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BOTULINOLYSIN, A THIOL-ACTIVATED HEMOLYSIN PRODUCED BY *CLOSTRIDIUM BOTULINUM*, INHIBITS ENDOTHELIUM-DEPENDENT RELAXATION OF RAT AORTIC RING

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N. Sugimoto, A. Haque, Y. Horiguchi and M. Matsuda. Botulinolysin, a thiol-activated hemolysin produced by *Clostridium botulinum*, inhibits endothelium-dependent vasorelaxation of rat aortic ring. *Toxicon* **35,** 1011–1023, 1997.—The effects of botulinolysin (Blyn), a thiol-activated hemolysin produced by *Clostridium botulinum*, on contractility of rat aortic ring were studied in order to clarify an underlying mechanism of vasoconstriction by the toxin observed previously as an increase in perfusion pressure in isolated rat organs. Blyn (30 hemolytic units/ml; HU/ml) itself did not elicit any apparent change in resting tension of the ring. Contractile tension elicited by a high concentration of phenylephrine in endothelium-intact rings increased significantly after treatment with Blyn (30 HU/ml), while phenylephrine-induced contraction of endothelium-denuded rings was not influenced by toxin treatment. In rings with intact endothelium, acetylcholine (ACh)-induced relaxation was significantly inhibited after treatment with Blyn (30, 10, 1 HU/ml). In contrast, relaxation of denuded rings by sodium nitroprusside was not affected by toxin treatment (30 HU/ml). Arginine (10^{-4} M) partly reversed the inhibition of ACh-induced relaxation by the toxin (1 HU/ml). Endothelium-dependent relaxation by histamine or adenosine triphosphate was also inhibited by Blyn (1 HU/ml), but the relaxation elicited by calcium ionophore A23187 was not influenced by the toxin. The results indicate that Blyn acts on endothelium and inhibits agonist-induced endothelium-dependent relaxation of blood vessels. © 1997 Elsevier Science Ltd

INTRODUCTION

Clostridium botulinum produces a hemolysin named botulinolysin (Blyn) in addition to the well-known botulinum neurotoxin. The hemolysin consists of a single peptide chain with a mol. wt of about 58,000 and estimated p*I* of 8.4. It lyses erythrocytes and other eukaryotic cells, and kills animals when administered i.v. These biological activities are inactivated by oxidation and reactivated by thiol-activating agents such as cysteine and dithiothreitol. A small amount of cholesterol inactivates its activity irreversibly. Based on these physicochemical and biological characteristics, Blyn is classified into a group known as the bacterial thiol-activated hemolysins (Alouf and Geoffroy, 1988; Freer, 1988). However, the exact mechanism of its action, especially of its lethal action, was not clarified because of the difficulty in obtaining a purified preparation of Blyn without contamination by the potent botulinum neurotoxin.

A method was recently established for the purification of Blyn and its physicochemical and biological characteristics as a member of bacterial thiol-activated hemolysins were confirmed with a purified preparation (Haque *et al.*, 1992). With purified Blyn, an increase in perfusion pressure in isolated rat hearts was observed, as reported in cases of streptolysin O. In addition, Blyn was found to increase perfusion pressure in isolated rat kidneys, lungs and livers (Sugimoto *et al.*, 1995), suggesting that vascular constriction is the primary feature of the toxin's action. In order to clarify the underlying mechanism of vascular constriction by the toxin, this study examined the effects of Blyn on the vascular contractility of the isolated rat thoracic aortic ring, and found that Blyn inhibits agonist-induced endothelium-dependent relaxation of the ring.

MATERIALS AND METHODS

Botulinolysin

Blyn was purified from the culture filtrate of *C. botulinum* type C, strain 203, as described previously (Haque *et al.*, 1992). In brief, stored sporangia of the bacterial strain were inoculated into fortified cooked meat medium (Kurazono *et al.*, 1985) and incubated at 37°C overnight under anaerobic conditions as a seed culture. An aliquot of the seed culture was inoculated into 750 ml of the fresh medium and incubated at 37°C for 14 hr under anaerobic conditions. After the incubation, the bacteria and cooked meat particles were removed by centrifugation at 10,000 ***g*** for 20 min. The supernatant was then fractionated by 60% saturation of ammonium sulfate. The toxin was extracted from the precipitate with 50 mM Tris–HCl buffer, pH 6.5, containing 5 mM cysteine. From the extract, Blyn was purified by a combination of anion-exchange chromatography with DEAE-Sepharose CL-6B, gel-filtration chromatography through Sephadex G-75 and cation-exchange chromatography with SP-Toyopearl 650 M. The purified preparation gave a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at the position corresponding to a mol. wt of 58,000. The preparation had a hemolytic activity of 2100 hemolytic units (HU) per μg of protein on rabbit erythrocytes and its 50% lethal dose in mice (20 g) was 310 ng. Because of its relatively rapid inactivation by oxidation (half-life of the activity is about 2 weeks), the amounts of toxin used in the experiments are expressed in HU throughout this paper.

Assay of hemolytic activity

Hemolytic activity of Blyn was routinely determined with rabbit erythrocytes. The toxin was diluted two-fold serially with gelatine Tris-buffered saline (GTBS). The composition of GTBS was: NaCl, 150 mM; KCl, 4.5 mM; $MgSO_4$, 2.0 mM; $CaCl_2$, 1 mM; Tris, 20 mM; gelatine, 0.1%; glucose, 10.0 mM. The diluted toxin (100 μl) was mixed with an equal volume of erythrocyte suspension (4×10^8 cells/ml) in GTBS, then the mixture was incubated at 37°C for 45 min. After the incubation, the mixture was centrifuged at 1000 ***g*** for 10 min and the absorbance of supernatant at 540 nm was measured. The absorbance value of supernatant obtained from the erythrocyte suspension treated with 0.1% Triton X-100 was used as a value of 100% hemolysis. One hemolytic unit (1 HU) was defined as the dose of hemolysin that causes 50% hemolysis under these conditions.

