gas gangrene, which is a life-threatening infection with fever, pain, edema, myonecrosis and gas production. Intramuscular injection of the toxin or *Bacillus subtilis* carrying the alpha-toxin gene causes myonecrosis and produces histopathological features of the disease. Immunization of mice with alpha-toxin or fragments of the toxin prevents gas gangrene caused by *C. perfringens*. The toxin possesses phospholipase C (PLC), sphingomyelinase (SMase) and biological activities causing hemolysis, lethality and dermonecrosis. These biological activities are closely related to PLC and/or SMase activities. However, there is yet some uncertainty about the biological activities induced by the PLC and SMase activities of alpha-toxin. Based on the isolation and characterization of the gene for alpha-toxin and a comparison of the toxin with enzymes of the PLC family, significant progress has been made in determining the function-structure of alpha-toxin and the mode of action of the toxin. To provide a better understanding of the role of alpha-toxin in tissue damage in gas gangrene, this article summarizes current knowledge of the characteristics and mode of action of alpha-toxin.**

Key words: *Clostridium perfringens* alpha-toxin, phospholipase C, sphingomyelinase, phospholipase C family, signal transduction.

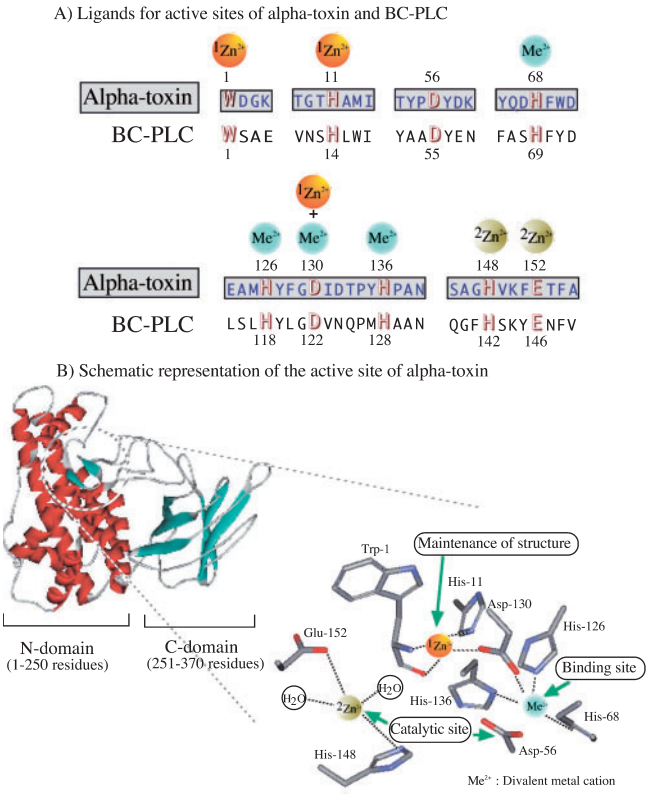
1. Characterization of alpha-toxin

The genes encoding alpha-toxin (*1*), *Bacillus cereus* PLC (BC-PLC) (*2*), and PLCs from *C. bifermentans* (*3*) and *Listeria monocytogenes* (*4*) 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. Alpha-toxin has an additional C-terminal domain (120 residues). From these findings, alpha-toxin was found to belong to the PLC family (*5*). BC-PLC has two tightly bound and one loosely bound zinc ions (*6*, *7*). On the basis of crystallographic data and the structure-function relationship of BC-PLC (*6*), a site-directed mutagenesis analysis was performed that revealed the relationship between the amino acid residues and zinc ions of alpha-toxin and its biological activity, as shown in Figure 1A; one zinc ion is tightly coordinated with His-11 and Asp-130, a second is coordinated tightly with His-148 and loosely with Glu-152, and a divalent cation is loosely associated with His-68, -126, -136 and Asp-130 (*8*, *9*). Asp-56 is essential for catalytic activity (*10*), so it is concluded that the catalytic site of the toxin is located in the N-terminal domain.

