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Some pharmacological properties of the venom, venom fractions and pure toxin of the yellow-bellied sea snake *Pelamis platurus*

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The effects of the crude venom, four partially purified venom fractions and pure toxin (Pelamis toxin *a*) from yellow-bellied sea snake, *Pelamis platurus*, on respiration, blood pressure, heart and skeletal muscle of rabbits have been examined. Results indicated that crude venom, a partially purified toxic fraction and Pelamis toxin *a* caused initial respiratory stimulant effects followed by respiratory paralysis. In most cases, respiratory paralysis occurred before a profound fall in arterial pressure. Depression of the twitch response to nerve stimulation was observed in the tibialis anterior muscle. No significant change in the electrocardiogram was seen. Three partially purified non-toxic fractions of the crude venom induced transient respiratory stimulant effects. It was concluded that the crude venom and Pelamis toxin *a* had an identical mode of action and that they caused respiratory paralysis in rabbits.

The yellow-bellied sea snake, *Pelamis platurus*, which is considered to be the most widespread sea snake in the world, is found commonly in the coastal waters of the Indo-Malayan region, and of Africa, Australia, Tasmania, Japan and southern Siberia. In the eastern Pacific, it is common in the Gulf of California, and in the coastal waters of southern Mexico, countries in Central America, Ecuador and Peru (Pickwell, 1972). The fatalities due to snakebites from this species have been reported in Mozambique and Central America (Swaroop & Grab, 1954). Death due to other species of sea snake are more common and well-documented by Reid (1963). Previous reports have shown that the crude venom of this snake has considerable toxicity in various animals: fowl and dogs (Fayrer, 1872); pigeon and mudfish (Rogers, 1903); guinea-pigs (Nauck, 1929); rats (Gail & Rageau, 1958); and mice (Barme, 1963).

Recently the purification of the crude venom of this snake has been attempted. Shipman & Pickwell (1973) obtained six partially purified fractions from the venom and the toxicity was tested in mice and two dogs (Pickwell, Vick & others, 1973). In our laboratory a pure toxin was isolated from the venom of *P. platurus*, through a two-step purification by column chromatography. The present study is concerned with the comparison of the effects of the crude venom, four venom fractions and pure toxin from this species on respiration, blood pressure, heart and skeletal muscle of rabbits.

MATERIALS AND METHODS

Venom

Sea snakes, *P. platurus*, captured off the west coast of Costa Rica in 1973, were decapitated and the venom glands excised. The dried glands of 3074 sea snakes were pulverized and the venom extracted with distilled water. After the insoluble tissue

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debris was removed by centrifugation, the supernatant liquid was lyophilized. The resultant amount of the crude venom obtained was 8·14 g in the dried form.

Purification procedures

1·5 g of the crude venom was applied to a Sephadex G-50 column (3·5 × 110 cm) previously equilibrated with 0·01 M potassium phosphate buffer (pH 6·5) containing 0·1 M NaCl. The eluate, collected in 3 ml aliquots, was monitored at 280 nm with an ISCO Model UA-2 dual beam ultraviolet analyser equipped with a recorder. The tubes from each protein peak were pooled and lyophilized. The samples were then desalted by passage through a Sephadex G-10 column (2·5 × 40 cm) and each was tested for toxicity.

The desalted, lyophilized, toxic fraction (Fraction IV) was dissolved in 2 ml of 0·01 M phosphate buffer (pH 7·8) and applied to a CM-cellulose column (2·5 × 70 cm). Elution was carried out by a stepwise increase in NaCl concentration, the flow rate being 9 ml h^{-1}. Individual fractions in each peak were combined. Each combined fraction was tested for toxicity after desalting with a Sephadex G-10 column.

All of the purification procedures were at 4°. For the determination of purity, discontinuous polyacrylamide gel electrophoresis was used (Hoefer Scientific Instruments). After the electrophoresis, the gels were removed and immersed in 12·5% trichloracetic acid for 1 h, stained by 0·025% Coomassie Blue for 2 h and destained with 7% acetic acid. The purity of the toxin was further determined by isoelectric focusing. A sucrose density gradient containing pH 9 to 11 ampholytes was established in the 110 ml LKB column. After applying 400 V for 60 h, the solution was collected in a fraction collector. The pH and the absorbance of the fraction were measured. The crude venom, venom fractions and pure toxin (toxin *a*) were stored in the dark at −12°.