Organ chamber experiments

All experiments were performed under the guidelines for animal experiments of the International Society on Toxinology. Sprague–Dawley rats of either gender weighing 230–300 g were anesthetized with sodium pentobarbital (35 mg/kg, i.p.). The animals were heparinized by i.v. injection (200 IU) and killed by bleeding. Their thoracic aortae were dissected free and immersed in Krebs solution prewarmed to 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The composition of Krebs solution (mM) was: NaCl, 124; KCl, 5; KH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; Na_2EDTA, 0.03; glucose, 10. After removal of visible fat and connective tissue, the aorta was cut into rings 4–5 mm long with special care taken not to touch the luminal surface. In some rings, endothelium was removed by inserting the tip of forceps into the lumen of the rings and rolling the rings over filter paper wetted with Krebs solution. Each ring was mounted in an organ chamber containing 10 ml of Krebs solution kept at 37°C and continuously bubbled with a 95% O_2–5% CO_2 mixture. Two horizontally arranged stainless-steel pins (0.2 mm diameter) were passed through the lumen of the vessel ring. One pin was fixed to a tissue holder and the other was connected vertically to an isometric tension transducer (TB-651T, Nihonkohden, Tokyo, Japan). The rings were subjected to an optimal resting tension of about 3.0 g. The isometric tension of the ring was recorded continuously on paper with an amplifier (EF-601G, Nihonkohden, Tokyo, Japan) and a pen-recorder (RJG-4124, Nihonkohden, Tokyo, Japan) and stored in magnetic tapes with an FM data recorder (XR-310, TEAC, Tokyo, Japan). During an equilibration period of 90 min, Krebs solution was changed and the resting tension was readjusted every 15 min. Preliminary experiments on contractions elicited by successive administrations of phenylephrine at intervals of 60 min to the rings showed that the contractile response to the second administration of phenylephrine was obviously larger than that to the first administration but thereafter responses to phenylephrine became stable. Therefore, the following two protocols were used in this study.

Protocol 1. After the equilibration period, the ring was first contracted by phenylephrine (10^{-6} M) and then ACh (3×10^{-7} or 10^{-6} M) was added to the bath to examine the functional integrity of endothelium. Rings showing almost complete relaxation by 3×10^{-7} M ACh were used as endothelium-intact rings and those showing no relaxation by 10^{-6} M ACh were used as endothelium-denuded rings. The ring was washed with fresh Krebs solution four times and allowed to recover for 30 min. Then, the tissue was exposed to a Krebs solution containing 80 mM KCl, in which NaCl was replaced by an equimolar amount of KCl, followed by four washes with fresh normal Krebs solution and a recovery period of 30 min. The maximum contractile response elicited by 80 mM KCl exposure was used as the standard response for all subsequent contractions. The ring was then subjected to control experiments of contraction or relaxation elicited by cumulative addition of an agonist to the organ bath. In relaxation experiments, the rings were precontracted by phenylephrine (10^{-6} M). After the control experiments, the rings were washed four times with fresh Krebs solution and allowed to recover for 30 min. Blyn appropriately diluted with Krebs solution was then added to the bath and incubated for 15 min. The toxin-treated ring was washed four times with toxin-free Krebs solution and rested for 30 min. After the resting period, the rings were subjected to the second contraction or relaxation by the same reagent as used in the control experiments.

Protocol 2. The integrity of endothelium and the response to 80 mM KCl in each ring were examined in the same manner as in Protocol 1, and then the rings were divided into two groups. Rings in one group were incubated with Blyn for 15 min and those in the other group were kept in toxin-free Krebs solution. After the incubation, all rings were washed four times with fresh Krebs solution and rested for 30 min. The rings were then subjected to the experiments of contraction or relaxation elicited by reagents.

Results obtained from experiments on phenylephrine, ACh, histamine and ATP were essentially same in Protocols 1 and 2. However, Protocol 1 was not used in experiments of A23187-induced relaxation of the rings because successive application of the reagent failed to relax the rings. Therefore, the results of A23187-induced relaxation were obtained only from Protocol 2.

Reagents

The following reagents were purchased from Wako Pure Chemical Industries (Tokyo, Japan): L-phenylephrine hydrochloride, sodium nitroprusside dihydrate, L-arginine, histamine, A23187, 5-hydroxytryptamine–creatinine sulfate complex, bradykinin and prostaglandin F2α. Acetylcholine chloride was from Nacalai Tesque (Kyoto, Japan), and adenosine triphosphate, Na-benzoyl-L-arginine ethyl ester (BAEE) and L-N^G-nitro arginine methylester (L-NAME) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Drug solutions were made in distilled water, except A23187 and prostaglandin F2α. Dimethylsulfoxide or ethanol was used to dissolve A23187 or prostaglandin F2α, respectively. The maximum concentration of dimethylsulfoxide or ethanol in the organ bath during the experiments was 0.07% or 0.5%, respectively. All solutions were kept in dark and stored in a freezer (− 70°C).

Statistical analysis

Results are expressed as means ± S.E. of number (n) of aortic rings used in experiments. Statistical differences between means were assessed using a paired two-tailed Student's t-test. A probability value less than 0.05 was considered statistically significant. The negative logarithm of the effective molar concentration of reagent causing 50% of maximum response (EC_{50}) was calculated for each concentration–response curve and the means of these values are presented.

