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CORONARY VASOCONSTRICTION IS THE MOST PROBABLE CAUSE OF DEATH OF RATS INTOXICATED WITH BOTULINOLYSIN, A HEMOLYSIN PRODUCED BY *CLOSTRIDIUM BOTULINUM*

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N. Sugimoto, A. Haque, Y. Horiguchi and M. Matsuda. Coronary vasoconstriction is the most probable cause of death of rats intoxicated with botulinolysin, a hemolysin produced by *Clostridium botulinum*. *Toxicon* **33,** 1215–1230, 1995.—The pathophysiology of lethal intoxication by botulinolysin (Blyn) was studied using anesthetized rats and isolated rat organs. Intravenous injection of 10,000 and 1000 hemolytic units (HU) of Blyn killed rats rapidly while 100 HU of the toxin did not. Congestion and edema of lungs were observed at autopsies of the rats killed by intoxication. Hemoglobinemia was obvious in rats injected with 1000 HU of Blyn but not in rats with 10,000 HU. Electrocardiograms of the intoxicated rats showed depression of T waves but not changes characteristic of hyperpotassemia. All the rats injected with the above doses of Blyn showed a rapid fall in arterial blood pressure (BP) immediately after the toxin injection, and BP soon recovered in rats injected with 100 HU, partially and transiently in rats with 1000 HU, and not in rats with 10,000 HU of Blyn. Perfusion of Blyn (1 HU/ml) to isolated rat hearts caused a rapid and marked increase in perfusion pressure and cessation of spontaneous heart beat. Acetylsalicylic acid (10^{-3} M) and quinacrine dihydrochloride (10^{-5} M) did not essentially influence the effects of Blyn on the isolated hearts, but verapamil (10^{-6} M) inhibited at least the initial increase in perfusion pressure elicited by Blyn. Spontaneous contractions of the isolated atria were little influenced by Blyn (60 HU/ml). Perfusion pressures of isolated kidneys, lungs and livers were also increased by Blyn (1 HU/ml). The results indicate that Blyn caused vasoconstriction but had little direct effect on myocardium. Based on the above findings, we conclude that coronary vasoconstriction elicited by direct action of Blyn causes acute cardiac dysfunction leading to systemic hypotension and death of the intoxicated animals.

INTRODUCTION

Clostridium botulinum produces a hemolysin, named botulinolysin (Blyn), in addition to the well-known neurotoxin which inhibits neuromuscular transmission in animals. The

botulinum neurotoxin has been studied intensively because of its extraordinarily potent toxicity and the uniqueness of its site of action (see reviews by Cull-Candy *et al.*, 1976; Simpson, 1981; DasGupta, 1993). In contrast, little is known about Blyn. Production of Blyn by the bacteria is easily recognized by the β-hemolytic halos around the colonies of the organisms on blood agar plates and by rapid lysis of erythrocytes incubated with the culture supernatant of liquid culture of *C. botulinum*. The hemolytic activity of the culture supernatant is lost spontaneously and reactivated by thiol-reducing reagents such as dithiothreitol and cysteine. Addition of a small amount of cholesterol inactivates the hemolytic activity irreversibly. Based on these characteristics of hemolytic activity, Blyn is classified into a group of thiol-activated bacterial hemolysins (Smyth and Duncan, 1978; Bernheimer and Rudy, 1986; Freer, 1988). The group consists of 15–17 polypeptides produced by pathogenic strains of four genera of bacteria, *Streptococcus*, *Clostridium*, *Bacillus* and *Listeria*. The hemolysins in this group are all lethal to experimental animals, but their pathogenic roles in the disorders elicited by the infection by those bacteria are still unclear. Since streptolysin O (SLO), a representative of this group, was reported to be cardiotoxic (Bernheimer and Cantoni, 1945, 1947; Cantoni and Bernheimer, 1945; Kellner *et al.*, 1956; Halbert *et al.*, 1961; Reitz *et al.*, 1968), by analogy, some reviews have claimed that Blyn is also cardiotoxic (Smyth and Duncan, 1978; Bernheimer and Rudy, 1986; Freer, 1988). However, no direct evidence showing the cardiotoxicity of Blyn has been reported, probably because the purified preparation of Blyn, without contamination by the potent botulinum neurotoxin, was not available.

Recently, we established a method for purification of Blyn from the culture supernatant of *C. botulinum* type C (Haque *et al.*, 1992). The purified Blyn is a single protein with a mol. wt of about 58,000, having hemolytic activity of 2100 hemolytic units (HU) per microgram of protein. This enabled us to study the action of Blyn without interference by the potent neurotoxin. The purified preparation of Blyn kills mice within 30 min without signs characteristic of botulism. The LD_{50} of the preparation is estimated to be 310 ng of protein for a 20 g mouse. This relatively high lethal toxicity of Blyn prompted us to study the pathophysiology of the lethal intoxication by the toxin and examine whether Blyn is actually a cardiotoxin as predicted by analogy with SLO.

MATERIALS AND METHODS

Botulinolysin

Botulinolysin was purified from the culture supernatant of *C. botulinum* type C strain 203 as described previously (Haque *et al.*, 1992). Briefly, the culture supernatant of 16 hr culture of the bacteria in a fortified cooked meat medium (Kurazono *et al.*, 1985) was fractionated by 60% saturation of ammonium sulfate. The toxin was extracted from the precipitate and purified by a combination of anion-exchange chromatography on DEAE-Sepharose CL-6B, gel-permeation chromatography through Sephadex G-75, and cation-exchange chromatography on SP-Toyopearl 650M. The purified preparation of the toxin gave a single protein band at a position corresponding to a mol. wt of 58,000 on 10% polyacrylamide gel electrophoresis with sodium dodecylsulfate. Fresh preparation of the toxin had specific hemolytic activity of about 2100 HU/μg of protein for rabbit erythrocytes, and its mouse (20 g) LD_{50} by i.v. injection was calculated as 310 ng. The purified preparation was filtered through a membrane filter with a pore size of 0.2 μm (Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A.) and kept aseptically in a refrigerator. Because of relatively rapid inactivation of the toxin preparation (half-life of the activity is about 2 weeks), hemolytic activities of the toxin preparations were determined just before the experiments.

Assay of hemolytic activity

Hemolytic activity of Blyn was routinely determined with rabbit erythrocytes. The toxin was diluted two-fold serially with gelatin Tris-buffered saline (GTBS). The composition of GTBS is: NaCl, 150 mM; KCl, 4.5 mM;

$MgSO_4$, 2.0 mM; $CaCl_2$, 1.0 mM; Tris, 20 mM; gelatin, 0.1%; glucose, 10 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 $1000 \times g$ for 10 min and the absorbance of the supernatant at 540 nm was measured. The absorbance value of supernatant obtained from erythrocyte suspension treated with 0.1% Triton X-100 in GTBS was used as a value of 100% hemolysis. One hemolytic unit was defined as the amount of hemolysin that causes 50% hemolysis under these conditions.