A crystallographic study of alpha-toxin revealed that the structure is divided into two domains (*11*): the N-domain, consisting of nine tightly packed α-helices, and the C-domain, consisting of an eight-stranded antiparallel β-sandwich motif (Fig. 1B). This finding confirms that the N-domain has a structural topology similar to the entire BC-PLC (*6*) and contains three divalent cations containing zinc ions in the active site, and that amino acid residues involved in zinc-coordination are essential for the enzymatic activities. Mixing the individual N-domain and C-domain restores the hemolytic activity (*12*). This observation suggests that the C-domain affects the activity of the N-domain. Guillouard *et al.* (*13*) and Naylor *et al.* (*11*) reported that the fold of the C-domain is similar to those of the "C2" and "C2-like" domains, present in eukaryotic proteins involved in signal transduction, of eukaryotic phospholipid-binding proteins such as synaptotagmin. Guillouard *et al.* (*13*) reported that Asp-269 and -336 play a role in the specific interaction with calcium ions. Furthermore, Alape-Giron *et al.* (*14*) reported that Tyr-275, -307 and -331 residues are critical for binding of the toxin. It is likely that the C-domain plays a role in binding to membranes.

The toxin induces carboxyfluorescein (CF) leakage and phosphorylcholine release from liposomes composed of PC or SM. The toxin-induced CF release decreases with an increase in the phase transition temperature (*Tm*) of the PC used, indicating that the sensitivity of liposomes to the toxin correlates well with the *Tm* of the PC (*15*). Furthermore, both toxin binding to liposomes and the hydrolysis of PC in liposomes by the toxin are also related to the *Tm* of the PC in liposomes. Accordingly, the membrane-damaging action of the toxin appears to be closely related to membrane fluidity in liposomes. We have reported that acrylodan-labeled C-domain variants (S263C and S365C) bind to liposomes and exhibit a

*To whom correspondence should be addressed. Tel: +81-88-622-9611, Fax: +81-88-655-3051, E-mail: sakurai@ph.bunri-u.ac.jp



The figure is based on those presented in references (24,26,31).

Fig. 1. **The relationship between amino acid residues and metal ions in alpha-toxin and *Bacillus cereus* phospholipase C (BC-PLC).**

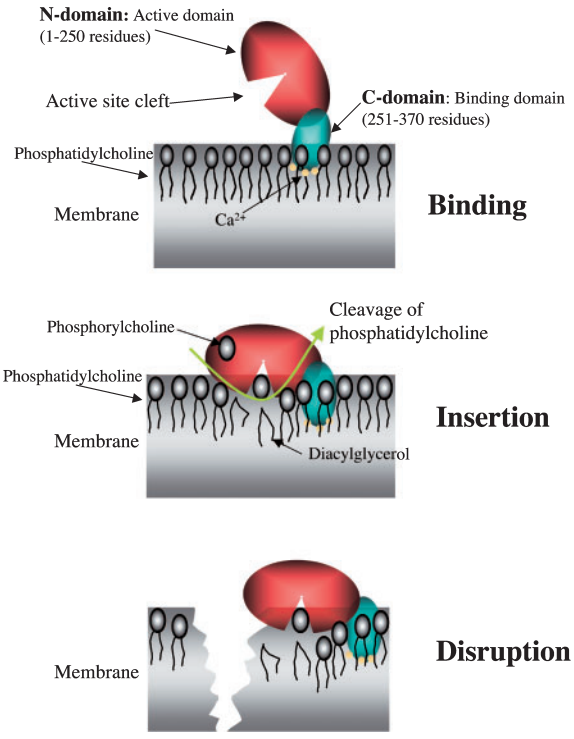


Fig. 2. **Action of alpha-toxin on artificial and biological membranes.**

marked blue shift, indicating internalization of the C-domain into the hydrophobic environment in liposomes (*12*). Given that the membrane-damaging action of alpha-toxin is dependent on membrane fluidity, invasion of the C-domain into the bilayer membrane may play an important role in its action (Fig. 2).

2. Action of alpha-toxin on cells and biological membranes

Alpha-toxin at high concentrations induces massive degradation of PC and SM in membranes, followed by membrane disruption. However, small amounts of toxin cause limited hydrolysis of PC and SM, generating diacylglycerol and ceramide, respectively. These events activate various signal transduction pathways, which lead to the uncontrolled production of several intercellular mediators as follows. The toxin causes contraction of isolated rat ileum and aorta tissue through the activation of phospholipid metabolism in membranes (*16*, *17*). Notably, the toxin activates the arachidonic acid cascade in isolated rat aorta (*18*). It was found that the toxin-induced contraction is related to the production of thromboxane A_2 from arachidonic acid. Later, similar results were reported for PLC produced by other microorganisms (*19*). Therefore, it is likely that bacterial PLC mimics the actions of endogenous PLC in eukaryotic cell membranes.