RESULTS

Effects of botulinolysin on resting and contractile tension of rat aortic rings

Under the conditions of the present experiments, Blyn (1–30 HU/ml) did not cause any apparent change in resting tension in aortic rings with or without endothelium. The presence of acetyl salicylate (10^{-3} M), a cyclooxygenase inhibitor, BAEE (3.25×10^{-7} M), an *N*-substituted arginine compound, or L-NAME (10^{-4} M), an inhibitor of nitric oxide synthase, in the bathing solution did not influence the effects of Blyn on the resting tension of the rings. Addition of phenylephrine or prostaglandin F2α to the bathing solution elicited a concentration-dependent increase in contractile tension of the rings with or without endothelium before and after treatment with Blyn (1–30 HU/ml). A representative record of tension of the ring with intact endothelium before, during and after treatment with Blyn (30 HU/ml) is shown in Fig. 1. The maximum tensions elicited by 10^{-5} M phenylephrine before and after treatment with the toxin were 1.96 ± 0.24 g and 2.40 ± 0.34 g (mean $\pm$ S.E., $n = 5$), respectively. A summary of the effects of Blyn (30 HU/ml) on phenylephrine-induced contraction of the rings with intact endothelium is illustrated in Fig. 2. Estimated EC_{50}s of phenylephrine before and after toxin treatment were 1.0×10^{-7} M and 1.4×10^{-7} M, respectively. Minimum concentrations of phenylephrine

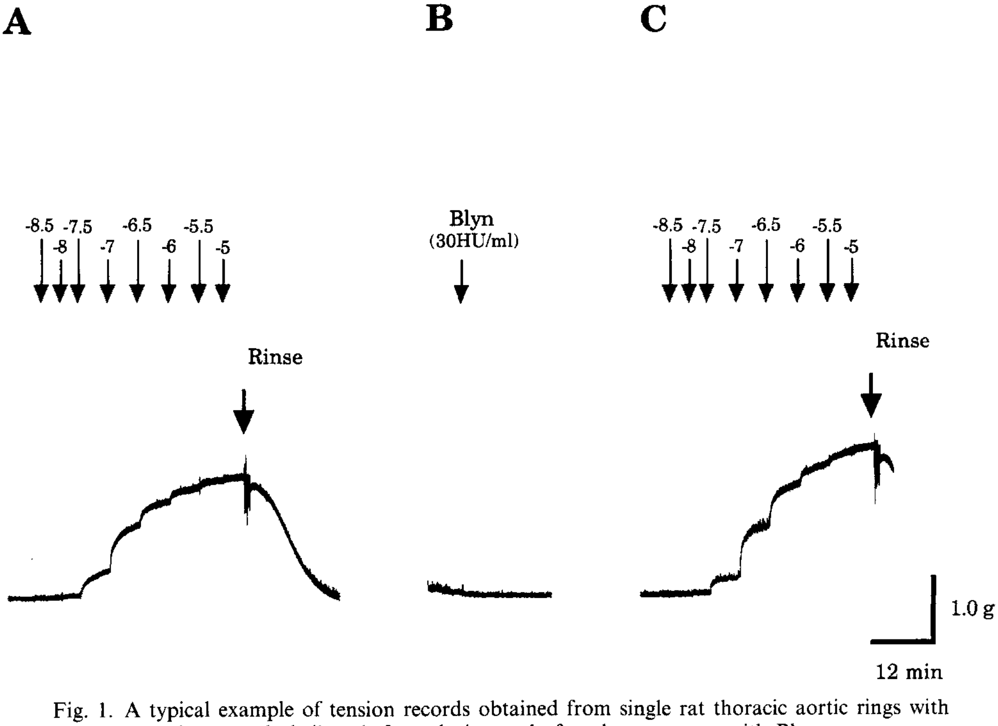

Fig. 1. A typical example of tension records obtained from single rat thoracic aortic rings with intact endothelium before, during and after the treatment with Blyn.

An aortic ring was suspended in an organ bath containing 10 ml of Krebs solution, and loaded 3.0 g as a resting tension. According to Protocol 1, the ring was contracted by cumulative addition of phenylephrine to the bath (A) after the examination of functional integrity of its endothelium and response to 80 mM KCl. The ring was then exposed to 30 HU/ml of Blyn for 15 min (B). After washing four times with fresh Krebs solution and a resting period of 30 min, the ring was again contracted by cumulative addition of phenylephrine (C). Arrows with numbers above the traces show the times and logarithmic concentrations of phenylephrine added to the organ bath. Calibration bars show 1.0 g (vertical) and 12 min (horizontal).

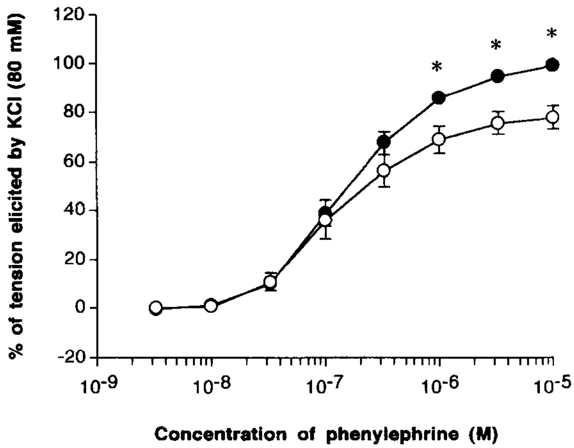

Fig. 2. Concentration–response curves of phenylephrine-induced contraction of endothelium-intact rings before and after the treatment with Blyn (30 HU/ml).
Contractile tensions elicited by cumulative addition of phenylephrine to the bath were measured as described in the legend for Fig. 1. The values of tension elicited by various concentrations of phenylephrine were expressed as percentages of that elicited by 80 mM KCl. ○, Tensions before toxin treatment; ●, tensions after treatment. Values are means ± S.E. ($n = 5$). The values of tensions after toxin treatment were compared with those before the treatment by Student's t-test (paired two-tailed). $*P < 0.05$.