Recording of carotid blood pressure, electrocardiogram and ventilation air flow in rats

The method used is essentially the same as described previously (Sugimoto *et al.*, 1991). In brief, female Sprague–Dawley rats weighing 250–350 g were anesthetized by i.p. injection of pentobarbital sodium (50 mg/kg) prior to surgical procedure. The trachea was cannulated with a glass cannula connected to an air resistance tube (TV-241T, Nihonkohden, Tokyo, Japan) to monitor ventilation air flow (VAF) with a differential pressure transducer (TP-602T, Nihonkohden, Tokyo, Japan) and an amplifier (AR-601G, Nihonkohden, Tokyo, Japan). The right common carotid artery was cannulated with a polyethylene tube filled with heparinized saline and connected to a pressure transducer (TP-200T, Nihonkohden, Tokyo, Japan) to record carotid blood pressure with an amplifier (AP-601G, Nihonkohden, Tokyo, Japan). The standard lead II electrocardiogram (ECG) was recorded by inserting needle electrodes into the right forelimb and the left hindlimb. Blyn was diluted to an appropriate concentration with Krebs bicarbonate buffer (Krebs solution; see below for composition) containing 0.2% gelatin. The diluted toxin (0.5 ml) was injected through a cannula inserted into a right femoral vein.

In vitro *perfusion experiments on isolated rat organs*

Female Sprague–Dawley rats anesthetized as described above were heparinized by i.v. injection of 200 IU. The rats were then killed by bleeding. Heart, liver, lung and left kidney were each dissected out and cleaned from surrounding tissue after cannulation of aorta, portal vein, pulmonary artery and left renal artery, respectively. Each organ was perfused by a peristaltic pump (Minipuls 2, Gilson, Ganbetta, France) with Krebs solution prewarmed to 37°C and oxygenized by continuous bubbling with a mixture of 95% O_2 and 5% CO_2. A constant flow rate of 3.6 or 3.0 ml/min was used for the perfusion of isolated heart, lung and liver. For the perfusion of kidney, we chose a flow rate of 1.8 ml/min, because the resistance of kidney was too high to perfuse at a flow rate of 3.6 ml/min. Perfusion pressure was recorded by an amplifier (AP-601G, Nihonkohden, Tokyo, Japan) via a pressure transducer (TP-200T, Nihonkohden, Tokyo, Japan) connected to a side arm of the organ cannula. Tension elicited by spontaneous contraction of the isolated heart was recorded via a thread attached to the apex of the heart and connected to an isometric tension transducer (TB-651T, Nihonkohden, Tokyo, Japan) under a preload of 2.0 g. Organs were equilibrated for at least 30 min to the perfusion with Krebs solution. In experiments with acetylsalicylic acid or quinacrine, perfusion fluid was switched to the Krebs solution containing the reagent and the organs were equilibrated to the perfusion fluid for an additional 30 min. Botulinolysin was first diluted appropriately with Krebs solution containing 0.2% gelatin, and 100 μl of the diluted toxin was mixed with the perfusion fluid without gelatin. Then the toxin solution was perfused to the organs after the equilibration period using two three-way valves to avoid air embolism in the organs. The perfused fluid was collected every 2 min by fraction collector (FRAC-100, Pharmacia, Uppsala, Sweden) with a suitable delay. Concentration of potassium and activity of creatine kinase in the fractions of the perfused fluid were measured by a flame photometer (Model 750, Hitachi Ltd, Tokyo, Japan) and an automatic analyzer (Model 736-40, Hitachi Ltd, Tokyo, Japan), respectively. In experiments with verapamil (10^{-6} M), the reagent was added to the toxin solution, because long-term perfusion of verapamil at the concentration caused gradual increase in perfusion pressure of the isolated heart.

Contraction of isolated atrial preparations of rats

Hearts were isolated from anesthetized rats as described above, then atria were freed from ventricular tissue in Krebs solution prewarmed to 37°C and oxygenized with bubbling with mixed gas of 95% O_2 and 5% CO_2. The tip of the left auricle of the atrial preparation was fixed to a tissue holder and the preparation was transferred to a 10 ml water-jacketed tissue bath containing Krebs solution kept at 37°C and bubbled continuously with the mixed gas. The tip of right auricle of the preparation was connected to an isometric tension transducer (TB-651T, Nihonkohden, Tokyo, Japan) with a thread. The tissue was allowed to equilibrate at a resting tension of 1.0 g in the bath for at least 60 min, and Krebs solution in the bath was changed every 15 min during the equilibration period. After the equilibration period, 100 μl of Blyn solution diluted appropriately with Krebs solution containing 0.2% gelatin was added directly to the bathing solution. After incubation with Blyn for 14 min, the atrial preparations were washed four times with toxin-free Krebs solution. In the experiments to determine the inotropic and chronotropic responses of the atrial preparations to isoproterenol, the preparations were exposed to a certain concentration of the reagent after the equilibration period to obtain a control value of the responses. Then the preparations were washed four times with fresh Krebs solution and allowed to recover for 30 min. After the recovery time, the preparations were treated with Blyn for 14 min followed by washing four times with

toxin-free Krebs solution and a resting period of 20 min. Then the preparations were exposed to the same concentration of isoproterenol as used before the toxin treatment.

Statistical analysis

All the data obtained in the experiments were recorded on paper using a pen recorder (RJG-4124, Nihonkohden, Tokyo, Japan) and also stored on magnetic tapes using an FM data recorder (XR-310, TEAC, Tokyo, Japan). In some experiments, changes in perfusion pressure of isolated rat organ elicited by perfusion of Blyn were expressed as percentages of the pressure before the toxin treatment. Others are expressed in absolute terms. Values are presented as mean ± S.E. and n indicates the number of animals, organs or tissues in each group.

Reagents

The concentrations of the constituents of Krebs solution in mM were NaCl, 124; KCl, 5; KH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; glucose, 10; EDTA, 0.03. Acetylsalicylic acid and DL-isoproterenol were purchased from Wako Pure Chemicals Industry (Tokyo, Japan). Quinacrine dihydrochloride and verapamil were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Acetylsalicylic acid and quinacrine were dissolved directly in Krebs solution and the pH of the solution was adjusted with NaOH solution. Isoproterenol and verapamil were dissolved in distilled water at concentrations of 5×10^{-2} M and stored at −70°C.