Alpha-toxin induces the production of intercellular mediators in endothelial cells, intercellular adhesion molecule 1, interleukin-8, TNF-α, platelet-activating factor and the endothelial leukocyte adhesion molecule (*20*). It appears that these events contribute to the increased vascular permeability and edema (*21*). The toxin induces translocation of the platelet fibrinogen receptor from internal stores to the membrane in platelets, inducing the formation of platelet/platelet aggregates (*22*, *23*). Furthermore, cells exposed to the toxin undergo morphological changes similar to those induced by exposure to TNF-α or IFN-γ (*20*).

Exposure of rabbit neutrophils to alpha-toxin induces firm adhesion of the cells to fibrinogen and fibronectin, and O_2^- production (*24*). Incubation of neutrophils or their cell lysates with alpha-toxin leads to the production of 1,2-diacylglycerol (DG) and phosphatidic acid (PA). The toxin-induced DG and PA formation precedes the toxin-induced adhesion and production of O_2^-. Pertussis toxin (PT) inhibits the toxin-induced formation of PA, adhesion to fibrinogen and O_2^- production. GTPγS enhances these events induced by the toxin, but GDPβS inhibits them. The toxin stimulates the phosphorylation of a protein with a molecular mass of about 40 kDa. In addition, treatment of the cells with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and phorbol-12, 13-dibutyrate stimulates cell adhesion, production of O_2^- and phosphorylation of a 40-kDa protein, but has no effect on the formation of PA. Furthermore, these events induced by a combination of OAG and PDBu are not inhibited by treatment with PT. protein kinase C (PKC) inhibitors, staurosporine and chelerythrine, block the toxin-induced adhesion, production of O_2^- and phosphorylation of the 40-kDa protein. These observations suggest that the toxin activates PKC *via* DG, which is produced by PLC activated by PT-sensi-