required to elicit a significant increase in tension ($P < 0.05$) of the rings were 3.25×10^{-8} M in both cases. However, the tension elicited by high concentration of phenylephrine after toxin treatment was significantly ($P < 0.05$) larger than that before treatment. In contrast, in endothelium-denuded rings, the concentration–response curve of phenylephrine-induced contraction was not influenced by treatment with Blyn (30 HU/ml) (Fig. 3). Estimated EC_{50}s of phenylephrine before and after treatment were 5.1×10^{-8} M and 7.1×10^{-8} M, respectively. The maximum tensions elicited in endothelium-denuded rings before and after treatment with the toxin were 2.40 ± 0.23 g and 2.41 ± 0.27 g (mean ± S.E., $n = 6$), respectively, which coincide with those of rings with endothelium after treatment with the toxin. Similar results were obtained from the experiments on the influence of Blyn to the contractile response of the rings to prostaglandin F2α.

Effects of botulinolysin on relaxation of rat aortic ring

A representative record of acetylcholine (ACh)-induced relaxation of a rat aortic ring precontracted with 10^{-5} M phenylephrine before and after treatment with Blyn (10 HU/ml) is shown in Fig. 4. After toxin treatment, the concentration of ACh required to relax the ring increased remarkably. The effects of Blyn on ACh-induced relaxation of the aortic rings with intact endothelium are summarized in Fig. 5, where it can be seen that relaxation of the rings by ACh almost disappeared after treatment with 30 HU/ml of Blyn. Treatment with Blyn (1 HU/ml) caused an obvious rightward shift in the concentration–response curve of ACh-induced relaxation. Estimated EC_{50}s of ACh in rings before and after treatment with Blyn (1 HU/ml) were 6.0×10^{-9} M and 1.3×10^{-7} M, respectively. As the concentration of Blyn increased, the degree of maximum relaxation by ACh decreased. Relative values of the maximum relaxation against the contractile force elicited by 10^{-5} M

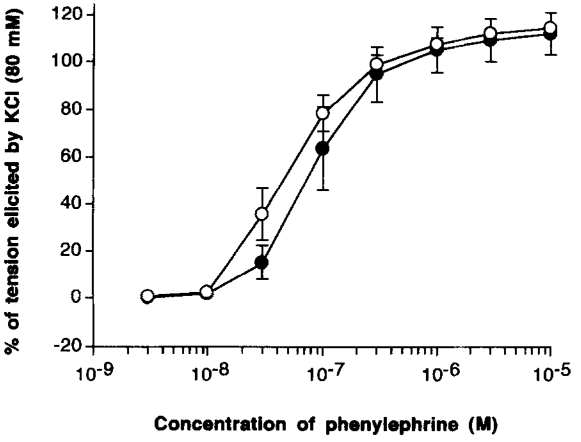

Fig. 3. Concentration–response curves of phenylephrine-induced contraction of endothelium-denuded rat aortic rings before and after the treatment with Blyn (30 HU/ml).
Rat aortic rings were denuded and the effectiveness of denudation was confirmed by the absence of ACh-induced relaxation in these rings. Contractile tensions elicited by cumulative addition of phenylephrine in the rings before and after the toxin treatment were recorded according to Protocol 1 as described in the legend for Fig. 1. Values are expressed as percentages of those elicited by 80 mM KCl. ○, Tensions before toxin treatment; ●, tensions after treatment. Values are means ± S.E. ($n = 6$).