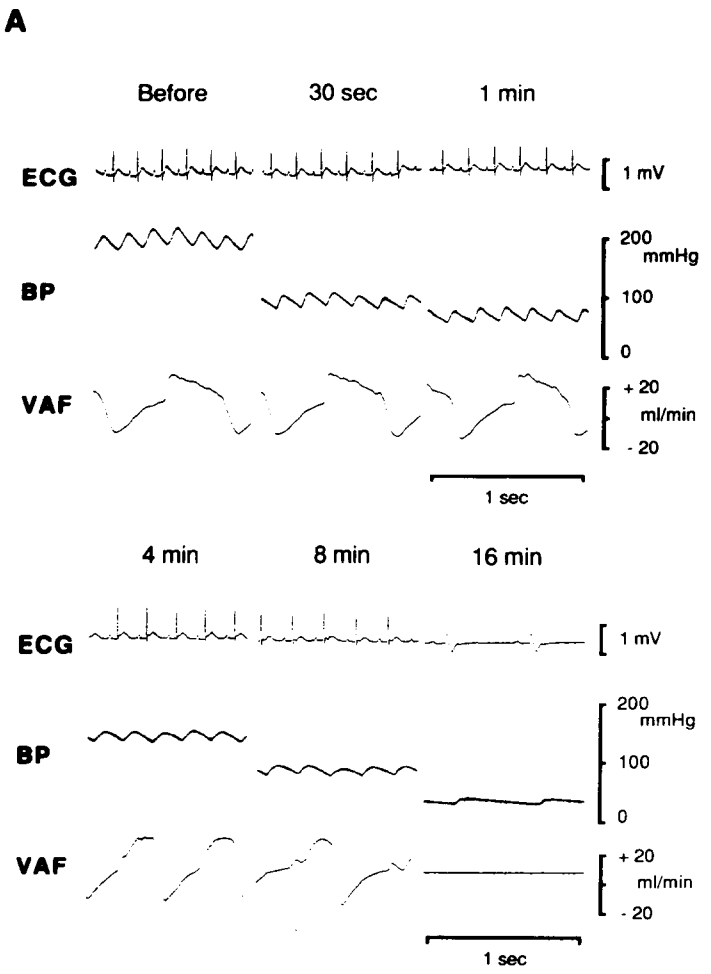

Fig. 1A

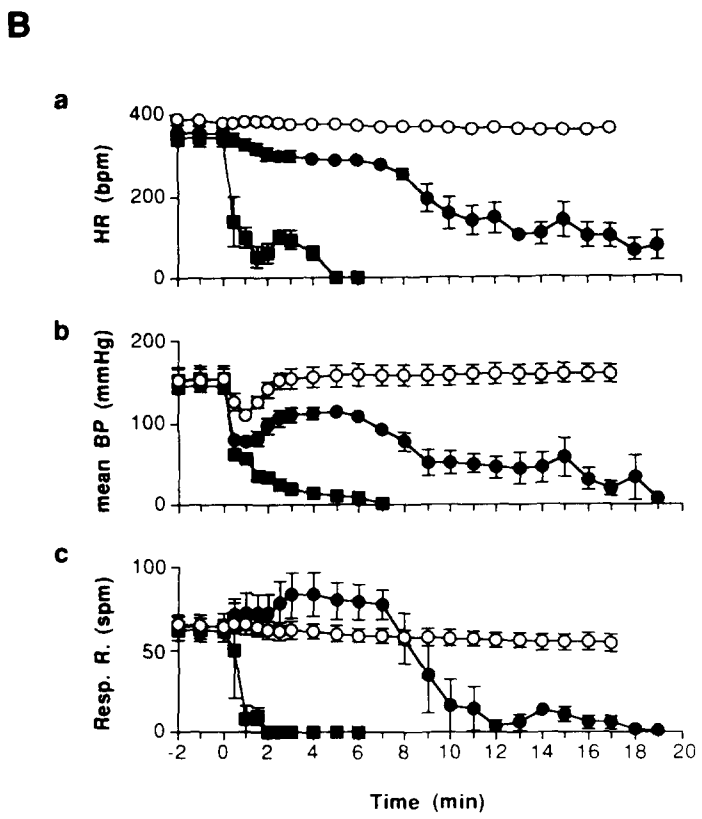

Fig. 1. Effects of intravenous injection of Blyn on electrocardiogram (ECG), carotid arterial blood pressure (BP) and ventilation air flow (VAF) of anesthetized rats.

(A) Representative traces of ECG, BP and VAF recorded from a rat injected i.v. with 1000 HU of Blyn. The rat ceased respiration 14.1 min after the toxin injection. Note that ECG pattern characteristic to hyperpotassemia, such as depressed P wave, prolonged QRS complex and peaked T wave, did not appear while the animal was alive. (B) Summary of the effects of Blyn on vital indices of rats injected with 10,000 HU (■), 1000 HU (●), and 100 HU (○) of the toxin. Heart rates in beats per minute (bpm), mean carotid blood pressures in mmHg, and respiration rates in strokes per minutes (spm) are presented in (a), (b), and (c), respectively. Values are mean ± S.E. ($n = 3$–5).

RESULTS

In vivo *intoxication by intravenous injection of Blyn*

Rats intoxicated with 10,000 HU and those intoxicated with 1000 HU of Blyn by i.v. injection ceased respiration 2.0 ± 0.6 and 15.6 ± 2.0 min (mean ± S.E. $n = 3$ and 5) after the injection, respectively. Rats intoxicated with 100 HU of Blyn survived. Figure 1A shows typical examples of ECG, carotid artery BP and VAF recorded from a rat before and after the injection of 1000 HU of Blyn. As shown in Fig. 1, ECG of the intoxicated rat showed progressive bradycardia and depression of T wave. The changes in ECG characteristic of hyperpotassemia, such as depressed P wave, prolonged QRS complex and peaked T wave, were not observed while the rat was alive, although prolongation of QRS complex appeared after the cessation of respiration. The injection of Blyn elicited an

abrupt fall in BP just after the injection, and then BP recovered partially and transiently. Respiration rate increased during the period of the partial recovery of BP, but thereafter it decreased gradually and became irregular, then finally stopped. Figure 1B shows the summary of changes in heart rate (HR), mean carotid BP and respiration rate (Resp. R.) of rats intoxicated with 10,000, 1000 and 100 HU of Blyn. Changes in HR and Resp. R. of the intoxicated rats differed in their patterns depending on doses of the toxin. Heart rates decreased rapidly in rats intoxicated with 10,000 HU and gradually in rats intoxicated with 1000 HU of Blyn. However, rats intoxicated with 100 HU of Blyn showed no remarkable changes in HR. Respiration rate was also stable in rats intoxicated with 100 HU of Blyn, while it decreased rapidly in rats intoxicated with 10,000 HU, and first increased then decreased gradually in rats with 1000 HU of Blyn. An abrupt fall in BP immediately after the injection of the toxin was observed in all the intoxicated rats. Blood pressure thereafter recovered completely in rats intoxicated with 100 HU, recovered partially and transiently in rats with 1000 HU, and did not recover in rats with 10,000 HU of Blyn.