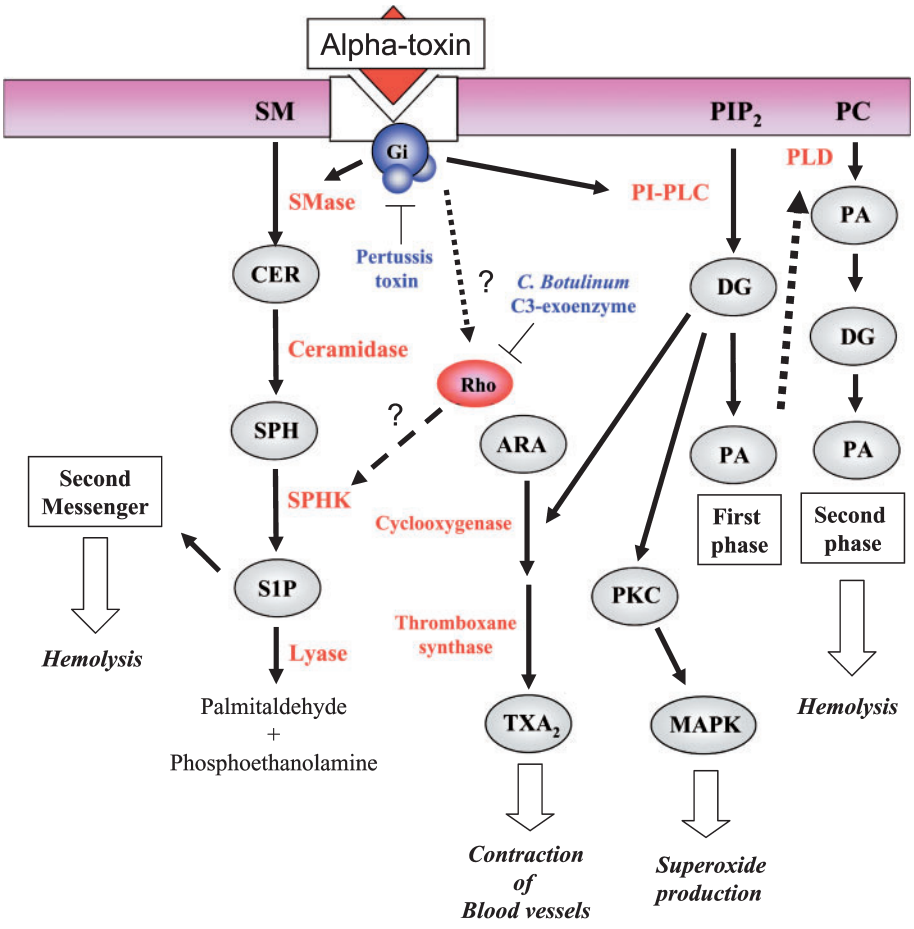


Fig. 3. **Signalling pathways for alpha-toxin-induced hemolysis, O_2^- production, and the contraction of isolated aorta.**

SM: sphingomyelin, CER: ceramide, SPH: sphingosine, S1P: sphingosine 1-phosphate, PC: phosphatidylcholine, PIP_2: phosphatidylinositol 4,5-biphosphate, DG: diacylglycerol, PA: phosphatidic acid, ARA: arachidonic acid, TXA_2: Thromboxane A_2, SMase: sphingomyelinase, SPHK: sphingosine kinase, PI-PLC: phosphatidylinositol phospholipase C, PKC: protein kinase C, PLD: phospholipase D, MAPK: mitogen-activated protein kinase.

tive GTP-binding protein. It, therefore, is likely that the alpha-toxin-stimulated adhesion to the matrix and O_2^- production are due to the activation of PKC in neutrophils (*25*). In addition, we find that the alpha-toxin-stimulated O_2^- production is dependent on the activation of the mitogen-activated protein kinase (MAPK) system *via* the activation of PKC (unpublished data).

The toxin induces hot-cold hemolysis of rabbit erythrocytes. When erythrocyte membranes are incubated with the toxin in the presence of [γ-^{32}P]ATP at 37°C, the formation of [^{32}P]PA is biphasic, the first phase being about 30 s long and the second phase about 20 min long (*26*). The formation of PA in the first phase is closely related to the generation of diacylglycerol and inositol 1,4,5-phosphate (IP_3) from phosphatidyl-inositol 4,5-bisphosphate (PIP_2) in membranes. The toxin can not hydrolyze PIP_2, indicating that a PIP_2-specific PLC is activated by treatment of the cells with the toxin. Furthermore, the formation of PA in the first and second phases is stimulated by AlF_4^- and/or GTP[γS]. It, therefore, seems that the toxin-induced PA formation in the first phase is due to the activation of an endogenous PIP_2-specific PLC by the GTP-binding protein. In the presence of ethanol, the late PA formation is inhibited and phosphatidylethanol production is stimulated, indicating that the formation of PA is due to the activation of endogenous phospholipase D (PLD). It, therefore, appears that the formation of PA in the second phase is related to the generation of DG *via* PA formed from the degradation of PC in membranes, as shown in Fig. 3. Furthermore, GTP[γS] stimulates PA formation and hemolysis, and GDP[βS] inhibits them in a dose-dependent manner. Accordingly, the toxin-induced formation of PA seems to be tightly linked to the toxin-elicited hemolysis. From these findings, it is likely that the hydrolysis of membrane phospholipids by the PLC activity of the toxin causes the activation of PT-sensitive GTP-binding. Second, phospholipid metabolism through the activation of endogenous PLC by a PT-sensitive GTP-binding protein results in the activation of endogenous PLD. These events seem to lead to hemolysis in rabbit erythrocytes (*27*, *28*) (Fig. 3).

Nelson (*29*) reported that in rabbit erythrocytes, PC constitutes approximately 34% of the total phospholipids, while in sheep erythrocytes, no PC is detected and SM accounts for about 50% of all phospholipids. Alpha-toxin also induces the hot-cold hemolysis of sheep erythrocytes.