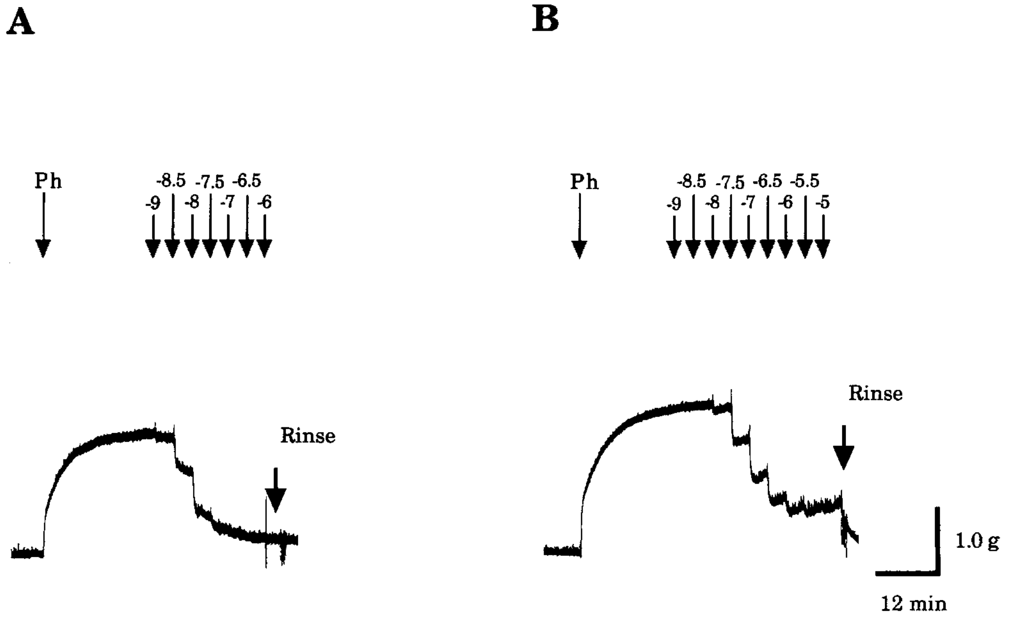

Fig. 4. Representative traces of ACh-induced relaxation of isometric tension of an endothelium-intact rat aortic ring before and after the treatment with Blyn (10 HU/ml).
ACh-induced relaxation of an aortic ring showing functional integrity of its endothelium was recorded according to Protocol 1. The ring was contracted with phenylephrine (10^{-6} M; shown in arrows and Ph). After the tension of the ring reached plateau, ACh was added cumulatively to the bath (A). The ring was then washed four times with fresh Krebs solution and rested for 30 min. Blyn was added to the bath and incubated for 15 min followed by four washes with toxin-free Krebs solution and a resting period of 30 min. ACh-induced relaxation of the ring precontracted by 10^{-5} M of phenylephrine was recorded again (B). Arrows with numbers above the traces show the times and logarithmic concentrations of ACh added to the organ bath. Calibration bars show 1.0 g (vertical) and 12 min (horizontal).

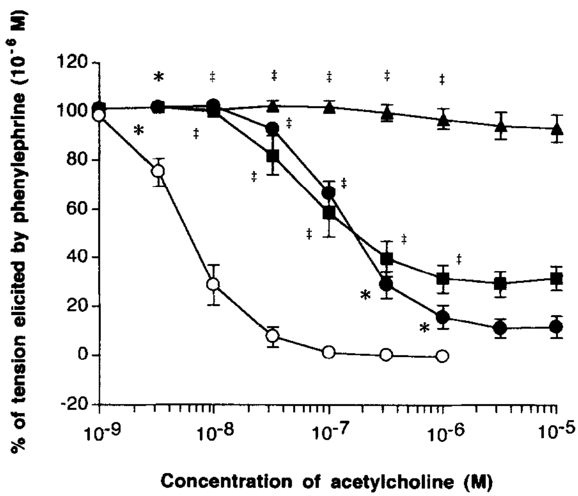

Fig. 5. Effects of Blyn on concentration–response curve of ACh-induced relaxation of rat aortic rings.

Before and after the treatment of endothelium-intact aortic rings with Blyn (1, 10, 30 HU/ml), ACh-induced relaxation of the rings were recorded as described in the legend for Fig. 4. The minimum values of tension (= maximum relaxation) of a ring recorded after the addition of ACh in each concentration were expressed as percentages of maximum tension elicited by phenylephrine (10^{-6} M) in the ring. Values are means ± S.E. ($n = 7$, 4 and 3 for 1, 10 and 30 HU/ml of Blyn, respectively). ○, Tensions before toxin treatment; ●, ■, ▲, tensions after treatment with 1, 10 and 30 HU/ml of Blyn, respectively. The values of tensions after toxin treatment were compared with those before the treatment by Student's t-test (paired two-tailed). $^*P < 0.05$, $^{**}P < 0.01$.

phenylephrine were 88.4 ± 4.2%, 68.8 ± 5.0% and 0.7 ± 5.5% (means ± S.E., $n = 7$, 4 and 3, respectively) in the rings treated with 1 HU/ml, 10 HU/ml and 30 HU/ml, respectively. In contrast, relaxation elicited by sodium nitroprusside in the endothelium-denuded rings was not affected by treatment with Blyn (30 HU/ml) (Fig. 6). The relative values of maximum relaxation before and after toxin treatment against the contractile force elicited by 10^{-5} M phenylephrine were 106.9 ± 3.5% and 98.7 ± 1.5%, respectively (means ± S.E., $n = 3$ in each). The estimated EC_{50}s of sodium nitroprusside were 2.3×10^{-9} M and 2.0×10^{-9} M, respectively. Addition of arginine (10^{-4} M) to the bathing solution of aortic rings partly reversed the rightward shift in the concentration–response curve of ACh-induced relaxation after treatment with Blyn (1 HU/ml) (Fig. 7). The maximum relaxation by ACh increased from 88.9 ± 4.0% to 96.9 ± 3.1% (means ± S.E., $n = 7$ in each) in the presence of arginine (10^{-4} M), while that before treatment was 107.4 ± 2.1%. The EC_{50} of ACh after toxin treatment decreased from 1.4×10^{-7} M to 6.0×10^{-8} M by addition of arginine (10^{-4} M) to the bathing solution, whereas that before treatment was 1.1×10^{-8} M.

Selectivity of botulinolysin action on endothelium-dependent relaxation elicited by various vasoreactive agents

Using another experimental design (Protocol 2), the effects of Blyn on ACh-induced relaxation of rat aorta were confirmed, and the specificity of the action of Blyn on endothelium-dependent relaxation elicited by various vasoreactive agents was examined. Among the reagents examined, 5-hydroxytryptamine and bradykinin did not relax the rat rings precontracted with phenylephrine. However, ATP, histamine and calcium ionophore A23187, in addition to ACh, decreased the tension of endothelium-intact rings precontracted with 10^{-6} M phenylephrine, in a concentration-dependent manner. The results are summarized in Fig. 8. Treatment with Blyn (3 HU/ml) also caused a significant rightward shift in the concentration–response curve of ACh-induced relaxation of rat aortic rings under the conditions of Protocol 2 [Fig. 8(A)]. Estimated ED_{50}s of ACh were 1.5×10^{-8} M in control rings and 6.8×10^{-8} M in the rings treated with Blyn. Rightward shifts in the concentration–response curves by treatment with Blyn were also observed in the relaxation of the toxin-treated rings by ATP and histamine [Fig. 8(B,C)]. Estimated EC_{50}s of ATP and histamine were 4.1×10^{-6} M and 1.2×10^{-6} M in control rings, and 3.7×10^{-5} M and 1.2×10^{-5} M in the rings treated with Blyn, respectively. In contrast, treatment of the rings with Blyn did not shift the concentration–response curve of the relaxation by calcium ionophore A23187 [Fig. 8(D)]. Estimated EC_{50}s were 1.7×10^{-8} M and 1.4×10^{-8} M in the control and toxin-treated rings, respectively.