Pink frothy secretions were in some cases observed in the glass cannula inserted into the trachea of the rats intoxicated with 1000 HU of Blyn after their respiration became irregular. Marked congestion and edema in both lungs were observed at autopsies performed immediately after the cessation of respiration. Congestion of other organs or bleeding in abdominal and thoracic cavities was not apparent. The blood obtained from the rats intoxicated with 1000 HU of Blyn after the cessation of respiration was transparent, indicating intravascular hemolysis. However, the blood obtained from the rats intoxicated with 10,000 HU was still turbid, although it became transparent after an incubation for about 10 min at room temperature.

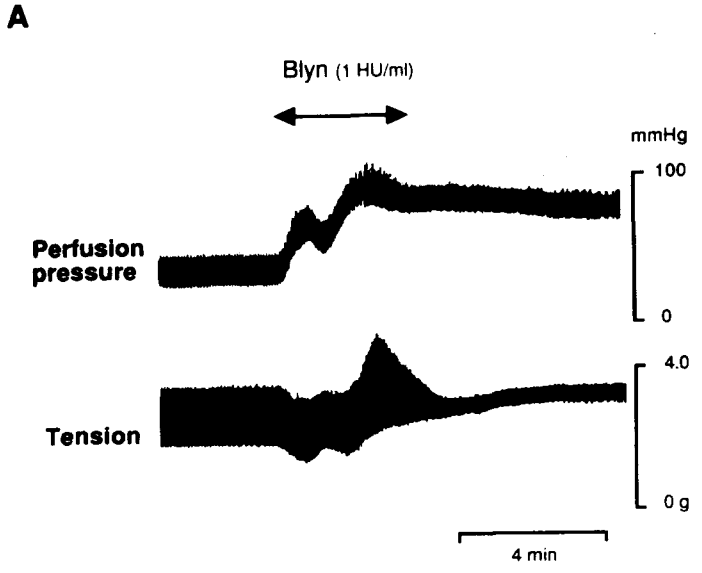

Fig. 2A

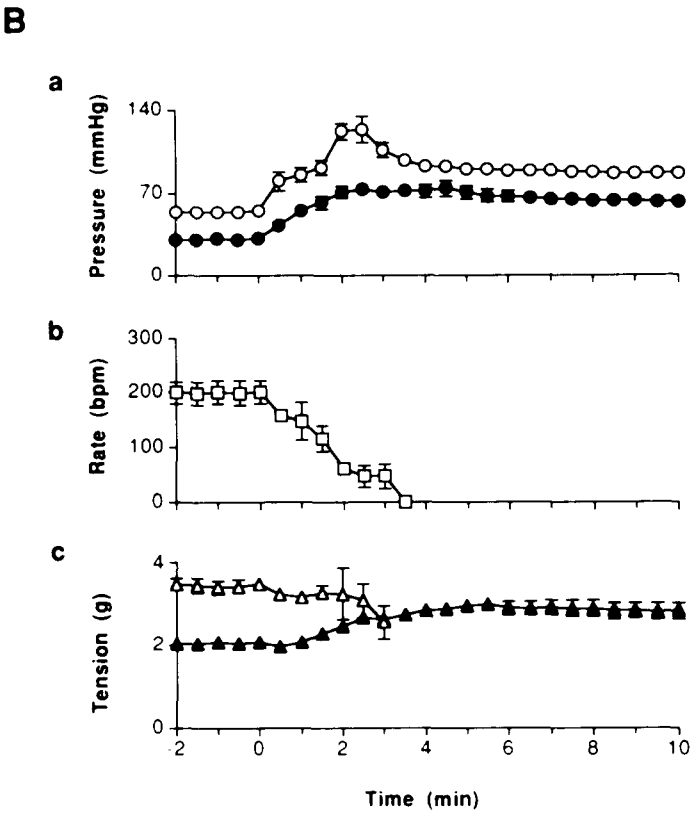

Fig. 2. Effects of perfusion of isolated rat hearts with Blyn.
Hearts were dissected out from the heparinized and exsanguinated rats, and were perfused via a cannula inserted into an ascending aorta with Krebs solution at a constant flow of 3.6 ml/min. the perfusion pressure and the tension of the apex as the index of spontaneous contraction movements of the preparation were recorded. (A) Representative traces of the perfusion and the apical tension of an isolated rat heart before, during and after the perfusion of Blyn (1 HU/ml, 10 ml). The horizontal line with arrows above the traces in the figure indicates the period of the toxin perfusion. (B) Summary of the perfusion experiments on isolated rat hearts. Time 0 on the abscissa indicates the beginning of the perfusion of Blyn (1 HU/ml, 10 ml). (a) Perfusion pressures in mmHg. ○, Peak (systolic) pressure; ●, basal (diastolic) pressure. (b) Rates of spontaneous contractions (□) in beats per minute (bpm). (c) Tension of apices of the hearts in grams (g). △ Peak (systolic) tension; ▲, basal (diastolic) tension. Values are mean ± S.E. ($n = 7$).

In vitro *perfusion of Blyn to isolated rat heart*

Figure 2A shows representative traces of a perfusion pressure and an apical tension before, during and after the perfusion of Blyn (1 HU/ml, 10 ml) to an isolated rat heart, and results obtained from similar experiments are summarized in Fig. 2B. Under the experimental conditions, isolated hearts spontaneously contracted regularly. The rate of contractile movements monitored as changes in apical tension was 200 ± 21/min (mean ± S.E., $n = 7$) before the toxin treatment. Perfusion of Blyn (1 HU/ml, 10 ml) decreased the rate of spontaneous contraction and the contraction ceased 3.0 ± 0.4 min (mean ± S.E., $n = 7$) after the beginning of perfusion of the toxin. Basal (diastolic) tensions of the apices of the hearts increased gradually from the beginning of the perfusion of Blyn