Recently, activation of the SM cycle, analogous to the glycerophospholipid cycles, has been recognized as a key event in the signal transduction cascade involved in cellular proliferation, differentiation and apoptosis (*30*, *31*). Ceramide causes the arrest of cell growth and apoptosis (*30*, *31*). Sphingosine was found to be a potent inhibitor of protein kinase C (*32*), and to inhibit cell growth and induce apoptosis (*31*). Sphingosine 1-phosphate (S1P) has been reported to promote cell growth and inhibit apoptosis (*31*, *33*). Several studies have reported that bacterial SMases hydrolyze cell surface SM leading to increased levels of ceramide, mimicking the effects of the activation of endogenous neutral SMase induced by physiological stimuli (*34–36*). Olivera et al. reported that exogenous SMase induces the synthesis of DNA and potentiates the actions of known growth factors in Swiss 3T3 fibroblasts (*37*). The SM metabolites have been shown to play important roles in such fundamental biological processes (*30*).

Treatment of sheep erythrocyte lysates with the toxin stimulates the formation of DG and IP_3, suggesting that the toxin activates PIP_2-specific PLC in the cells. U73122, an endogenous PLC inhibitor, inhibits the toxin-induced formation of DG and IP_3 in a dose-dependent manner, but not toxin-induced hemolysis under the same conditions, suggesting that the toxin-induced hemolysis of sheep erythrocytes is independent of the toxin-activated PIP_2-specific PLC (*38*). Incubation of sheep erythrocytes with the toxin simultaneously induces hemolysis and a reduction in the levels of SM and formation of ceramide and S1P. Little sphingosine is detected in the toxin-treated cells. The extent of the hemolysis decreases as the dose of *N*-oleoylethanolamine, a ceramidase inhibitor, increases. The ceramide levels in the cells increase with an increase in the amount of the agent, suggesting that *N*-oleoylethanolamine specifically blocks the toxin-stimulated deamidation of ceramide to sphingosine, resulting in the accumulation of ceramide and suppression of hemolysis induced by the toxin. It, therefore, is likely that sphingosine is also rapidly metabolized by treatment with the toxin. Sphingosine is phosphorylated by sphingosine kinase. DL-*threo*-dihydrosphingosine and B-5354c, isolated from a novel marine bacterium (*39*), both sphingosine kinase inhibitors, block the toxin-induced hemolysis and production of S1P, and cause sphingosine to accumulate. These observations suggest that the toxin-induced activation of the SM metabolic system is closely related to hemolysis. The incubation of sheep erythrocytes permeabilized by saponin with a sub-hemolytic dose of the toxin in the presence of S1P promotes hemolysis with an increase in the dose of S1P. Moreover, incubation of the saponin-permeabilized cells or intact cells with S1P in the absence of the toxin results in no hemolysis, indicating that S1P itself does not induce hemolysis of sheep erythrocytes permeabilized by saponin and intact erythrocytes (*38*). It has been reported that the biological activities elicited by stimuli depend on S1P released from the cells. There have been several reports that S1P is produced in cells, is secreted, and then binds to endothelial differentiation gene (EDG) family receptors such as EDG-1, EDG-3 and AGR16/H218 on the surface of the cell (*40*). It has been reported that the binding of S1P to the receptors results in the activation of MAPK (*41*, *42*), inhibition of cAMP production (*43*, *44*), and release of Ca^{2+} (*45*). It has been reported that S1P stimulates the release of Ca^{2+} from intracellular stores (*46*), that it acts intracellulary to regulate the endothelial-signal regulated kinase1/2 (ERK1/2) pathway (*42*, *47*), and that it controls mitogenesis (*48*) and apoptosis (*49*, *50*). In addition, the inhibition of ERK1/2 activation in Swiss 3T3 fibroblasts (*51*) and Ca^{2+} signals in rat mast cells by DL-*threo*-dihydrosphingosine has been reported to support a second messenger role for S1P in biological events (*52*). Accordingly, S1P has been proposed to play a role in intracellular and extracellular actions (*53*). Pyne and Pyne (*40*) reported that the key question is whether S1P can function as an intracellular second messenger, because it is possible that S1P is released from cells to act at EDG receptors. However, even when sheep erythrocytes are incubated with the toxin, no S1P is detected outside the cells, showing that S1P is not released from cells treated with the toxin. It therefore appears that S1P plays a role in the hemolysis of cells induced by the toxin as a second messenger (Fig. 3).