DISCUSSION

The results presented above indicate that Blyn, a hemolysin produced by *Clostridium botulinum*, increases the contractility of rat aortic ring by inhibiting the endothelium-dependent relaxation of rat thoracic aortic rings. The rings with or without endothelium contracted in response to phenylephrine or prostaglandin F2α. However, in

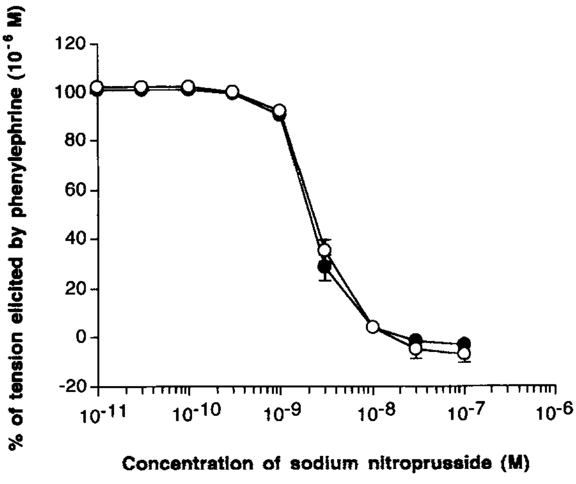

Fig. 6. Concentration–response curves of sodium nitroprusside-induced relaxation of endothelium-denuded rat aortic rings before and after the treatment with Blyn (30 HU/ml).
Rat aortic rings were denuded and suspended in an organ bath containing 10 ml of Krebs solution. Effectiveness of denudation was confirmed by the absence of ACh-induced relaxation in these rings. The rings were then contracted by phenylephrine (10^{-6} M). After the contractile tension reached a plateau, sodium nitroprusside was added cumulatively to the bath followed by four washes with fresh Krebs solution and a resting period of 30 min. Then the rings were treated with Blyn (30 HU/ml) for 15 min and washed four times with toxin-free Krebs solution. After a resting period of 30 min, sodium nitroprusside-induced relaxation of the rings was again recorded. The minimum values of tension (= maximum relaxation) of a ring recorded after the addition of sodium nitroprusside at each concentration were expressed as percentages of maximum tension elicited by phenylephrine (10^{-6} M) in the ring. Values are means ± S.E. ($n = 3$). ○, Tensions before toxin treatment; ●, tensions after treatment.

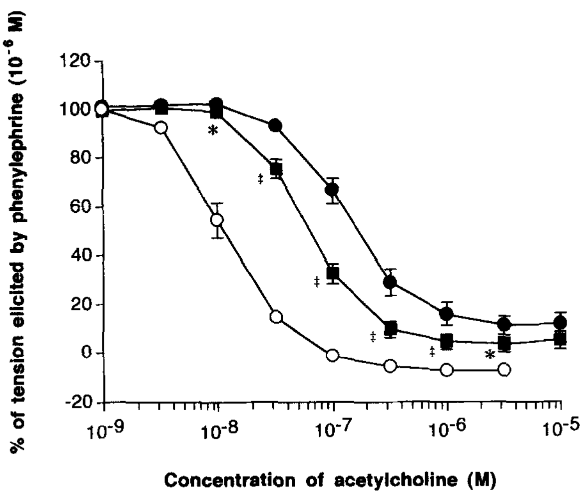

Fig. 7. Effects of arginine on the inhibition of ACh-induced relaxation by Blyn in rat aortic rings. Relaxation induced by ACh in rings with intact endothelium was recorded as described in the legend for Fig. 4. After four washes with fresh Krebs solution and a recovery period of 30 min, the rings were exposed to Blyn (1 HU/ml) for 15 min and washed four times with toxin-free Krebs solution. The rings were then divided into two groups. One group was equilibrated in Krebs solution containing 10^{-4} M of arginine, and the other in normal Krebs solution for 15 min. After the equilibration period, rings in both groups were contracted with phenylephrine (10^{-6} M) and ACh-induced relaxations of the rings were recorded. The minimum values of tension (= maximum relaxation) of a ring recorded after the addition of ACh at each concentration were expressed as percentages of maximum tension elicited by phenylephrine (10^{-6} M) in the ring. Values are means $\pm$ S.E. ($n = 7$). ○, Tensions before toxin treatment; ●, ■, tensions of rings in the absence and presence of 10^{-4} M arginine after toxin treatment, respectively. The values of tensions in the presence of arginine were compared with those in the absence of arginine by Student's t-test (paired two-tailed). $^*P < 0.05$, $^{**}P < 0.01$.

endothelium-intact aortic rings, treatment with Blyn caused an increase in contractile tension elicited by phenylephrine, although the toxin itself did not influence the resting tension of the rings. This indicates that Blyn influences the regulation of vascular contractility and increases the tonicity of blood vessels. A variety of mechanisms is known for the regulation of vascular contractility, but systemic nervous and hormonal factors and factors from platelets were excluded under the experimental conditions. Therefore, possible mechanisms of the increase in contractile tension of the rings after treatment with Blyn are: (1) an increase in the reactivity of vascular smooth muscle to phenylephrine; and (2) an inhibition of the local relaxation mechanism in the rings. However, the former possibility is made less likely because the increase in contractile tension was not due to the leftward shift in the concentration–response curve but was significant only at a high concentration of phenylephrine. Moreover, treatment of endothelium-denuded rings with Blyn did not alter the concentration–response curve of phenylephrine-induced contraction, indicating that reactivity of vascular smooth muscle to phenylephrine is not influenced by treatment. In contrast, relaxation elicited by ACh in endothelium-intact rings was remarkably affected by the toxin. Relaxation by ACh disappeared almost completely after treatment with 30 HU/ml of Blyn, and the concentration–response curve of the relaxation showed a significant rightward shift after treatment with even 1 HU/ml of the toxin. These results support the latter possibility as a mechanism of increase in contractile tension elicited by phenylephrine after treatment with Blyn.