and were maintained high after the switch of perfusion fluid from the toxin solution to the toxin-free Krebs solution. Spontaneous contraction of auricles remained noticeable even after the cessation of the contraction of the ventricles. Perfusion pressure of the isolated hearts fluctuated regularly according to spontaneous contraction of the hearts. Peak (systolic) pressure and basal (diastolic) pressure before the toxin treatment were 53.5 ± 6.4 mmHg and 30.7 ± 1.3 mmHg (mean ± S.E., $n = 4$), respectively, under a constant flow rate of 3.6 ml/min. As shown in Fig. 2A, as soon as Blyn reached the heart, both peak and basal perfusion pressures rapidly increased, and reached maxima of 123.5 ± 11.1 mmHg (peak) and 73.4 ± 5.0 mmHg (basal) about 2.5 min after the beginning of perfusion of the toxin. Varying with the preparations, a more or less pronounced 'fast' peak was noticeable about 0.5 min after the beginning of the toxin perfusion (Fig. 2A). Perfusion pressure was maintained at a high level even after the cessation of the spontaneous contractions of the hearts, and fluctuation due to pulsatile movement of the peristaltic pump became predominant in records. Figure 3 shows the effect of Blyn on the concentration of potassium and the activity of creatine kinase (CK) in the perfusion fluid in relation to the change of perfusion pressure. Concentration of potassium in perfusion fluid was little influenced by the addition of Blyn (1 HU/ml, 10 ml). The activity of CK in perfusion fluid began to increase about 6 min after the addition of Blyn and increased gradually thereafter, while the perfusion pressure increased immediately after the perfusion of the toxin (Fig. 3). Addition of 10^{-3} M of acetylsalicylic acid to the perfusion fluid did

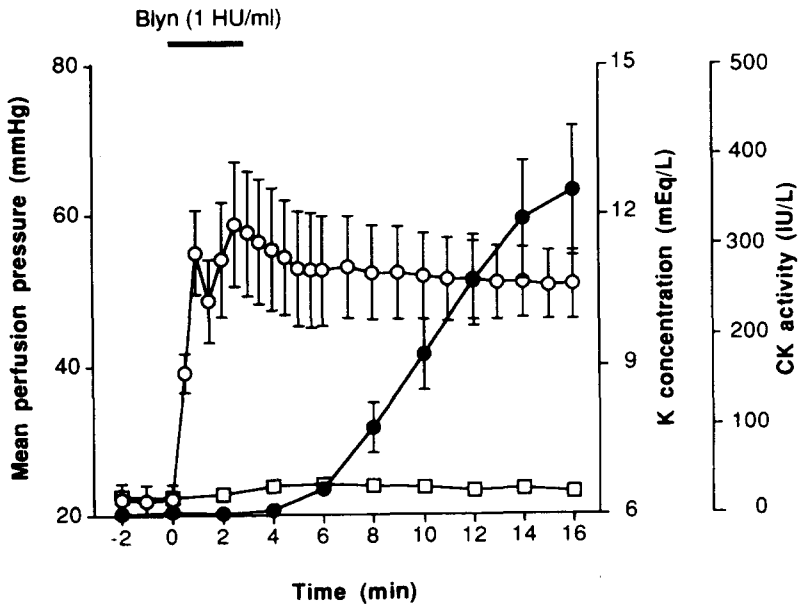

Fig. 3. Effects of Blyn on concentration of potassium and activity of creatine kinase in relation to the change of mean perfusion pressure.

The isolated rat hearts were perfused with Krebs solution at a constant flow rate of 3.0 ml/min. The perfusion pressure was recorded and perfused fluid was fractioned every 2 min. The concentration of potassium (K) and the activity of creatine kinase (CK) in each fraction were measured. ○, Mean perfusion pressure; ●, CK activity; □, concentration of K. From time 0 in the figure, Blyn (1 HU/ml, 10 ml) was perfused to the hearts as indicated by a horizontal line above the plot-frame. Values are mean ± S.E. ($n = 4$).

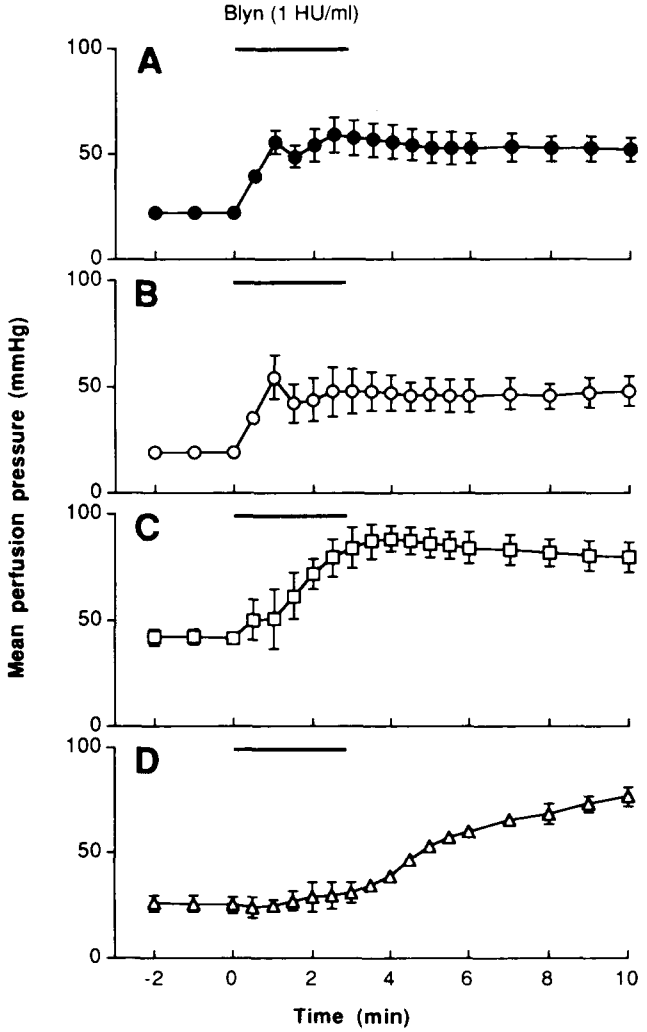

Fig. 4. Effects of acetylsalicyclic acid, quinacrine dihydrochloride and verapamil in the perfusion fluid on the Blyn-induced elevation of perfusion pressure in isolated rat hearts.
The conditions for perfusion of isolated rat hearts are the same as described in the legend for Fig. 3. (A) Krebs solution without drug; (B) with acetylsalicylic acid (10^{-3} M); (C) with quinacrine dihydrochloride (10^{-5} M); (D) with verapamil (10^{-6} M). From time 0 in the figures, Blyn (1 HU/ml, 10 ml) was perfused to the hearts as indicated by a horizontal line above each plot-frame. Values are mean $\pm$ S.E. ($n = 4$–6).

not influence the effects of Blyn on the perfusion pressure and spontaneous contraction of the heart (Fig. 4B). Quinacrine (10^{-5} M) itself increased the basal perfusion pressure and decreased the peak pressure and the apical tension of the preparations, but essentially did not influence the effects of Blyn (Fig. 4C). In contrast, as shown in Fig. 4D, verapamil (10^{-6} M) inhibited the initial rapid increase in perfusion pressure caused by Blyn, although perfusion pressure increased after the switch of perfusion fluid to Krebs solution without verapamil and the toxin.