GTPγS stimulates the toxin-induced hemolysis of sheep erythrocytes as well as rabbit erythrocytes. When lysates of sheep erythrocytes are incubated with the toxin in the presence of GTPγS, the formation of ceramide and S1P increases with an increase in the dose of GTPγS, suggesting that the toxin-induced hemolysis and SM metabolism are linked to the activation of GTP-binding proteins (*38*). Incubation of lysates treated with PT with alpha-toxin causes little change in the SM content. PT specifically blocks the alpha-toxin-stimulated conversion of SM to ceramide, suggesting that alpha-toxin activates endogenous SMase through a PT-sensitive GTP-binding protein. The *C. botulinum* C3 exoenzyme causes a dramatic accumulation of sphingosine, and inhibits the production of S1P (*38*). However, the C3 exoenzyme has no effect on the SMase activity of alpha-toxin. These results show that the C3 exoenzyme specifically inhibits the alpha-toxin-activated formation of S1P from sphingosine, showing that alpha-toxin activates sphingosine kinase through a small GTP-binding protein, RhoA (Fig. 3).

In conclusion, at small concentrations, the toxin induces biological activities inherent to tissues and cells as follows: 1) alpha-toxin binds to the binding site, 2) the C-domain of the toxin inserts into the membrane, 3) the N-domain of the toxin attacks and hydrolyses PC and/or SM in the membrane, and 4) this event stimulates signal transduction through the activation of endogenous PLC and/or SMase (Fig. 3).

REFERENCES

1. Titball, R.W., Hunter, S.E.C., Martin, K.L., Morris, B.C., Shuttleworth, A.D., Rubidge, T., Anderson, D.W., and Kelly, D.C. (1989) Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Infect. Immun.* **57**, 367–376
2. Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J., and Goebel, W. (1989) A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. *J. Bacteriol.* **171**, 744–753