Acetylcholine is reported to relax vascular smooth muscle by inducing the production of endothelium-derived relaxation factor (EDRF) in endothelial cells (Furchgott and Zawadzki, 1980; Kimura *et al.*, 1992; Alonso *et al.*, 1992), and nitric oxide (NO) is the most probable candidate for EDRF (Palmer *et al.*, 1987; Ignarro, 1989). Thus, a question arises as to whether Blyn affects EDRF production in endothelial cells or reduces the reactivity of smooth muscle to NO. NO is also produced from NO donors such as sodium nitroprusside in vascular smooth muscle cells and relaxes blood vessels directly (Kowaluk *et al.*, 1992). Therefore, the reactivity of vascular smooth muscle to NO can be examined by sodium nitroprusside in endothelium-denuded vascular rings. It can be shown that the reactivity of vascular smooth muscle to NO is not affected by treatment with Blyn, in that

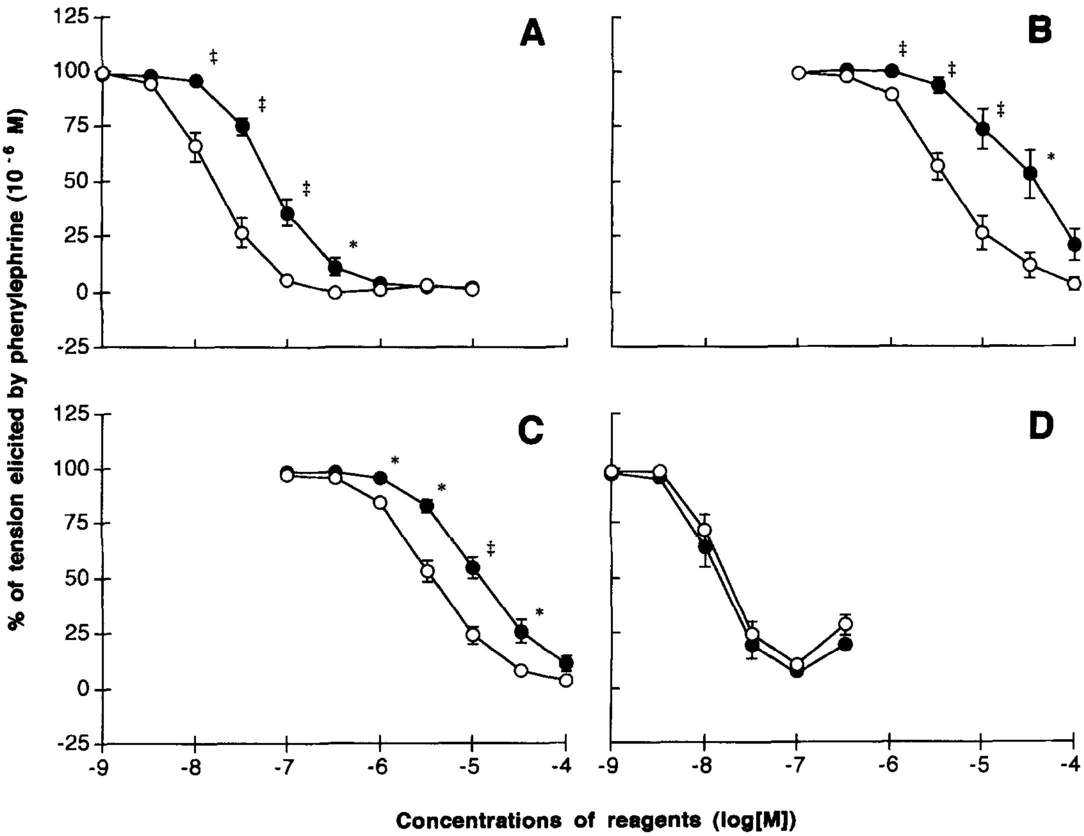

Fig. 8. Effects of Blyn on endothelium-dependent relaxation of rat aortic rings by various vasoactive reagents.

Rat aortic rings showing functional integrity of the endothelium were divided into two groups according to Protocol 2. One group was treated with Blyn (3 HU/ml), and the other was kept in toxin-free Krebs solution for 15 min. Rings in both groups were then washed four times with toxin-free Krebs solution and rested for 30 min, then contracted with phenylephrine (10^{-6} M). After the contractile tension reached a plateau, relaxations elicited by ACh (A), ATP (B), histamine (C), and A23187 (D) were examined. The minimum values of tension (= maximum relaxation) of a ring recorded after the addition of reagents in each concentration were expressed as percentages of maximum tension elicited by phenylephrine (10^{-6} M) in the ring. Values are means ± S.E. (n = 4–10). ○, Tensions before toxin treatment; ●, tensions after treatment. The values of tensions of the toxin-treated rings were compared with those of untreated rings by Student's t-test (paired two-tailed). $*P < 0.05$, $**P < 0.01$.

there was no difference between the concentration–response curves of sodium nitroprusside-induced relaxation of endothelium-denuded rings before and after toxin treatment. Thus, it is likely that Blyn affects EDRF synthesis in endothelial cells. This possibility is supported by the fact that the rightward shift in the concentration–response curve of ACh-induced relaxation of aortic rings caused by toxin treatment was partly reversed by the addition of arginine, a precursor of NO (Palmer *et al.*, 1988), to the bathing solution of the tissue. A question which remains is why the recovery of endothelium-dependent relaxation by arginine was not complete but only partial. This may reflect the heterogeneity of sensitivity in endothelial cells to Blyn or the presence of other substance(s) than NO in EDRF.