Effects of Blyn on isolated atrial preparations

To examine the direct effects of Blyn on the myocardium, isolated and spontaneously beating atrial preparations were used. Tension and frequency of spontaneous contraction of the atria were little influenced by Blyn. No significant changes in tension and contraction rate were observed by the treatment of the atria with 1 HU/ml of Blyn. Treatment of the preparations with 60 HU/ml of Blyn caused slight and reversible increases in tension and rates of the spontaneous contractions (Fig. 5). However, inotropic and chronotropic responses of the atria to isoproterenol were not influenced significantly by the treatment with 60 HU/ml of Blyn (Fig. 6).

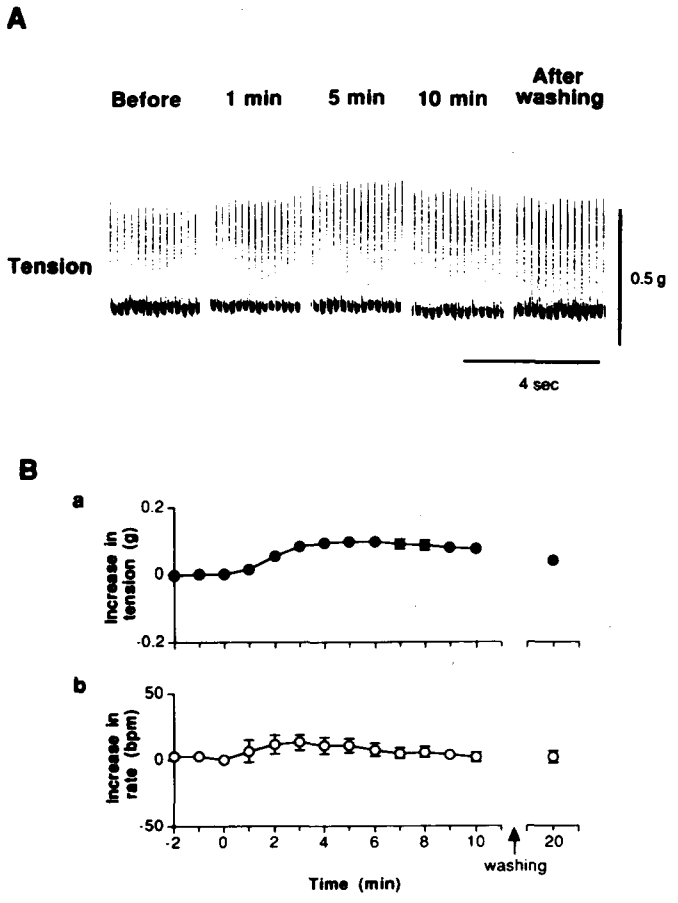

Fig. 5. Effects of Blyn on spontaneous contraction of isolated rat atrium.

Atria were dissected out from the isolated hearts of rats and tension elicited by spontaneous contraction of atrium was recorded. (A) Representative traces of spontaneous contract of an atrium before, during and after the addition of Blyn (60 HU/ml) into bathing solution of the preparation. (B) Summary of the effects of botulinolysin on tension and rate of spontaneous contraction of isolated rat atria. At time 0 in the figure, Blyn (60 HU/ml) was added to the bathing solution. (a) Increase in contractile tension; (b) increase in rate of spontaneous contraction. Values are mean $\pm$ S.E. ($n = 4$).

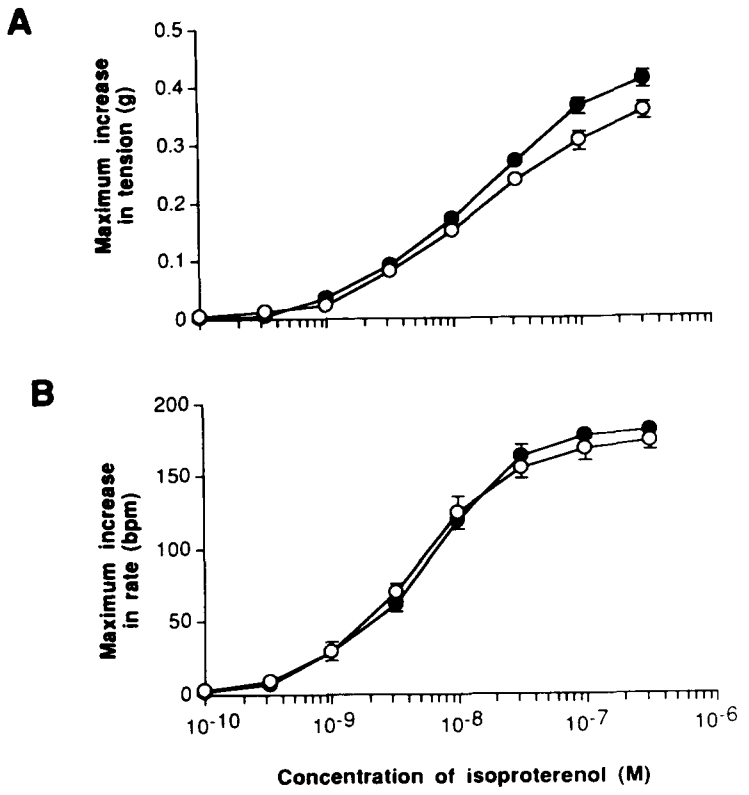

Fig. 6. Effects of Blyn on inotropic and chronotropic responses of isolated rat atria to isoproterenol.
Maximum increases in tension and rate of spontaneous contraction of isolated rat atrial preparations elicited by various concentration of isoproterenol were measured before (○) and after (●) treatment with 60 HU/ml of Blyn. Values are mean ± S.E. ($n = 4$).