Physiological methods

The LD50 in mice was calculated by the method of Litchfield & Wilcoxon (1949). Thirty-two New Zealand white male rabbits, 2·5 to 3·7 kg, were used.

The rabbits were anaesthetized intravenously with 30 mg kg^{-1} pentobarbitone sodium. The venom or Pelamis toxin *a* (see results) was dissolved in physiological saline shortly before use. The venom or toxin *a* and physiological saline were given in volumes of 0·2 ml kg^{-1} through the marginal vein of the ear. The injection of the venom or toxin *a* was carried out over 15 s and was preceded by a control injection of saline. Throughout the experiment, the room temperature was kept at 23 ± 1°. Four physiological events were recorded on a desk model physiograph DMP-4B (Narco Bio-Systems, Inc.). Respirations were recorded by measuring the impedance between two hypodermic needles applied to the thorax by means of an impedance pneumograph coupler. When necessary, artificial respiration was carried out with a Harvard respirator. This was done only towards the end of the experiment after the venom had produced respiratory paralysis. Blood pressure was recorded from the right carotid artery with a linear-core pressure transducer. Polyethylene tubing between the test animal and the recording device contained heparinized saline (4 mg 100 ml^{-1}). The electrocardiogram was recorded differentially from two hypodermic needles, one precordially, and the other in the left front leg. Contractions of the right tibialis anterior muscle of the rabbit, produced by delivering supramaximal 0·5 ms electrical stimuli once every 3 s with a Grass S48 stimulator to it's motor nerve, were

recorded on a myograph B transducer connected to a transducer coupler (type 7173). The electrocardiogram was recorded separately with the paper speed at 50 times that of the other physiological events. Immediately after the cessation of respiration and in the absence of blood pressure, the phrenic nerve, diaphragm, sciatic nerve and the tibialis anterior muscle were stimulated with square pulses of 2 ms duration, a frequency of 100 pulses s^{-1}, at a strength ranging from 1–100 V. Due to very limited amounts available, the physiological events of Fraction III and VI could not be studied, except for the LD50 value as determined in mice.

RESULTS

Fractionation

A major toxin, Pelamis toxin *a*, was isolated through two-step purification in Sephadex G-50 and CM-cellulose column chromatography (Fig. 1A, B). In the first fractionation (Fig. 1A), six fractions were obtained. The most toxic fraction was Fraction IV with an LD50 value of 0·093 mg kg^{-1} via the intravenous route compared to 0·44 for the crude venom in mice. The Fractions III and VI had LD50 values of 0·75 and 0·43 mg kg^{-1}, respectively. Other fractions (Nos. I, II, V_a and V_b) were determined to be non-toxic at the level of 10 mg kg^{-1} dosage. The most toxic fraction (No. IV) was further fractionated in a CM-cellulose column resulting in ten fraction

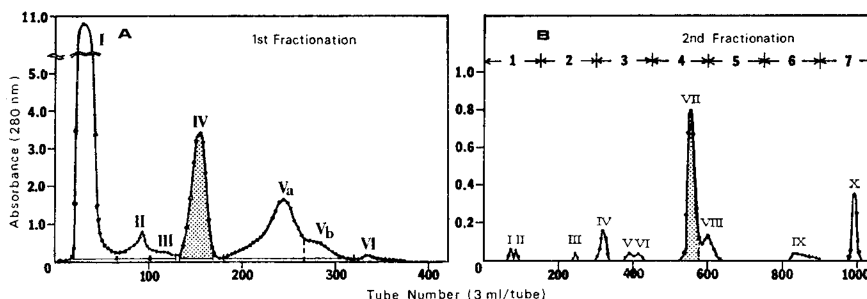

FIG. 1. Purification of Pelamis toxin *a* from the venom of *Pelamis platurus*. A. First step purification was made with Sephadex G-50 column. The most toxic fraction is indicated by the shaded area. B. Second step purification was made through CM-cellulose column. The fractionation was made through a step-wise increase of NaCl concentration in 0·01 M potassium phosphate buffer at pH 7·8. 1. No NaCl, 2. 0·03 M NaCl, 3. 0·05 M NaCl, 4. 0·075 M NaCl, 5. 0·1 M NaCl, 6. 0·15 M NaCl, 7. 0·40 M NaCl.