3. Tso, J.Y. and Siebel, C. (1989) Cloning and expression of the phospholipase C gene from *Clostridium perfringens* and *Clostridium bifermentans*. *Infect. Immun.* **57**, 468–476
4. Vazquez-Boland, J-.A., Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J., and Cossart, P. (1992) Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. *Infect. Immun.* **60**, 219–230
5. Titball, R.W. (1993) Bacterial phospholipases C. *Microbiol. Rev.* **57**, 347–366
6. Hough, E., Hansen, L.K., Birknes. B., Jynge, K., Hansen, S., Hordvik, A., Little, C., Dodson, E., and Derewenda, Z. (1989) High-resolution (1.5 Å) crystal structure of phospholipase C from *Bacillus cereus*. *Nature* (*London*) **338**, 357–360
7. Vallee, B.L. and Auld, D.S. (1993) New perspective on zinc biochemistry: cocatalytic sites in multi-zinc enzymes. *Biochemistry* **32**, 6493–6500
8. Nagahama, M., Okagawa, Y., Nakayama, T., Nishioka, E., and Sakurai, J. (1995) Site-directed mutagenesis of histidine residues in *Clostridium perfringens* alpha- toxin. *J. Bacteriol.* **177**, 1179–1185
9. Nagahama, M., Ochi, S., Kobayashi, K., and Sakurai, J. (1996) The relationship between histidine residues and various biological activities of *Clostridium perfringens* alpha toxin. *Ad. Exp. Med. Biol.* **391**, 251–255
10. Nagahama, M., Nakayama, T., Michiue, K., and Sakurai, J. (1997) Site-specific mutagenesis of *Clostridium perfringens* alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. *Infect. Immun.* **65**, 3489–3492
11. Naylor, C.E., Eaton, J.T., Howells, A., Justin, N., Moss, D.S., Titball, R.W., and Basak, A.K. (1998) Structure of the key toxin in gas gangrene. *Nat. Struct. Biol.* **5**, 738–746
12. Nagahama, M., Mukai, M., Morimitsu, S., Ochi, S., and Sakurai, J. (2002) Role of the C-domain in the biological activities of *Clostridium perfringens* alpha-toxin. *Microbiol. Immunol.* **46**, 647–655
13. Guillouard, I, Alzari, P.M., Saliou, B., and Cole, S.T. (1997) The carboxy-terminal C2-like domain of the alpha-toxin from *Clostridium perfringens* mediates calcium-dependent membrane recognition. *Mol. Microbiol.* **26**, 867–876
14. Alape-Giron, A., Flores-Diaz, M., Guillouard, I., Naylor, C.E., Titball, R.W., Rucavado, A., Lomonte, B., Basak, A.K., Gutierrez, J.M., Cole, S.T., and Thelestam, M. (2000) Identification of residues critical for toxicity in *Clostridium perfringens* phospholipase C, the key toxin in gas gangrene. *Eur. J. Biochem.* **267**, 5191–5197
15. Nagahama, M., Michiue, K., and Sakurai, J. (1996) Membrane-damaging action of *Clostridium perfringens* alpha-toxin on phospholipid liposomes. *Biochim. Biophys. Acta* **1280**, 120–126
16. Fujii, Y., Nomura, S., Oshita, Y., and Sakurai, J. (1986) Excitatory effect of *Clostridium perfringens* alpha toxin on the rat isolated aorta. *Br. J. Pharmacol.* **88**, 531–539
17. Sakurai, J., Fujii, Y., and Shirotani, M. (1990) Contraction induced by *Clostridium perfringens* alpha toxin in the isolated rat ileum. *Toxicon* **28**, 411–418
18. Fujii, Y. and Sakurai, J. (1989) Contraction of the rat isolated aorta caused by *Clostridium perfringens* alpha toxin (phospholipase C): evidence for the involvement of arachidonic acid metabolism. *Br. J. Pharmacol.* **97**, 119–124
19. Meyers, D.J. and Berk, R.S. (1990) Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. *Infect. Immun.* **58**, 659–666
20. Bryant, A.E. and Stevens, D.L. (1996) Phospholipase C and perfringolysin O from *Clostridium perfringens* upregulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce interleukin-8 synthesis in cultured human umbilical vein endothelial cells. *Infect. Immun.* **64**, 358–362
21. Sugahara, K., Tsuruda, K., Yamada, Y., and Kamihira, S. (1997) Support system for diagnosing hematologic malignancies. *Rinsho. Byori.* **45**, 1031–1037
22. Bryant, A.E., Chen, R.Y., Nagata, Y., Wang, Y., Lee, C.H., Finegold, S., Guth, P.H., and Stevens, D.L. (2000) Clostridial gas gangrene. II. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. *J. Infect. Dis.* **182**, 808–815
23. Bryant, A.E., Bayer, C.R., Hayes-Schroer, S.M., and Stevens, D.L. (2003) Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. *J. Infect. Dis.* **87**, 408–417
24. Patriarca, P., Zatti, M., Cramer, R., and Rossi, F. (1970) Stimulation of the respiration of polymorphonuclear leucocytes by phospholipase C. *Life Sci. I.* **9**, 841–849
25. Ochi, S., Miyawaki, T., Matsuda, H., Oda, M., Nagahama, M., and Sakurai, J. (2002) *Clostridium perfringens* α-toxin induces rabbit neutrophil adhesion. *Microbiology* **148**, 237–245
26. Sakurai, J., Ochi, S., and Tanaka, H. (1993) Evidence for coupling of *Clostridium perfringens* alpha-toxin-induced hemolysis to stimulated phosphatidic acid formation in rabbit erythrocytes. *Infect. Immun.* **61**, 3711–3718
27. Sakurai, J., Ochi, S., and Tanaka, H. (1994) Regulation of *Clostridium perfringens* alpha-toxin-activated phospholipase C in rabbit erythrocyte membranes. *Infect. Immun.* **62**, 717–721
28. Ochi, S., Hashimoto, K., Nagahama, M., and Sakurai, J. (1996) Phospholipid metabolism induced by *Clostridium perfringens* alpha-toxin elicits a hot-cold type of hemolysis in rabbit erythrocytes. *Infect. Immun.* **64**, 3930–3933
29. Nelson, G.J. (1967) Lipid composition of erythrocytes in various mammalian species. *Biochim. Biophys. Acta* **144**, 221–232
30. Ohanian, J. and Ohanian, V. (2001) Sphingolipids in mammalian cell signalling. *Cell. Mol. Life Sci.* **58**, 2053–2068
31. Maceyka, M., Payne, S.G., Milstien, S., and Spiegel, S. (2002) Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim. Biophys. Acta* **1585**, 193–201
32. Hannun, Y.A., Loomis, C.R., Merrill, A.H., Jr., and Bell, R.M. (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J. Biol. Chem.* **261**, 12604–12609
33. Katsuma, S., Hada, Y., Shiojima, S., Hirasawa, A., Tanoue, A., Takagaki, K., Ohgi, T., Yano, J., and Tsujimoto, G. (2003) Transcriptional profiling of gene expression patterns during sphingosine 1-phosphate-induced mesangial cell proliferation. *Biochem. Biophys. Res. Commun.* **300**, 577–584
34. Raines, M.A., Kolesnick, R.N., and Golde, D.W. (1993) Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. *J. Biol. Chem.* **268**, 14572–14575
35. Zhang, P., Liu, B., Jenkins, G.M., Hannun, Y.A., and Obeid, L.M. (1997) Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. *J. Biol. Chem.* **272**, 9609–9612
36. Birbes, H., El Bawab, S., Hannun, Y.A., and Obeid, L.M. (2001) Selective hydrolysis of a mitochondrial pool of sphingomyelin induces apoptosis. *FASEB J.* **15**, 2669–2679
37. Olivera, A., Buckley, N.E., and Spiegel, S. (1992) Sphingomyelinase and cell-permeable ceramide analogs stimulate cellular proliferation in quiescent Swiss 3T3 fibroblasts. *J. Biol. Chem.* **267**, 26121–26127
38. Ochi, S., Oda, M., Matsuda, H., Ikari, S., and Sakurai, J. (2004) *Clostridium perfringens* α-toxin activates the sphingomyelin metabolism system in sheep erythrocytes. *J. Biol. Chem.* **279**, 12181–12189
39. Kono, K., Tanaka, M., Ogita, T., and Kohama, T. (2000) Characterization of B-5354c, a new sphingosine kinase inhibitor, produced by a marine bacterium. *J. Antibiot.* **53**, 759–764
40. Pyne, S. and Pyne, N.J. (2000) Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**, 385–402
41. Guo, C., Zheng, C., Martin-Padura, I., Bian, Z.C., and Guan, J.L. (1998) Differential stimulation of proline-rich tyrosine kinase 2 and mitogen-activated protein kinase by sphingosine 1-phosphate. *Eur. J. Biochem.* **257**, 403–408