In vitro *perfusion of Blyn to kidneys, lungs and livers*

To observe the effects of Blyn on resistant blood vessels, Blyn was perfused to isolated rat kidneys, lungs and livers in addition to hearts. Pressures required for a constant perfusion flow rate were significantly different between organs. Livers and lungs showed low resistance. Under the constant flow rate of 3.6 ml/min, livers and lungs showed perfusion pressures of 5.8 ± 1.2 and 10.0 ± 1.2 mmHg (mean ± S.E., $n = 3$ and 4), respectively. Kidneys and hearts, however, showed high resistance. In particular, the pressure required for the perfusion of kidneys was so high that the flow had to be reduced to 1.8 ml/min, which produced 57.5 ± 7.3 mmHg ($n = 4$) of perfusion pressure. In spite of the differences of the basal perfusion pressures between organs, perfusion of Blyn (1 HU/ml) caused remarkable elevations of the perfusion pressure in all of these organs, although the degrees and patterns of the elevation were different between organs (Fig. 7). The increase in perfusion pressure by the toxin was quicker in kidneys and hearts than in lungs and livers. And, similarly to the Blyn-induced elevation of perfusion pressure in hearts, the increase in perfusion pressure in kidney by Blyn was composed of two peaks, a 'fast' peak appearing approx. 0.5 min after

the beginning of toxin perfusion and a subsequent 'slow' peak appearing approx. 3 min after the beginning of the toxin perfusion. In contrast, the increases in perfusion pressures in lungs and livers were composed of single peaks, which appeared about 3 min after the beginning of the toxin perfusion. The lungs became edematous after the perfusion of Blyn while other organs showed no such changes in appearance after the toxin treatment.

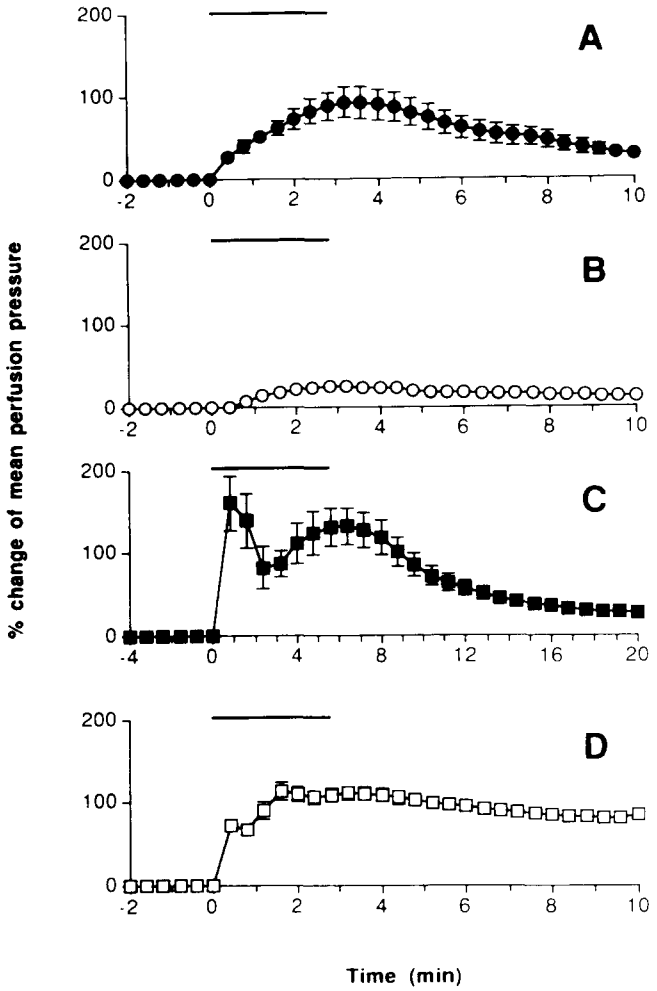

Fig. 7. Effects of Blyn on perfusion pressures of isolated rat lungs, livers, kidneys, and heart. Lungs, livers, left kidneys and hearts were dissected out from anesthetized and heparinized rats after cannulation of pulmonary arteries, portal veins, left renal arteries, and ascending aortae, respectively. Isolated organs were perfused with Krebs solution and mean perfusion pressure in each organ was recorded. A constant flow rate of 3.6 ml/min for lungs, livers and hearts, and a flow rate of 1.8 ml/min for kidneys were used. (A) lungs, (B) livers, (C) kidneys, (D) hearts. From time 0 in the figure, Blyn (1 HU/ml, 10 ml) was perfused to the organs as indicated by a horizontal line above each plot-frame. Mean perfusion pressures before, during, and after the toxin treatment in each organ are expressed as a percentage of those just before toxin treatment. ●, Lungs; ○, livers; ■, kidneys; □, hearts. Values are mean ± S.E. ($n = 3$–4).

DISCUSSION

The results presented above indicate that cardiac dysfunction elicited by coronary vasoconstriction is the most probable cause of death of the rats intoxicated by i.v. injection of botulinolysin, a thiol-activated hemolysin produced by *C. botulinum*. Intravenous injection of Blyn killed rats very quickly, suggesting that respiratory or circulatory system is the most possible *in vivo* target of the toxin. Rats intoxicated with i.v. injection of Blyn manifested a variety of signs of respiratory and circulatory systems including changes in ECG pattern, hypotension and respiratory malfunction. Among the signs, an abrupt fall in arterial BP immediately after the toxin injection was observed in all rats, including those intoxicated with a sublethal dose of Blyn. The results suggest that the primary feature of the intoxication with Blyn is hypotension. Possible mechanisms of the acute hypotension elicited by the toxin are: (1) a deficit of circulating blood volume due to massive bleeding; (2) an increase in peripheral blood pools caused by vascular collapse; and (3) cardiac dysfunction. The first possibility was excluded by the fact that no massive bleeding was observed in abdominal or thoracic cavities or parenchyma of large organs at autopsies. The second possibility of collapse of peripheral blood vessels is also not a likely cause of the hypotension because congestion was not remarkable in organs or tissues except lungs. In addition, perfusion of Blyn to isolated organs did not elicit a decrease but produced an increase in perfusion pressures of the organs. Thus, cardiac dysfunction is considered to be the most probable cause of the hypotension elicited by i.v. injection of Blyn to the rats.

Perfusion of Blyn (1 HU/ml $\approx$ 0.5 ng.ml) to isolated hearts caused a rapid and irreversible increase in perfusion pressure and cessation of spontaneous contractile movements. The amount of Blyn used to elicit *in vitro* toxicity in isolated hearts was as little as 1/100 of that used to cause animal death by *in vivo* toxicity of Blyn. The result indicates that Blyn is an extremely potent toxin with an apparently direct action on the heart. Hyperpotassemia due to intravascular hemolysis by the toxin is another possible mechanism of cardiac dysfunction. However, intravascular hemolysis was not apparent in the rats which died after i.v. injection of 10,000 HU of Blyn. In addition, changes in ECG pattern characteristic of hyperpotassemia were not observed in the rats intoxicated with 1000 HU of Blyn during the course of intoxication, although intravascular hemolysis was noticeable after the death of the animals. From these results, we conclude that cardiac dysfunction elicited by direct action of Blyn on the heart is the cause of death of the intoxicated rats.