Endothelium-dependent relaxation of blood vessels is reported to be elicited by a variety of agents in addition to ACh. To determine the site of action of Blyn, we investigated the effect of Blyn on endothelium-dependent relaxation elicited by some agents other than ACh (Boulanger and Vanhoutte, 1992; Vials and Burnstock, 1993; Weinheimer and Osswald, 1989). The results show that endothelium-dependent relaxations elicited by histamine and ATP were also sensitive to Blyn, while relaxation elicited by A23187 was not affected by the toxin. The exact signal transduction mechanism of production and release of EDRF is not yet known; however, it has been shown that the release of EDRF is calcium dependent and that NO synthase in endothelium is calcium and calmodulin dependent (Furchgott, 1983; Long and Stone, 1985; Singer and Peach, 1982). The receptor-mediated endothelium-dependent relaxation so far reported can be divided into two groups: pertussis toxin resistant and toxin sensitive. Relaxation elicited by ACh or ATP is reported to be pertussis toxin resistant, while that elicited by histamine is pertussis toxin sensitive (Flavahan *et al.*, 1989; Hohlfeld *et al.*, 1990; Liao and Homcy, 1992). The present results indicate that Blyn inhibits receptor-mediated EDRF release whether the process is pertussis toxin resistant or toxin sensitive, while non-receptor-mediated release of EDRF is not affected by the toxin. Flavahan *et al.* (1989) and Miller *et al.* (1991) postulated that there are two distinct processes in the release of EDRF: pertussis toxin-sensitive G-protein dependent and independent. If one accepts their hypothesis, then our results suggest that the site of Blyn action is below the step at which the two processes converge and above the step of an increase in intracellular calcium concentration.

Blyn belongs to a group of bacterial thiol-activated hemolysins. Polypeptides in this group, including streptolysin O, pneumolysin, perfringolysin O, alveolysin, cereolysin and listeriolysin, have some physicochemical and biological characteristics in common (Smyth and Duncan, 1978; Bernheimer and Rudy, 1986). They are all lethal to experimental animals by i.v. injection, and bacteria which produce these toxins are all pathogenic and sometimes cause systemic manifestations in addition to inflammatory reaction at the site of infection in humans and animals. Among these toxins, the action of streptolysin O was studied most intensively *in vivo* and *in vitro*. Streptolysin O was shown to cause electrocardiographic alterations in animals (Halbert *et al.*, 1961), and to diminish coronary flow and myocardial contractility in isolated and perfused hearts (Bernheimer and Cantoni, 1945; Reitz *et al.*, 1968). Since then it has been established that streptolysin O is a cardiotoxin, and this idea was extended to the action of other bacterial thiol-activated hemolysins. However, further study on the detailed mechanism of the cardiotoxicity of bacterial thiol-activated hemolysins has not been performed and the pathogenic role of these toxins remains unclear. We resently established the method for purification of Blyn and found that Blyn caused an increase in perfusion pressure not only in isolated heart but also in isolated kidney, lung and liver of rat (Sugimoto *et al.*, 1995), suggesting that

Blyn causes vascular constriction. The present study shows that Blyn increases vascular contractility by inhibiting receptor-mediated endothelium-dependent relaxation of blood vessels. Thus, it is likely that the inhibition of endothelium-dependent relaxation by the toxin causes constriction of blood vessels in organs, e.g. the coronary artery. Recently, staphylococcal α-toxin (Seeger and Suttorp, 1986; Seeger *et al.*, 1990), *Escherichia coli* hemolysin (Seeger *et al.*, 1989; Ermert *et al.*, 1992) and *Pseudomonas aeruginosa* cytotoxin (Seeger *et al.*, 1986; Grimminger *et al.*, 1991), which are distinct from bacterial thiol-activated hemolysins, were reported to cause vascular constriction in isolated and perfused rabbit lungs. The authors of these reports concluded that vascular constriction is caused by thromboxane generation by these bacterial toxins. However, Suttorp *et al.* (1993) reported that *E. coli* hemolysin and staphylococcal α-toxin induced the release of NO in cultured porcine endothelial cell. This result appears to be inconsistent with former reports because the release of NO could cause vascular relaxation. This apparent inconsistency may be due to differences among experimental systems. The present results do not exclude the possibility that Blyn affects other functions of endothelial cells such as the metabolism of arachidonic acid, in addition to the inhibition of EDRF release. However, the contribution of eicosanoids to Blyn-induced vasoconstriction is thought to be less important because the presence of acetyl salicylate did not influence the Blyn-induced increase in perfusion pressure of isolated rat hearts as reported previously (Sugimoto *et al.*, 1995). The entire picture of the effects of Blyn on endothelium and the molecular mechanism of the inhibition of endothelium-dependent relaxation of vessels by Blyn remain to be clarified.

In conclusion, Blyn, one of bacterial thiol-activated hemolysins, acts on endothelium and inhibits receptor-mediated endothelium-dependent relaxation of blood vessels. The results obtained in this study are consistent with the previous observation that Blyn causes vascular constriction in isolated and perfused rat organs. It is suggested that bacterial thiol-activated hemolysins affect endothelial functions, and that the cardiotoxicity reported previously is merely a part of their action on the circulatory system. The present results also suggest that this action of thiol-activated hemolysins on endothelium may contribute to some of the manifestations observed in infections by thiol-activated hemolysin-producing bacteria. Further experiments are underway to examine this possibility and to clarify the pathogenic role of bacterial thiol-activated hemolysins.

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