(Fig. 1B). The first three fractions were non-toxic at the 10 mg kg^{-1} dose level. The intravenous LD50 in mice for the fractions from IV to X were 0·15, 8·4, 7·4, 0·044, 0·31, 0·93 and 1·0 mg kg^{-1}, respectively. The most toxic fraction, No. VII, is called Pelamis toxin *a*. Homogeneity of the toxin was established by acrylamide disc gel electrophoresis and isoelectric focusing in a sucrose density gradient. Only one band was observed after electrophoresis of the toxin. The isoelectric profile obtained from the column indicated one band with an isoelectric point of 9·69, showing that the toxin is a highly basic protein (Tu, Lin & Bieber, 1975).

Effect of the crude venom

The injection of 0·01–0·1 mg kg^{-1} crude venom did not cause any cardiovascular or muscular effects. The injection of 0·5–1 mg kg^{-1} crude venom caused a gradual and marked increase of amplitude of respiration and a slight increase, no change or a slight

decrease in respiratory rate within 5 min after injection. The respiratory stimulant effect reached a maximum in 10–20 min. This was followed by respiratory paralysis. Death took place 15–40 min after injection.

When doses of the crude venom were increased to 2–3 mg kg^{-1}, respiratory stimulant effects became much more prominent. Animals died 12–15 min after injection, following the eventual respiratory paralysis. The changes of blood pressure, heart rate, ecg, the response of the tibialis anterior muscle to electrical stimuli (Fig. 2), and the effect of artificial respiration were much like those observed with the toxin.

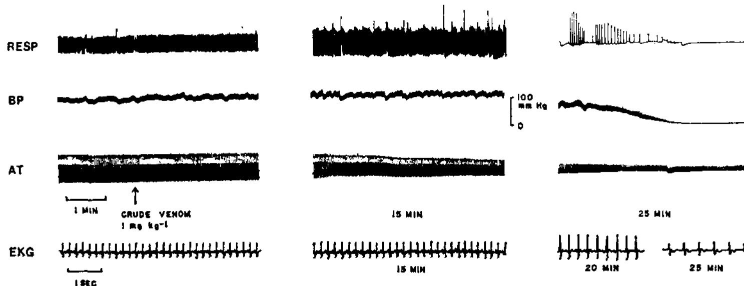

FIG. 2. Effects of crude venom (1 mg kg^{-1}) of *Pelamis platurus* in rabbit. RESP, respiration; BP, blood pressure; AT, twitch response of tibialis anterior muscle to stimulation of sciatic nerve; ecg, electrocardiogram. Note the different time base.

Effect of the partially purified toxic fraction (Fraction IV in the first fractionation)

The intravenous administration of 0·01 mg kg^{-1} Fraction IV produced no appreciable change in physiological events. With a dose of 0·05 mg kg^{-1} one out of three rabbits showed some changes in respiration and finally death. The intravenous administration of 0·1 mg kg^{-1} of Fraction IV caused changes in respiration, blood pressure, ecg and a response of the tibialis anterior muscle to indirect stimulation much like that observed with toxin.

Effect of the pure toxin

No appreciable change was observed in respiration, blood pressure, heart rate, ecg or skeletal muscle response of the rabbit after the intravenous administration of 0·001–0·01 mg kg^{-1} toxin *a*.