The cardiotoxicity of Blyn *in vitro* first appeared as an abrupt elevation of perfusion pressure of the isolated heart. The elevation of perfusion pressure indicates the increase in resistance of the coronary circulation. There are two possible mechanisms to increase the resistance of coronary circulation: a myocardial contracture and a coronary vasoconstriction. The increase in basal (diastolic) apical tension after the perfusion of Blyn suggests myocardial contracture. The increase in activity of CK in perfusion fluid indicates the damage of myocardium after the perfusion of Blyn. However, CK activity showed gradual increase with a latent period of 6 min while perfusion pressure showed a rapid increase immediately after the beginning of Blyn perfusion. This difference in kinetics suggests that myocardial damage and increase in perfusion pressure are not linked directly. The results obtained from the experiments using spontaneously contracting atrial preparations also indicate that direct action of Blyn on myocardium is less possible. One hemolytic unit per milliliter of Blyn, which causes a striking elevation of perfusion pressure in isolated hearts, affected neither tensions nor rates of spontaneous contractions of isolated atria. Botuli-

nolysin at a very high concentration (60 HU/ml) increased tension and rate of spontaneous contractions of atria, but these effects of the toxin were reversible. Moreover, the inotropic and chronotropic responses of the atria to isoproterenol, a β-adrenergic agonist, were little affected by the treatment of the preparations with 60 HU/ml of Blyn. These results suggest that the myocardium is resistant to the action of Blyn, although there still remains the possibility that atrial and ventricular myocardiums are different in sensitivity against Blyn. Perfusion of Blyn (1 HU/ml) to isolated rat kidneys, lungs and livers also elicited elevations of their perfusion pressure, indicating that the toxin also caused vasoconstriction in these organs. Blood vessels in these organs are not surrounded by contractile muscles to compress the vessels like coronary arteries in hearts. Therefore, the results suggest that Blyn acts directly on the blood vessels and produces vasoconstriction in these organs. Thus, it is likely that the direct action of Blyn on blood vessels also causes coronary vasoconstriction in hearts. From this circumstantial evidence, we conclude the following mechanism as the action of Blyn on the isolated hearts: the rapid increase in perfusion resistance of the hearts is caused by the direct vasoconstrictor action of the toxin on the coronary arteries, and the gradual increase in basal (diastolic) apical tensions, cessation of spontaneous beats of the hearts and increase in CK activity in perfusion fluid are probably caused by anoxia resulting from the coronary vasoconstriction. This is supported by the fact that verapamil, a reagent known as a coronary vasodilator, inhibited at least in part the elevation of perfusion pressure elicited by Blyn. This may suggest the involvement of voltage-dependent calcium channels of the vascular tissue in the toxin action. The exact mechanism of antagonistic effects of verapamil on the action of Blyn remained to be clarified. Here arises an apparent inconsistency between the hypotension observed in *in vivo* intoxication and the vascular constriction found in *in vitro* perfusion experiments. However, BP *in vivo* is a product of the cardiac output and the peripheral resistance. If the cardiac output is markedly reduced, the animal shows hypotension no matter how the resistance of blood vessels in periphery increases. Thus, it is possible that the increase in resistance of peripheral blood vessels by Blyn is overwhelmed by the cardiac dysfunction elicited by the coronary vasoconstriction in the intoxicated rats.

Botulinolysin belongs to a group of bacterial thio-activated (oxygen-labile) hemolysins (Smyth and Duncan, 1978; Bernheimer and Rudy, 1986; Freer, 1988). The bacteria which produce these hemolysins are all pathogenic to humans and animals and sometimes cause systemic manifestations in addition to the local inflammations at the sites of infection. However, the roles of these hemolysins in pathogenesis of the infectious disorders are still unknown. Since streptolysin O (SLO), a representative of the thiol-activated bacterial hemolysins, was reported to be cardiotoxic (Bernheimer and Cantoni, 1945, 1947; Cantoni and Bernheimer, 1945; Kellner *et al.*, 1956; Halbert *et al.*, 1961; Reitz *et al.*, 1968), Blyn has been a priori thought to be also cardiotoxic without any direct evidence. Our present study disclosed a lot of similarities between the *in vivo* and *in vitro* toxicity of Blyn and of SLO. An abrupt fall in arterial blood pressure, changes in ECG pattern and congestion of lungs were also observed in the animals intoxicated with SLO (Barnard and Todd, 1940; Halbert *et al.*, 1961, 1963; Halpern and Rahman, 1968; Halpern *et al.*, 1969). Perfusions of SLO to isolated hearts of guinea-pigs and rats were reported to cause rapid reduction of coronary flow (Reitz *et al.*, 1968; Halpern and Rahman, 1968), which is similar to our observation in the experiment of perfusion of Blyn to the isolated rat hearts. Thus, actions of Blyn and SLO on animals and organs seem to be identical or at least very similar. The mechanism of cardiotoxic action of SLO is uncertain. Thompson *et al.* (1970) reported direct myocardial damage by SLO in beating mammalian heart cell in culture. However,

Goullet *et al.* (1963) and Reitz *et al.* (1968) reported that the isolated atrium and ventricular strips were mechanically and electrophysiologically insensitive to SLO. Our present study on Blyn may provide a clue to bring the argument to an end. Botulinolysin caused an elevation of perfusion pressures not only of isolated hearts but also of isolated kidneys, lungs and livers, although patterns and degrees of the elevation were different between organs. The results indicate that the vascular system is the actual target of Blyn and that cardiotoxicity is only a part of the toxin action. It is likely that SLO also affects the vascular system. Recently, staphylococcal α-toxin, *Pseudomonas aeruginosa* cytotoxin and *Escherichia coli* hemolysin, which are hemolytic bacterial toxins but are not members of the group of bacterial thiol-activated hemolysins, were reported to cause vascular constrictions and edema in lungs via production of eicosanoids in endothelial cells (Seeger *et al.*, 1986, 1989, 1990; Seeger and Suttorp, 1987; Grimminger *et al.*, 1991; Ermert *et al.*, 1992). In our experiments on isolated lungs, Blyn caused marked edema in addition to the elevation of the perfusion pressure. Thus, thromboxane and its related eicosanoids produced by the endothelium are the possible candidates for the second messenger of the toxin action. However, acetylsalicylic acid (1 mM), an inhibitor of cyclooxygenase, or quinacrine (10^{-5} M), an inhibitor of phospholipase A_2, in perfusion fluid did not inhibit the elevation of coronary perfusion pressure by Blyn. The exact mechanism of vascular constriction by Blyn remains to be clarified in further investigations, and such work is in progress in our laboratory.

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