42. Niedernberg, A., Blaukat, A., Schoneberg, T., and Kostenis, E. (2003) Regulated and constitutive activation of specific signalling pathways by the human S1P5 receptor. *Br. J. Pharmacol.* **138**, 481–493
43. Okamoto, H., Takuwa, N., Yatomi, Y., Gonda, K., Shigematsu, H., and Takuwa, Y. (1999) EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem. Biophys. Res. Commun.* **260**, 203–208
44. Im, D.S., Heise, C.E., Ancellin, N., O′Dowd, B.F., Shei, G.J., Heavens, R.P., Rigby, M.R., Hla, T., Mandala, S., McAllister, G., George, S.R., and Lynch, K.R. (2000) Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* **275**, 14281–14286
45. Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and Takuwa, Y. (1998) EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**, 27104–27110
46. Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H., Okajima, F., and Ohta, H. (2000) Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca^{2+} signaling pathway. *Biochem. Biophys. Res. Commun.* **268**, 583–589
47. Pyne, S. and Pyne, N.J. (1996) The differential regulation of cyclic AMP by sphingomyelin-derived lipids and the modulation of sphingolipid-stimulated extracellular signal regulated kinase-2 in airway smooth muscle. *Biochem. J.* **315**, 917–923
48. Gonda, K., Okamoto, H., Takuwa, N., Yatomi, Y., Okazaki, H., Sakurai, T., Kimura, S., Sillard, R., Harii, K., and Takuwa, Y. (1999) The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem. J.* **337**, 67–75
49. Cuvillier, O., Rosenthal, D.S., Smulson, M.E., and Spiegel, S. (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.* **273**, 2910–2916
50. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutkind, S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800–803
51. Rani, C.S., Wang, F., Fuior, E., Berger, A., Wu, J., Sturgill, T.W., Beitner-Johnson, D., LeRoith, D., Varticovski, L., and Spiegel, S. (1997) Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. Involvement of sphingosine 1-phosphate in PDGF but not EGF signaling. *J. Biol. Chem.* **272**, 10777–10783
52. Choi, O.H., Kim, J.H., and Kinet, J.P. (1996) Calcium mobilization via sphingosine kinase in signalling by the Fc epsilon RI antigen receptor. *Nature* **380**, 634–636
53. Spiegel, S. and Milstien, S. (2000) Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476**, 55–57