Respiration was unaffected during the first 5–10 min following the injection of 0·05–0·1 mg kg^{-1} toxin *a*. The amplitude of respiration, both inspiration and expiration, gradually increased and reached a maximum 15–20 min after injection. Respiratory rate showed either no change or a slight increase. This was followed by a gradual decrease of amplitude of respiration and a slow, progressive decrease in respiratory rate. The ensuing respirations were often shallow, irregular and tended to be abdominal in type. The animals died in approximately 35–60 min.

Blood pressure showed no change during the first 20–30 min, but later gradually increased. A rise (20–30 mm Hg) in blood pressure induced by asphyxia became apparent when respiration was severely depressed. At this time an increase in pulse pressure was observed. Blood pressure fell gradually after respiration was markedly impaired. Then, blood pressure declined at death shortly after complete cessation of respiration occurred.

The twitch response of the tibialis anterior muscle to nerve stimulation was gradually depressed to 70% of the maximal muscle contraction. No significant change in

ecg was noted until severe respiratory depression became apparent. When respiration was severely depressed, the tracing showed a marked bradycardia, decreased P-wave amplitude, increased T-wave amplitude and increased voltage followed by decreased voltage of the QRS (Fig. 3).

These changes appeared to be caused by hypoxia and bradycardia became progressively more severe before death. It was noted that the heart remained beating for a short period after complete respiratory arrest.

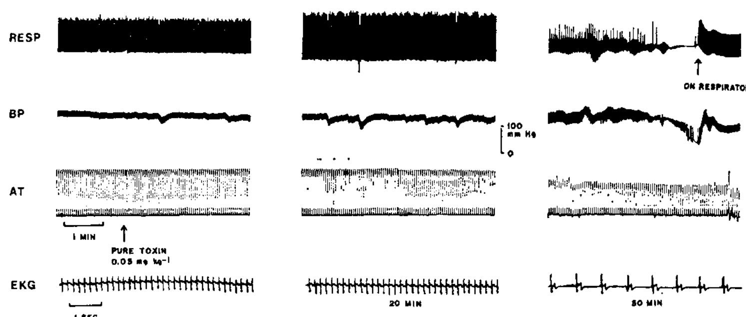

FIG. 3. Effect of pure toxin (0·05 mg kg^{-1}) of *Pelamis platurus* in rabbit. RESP, respiration; BP, blood pressure; AT, twitch response of tibialis anterior muscle to stimulation of sciatic nerve; ecg, electrocardiogram. Note the different time base. In the third panel, the rabbit was respired artificially.

When artificial respiration was carried out immediately after the cessation of respiration, declining blood pressure rose to nearly normal level, and the animal survived an additional 60–120 min.

Immediately after death occurred, the chest of the rabbit was opened by incision of the skin. The left and right phrenic nerves were separated from surrounding fatty tissues and the diaphragm exposed. Each nerve and the part of the diaphragm innervated by it was then electrically stimulated. In addition, electrical stimulations were given to the left and right sciatic nerves and the tibialis anterior muscles. The left and right phrenic nerve stimulation at a strength ranging from 1–100 V did not cause any response of the diaphragm, while the muscle of the diaphragm produced a contraction by direct stimulation at a strength of 2 to 3 V. Indirect stimulation of the sciatic nerve and direct stimulation of the tibialis anterior muscle induced responses. Macroscopically, no appreciable changes were found after death at the injection site or in any other tissue of the rabbit, except for cyanosis around the mouth.

Effect of the non-toxic fractions (Fractions I, II, V_a & V_b in the First Fractionation)

The injection of a 1 mg kg^{-1} dose of Fractions I, II or V_a resulted in remarkable respiratory stimulation within 10–30 min after injection. The amplitude of inspiration and expiration markedly increased and respiratory rate also increased. However, in some cases, respiratory rate decreased when the amplitude of respiration increased extensively. These stimulatory effects on respiration lasted approximately 20–40 min. Recovery took place within 1–1·5 h. Fig. 4 shows the respiratory changes produced. No significant change in blood pressure, heart rate, ecg or the twitch response of the skelatal muscle was observed. The injection of 0·01–1 mg kg^{-1} Fraction V_b did not induce any change in the physiological parameters measured.

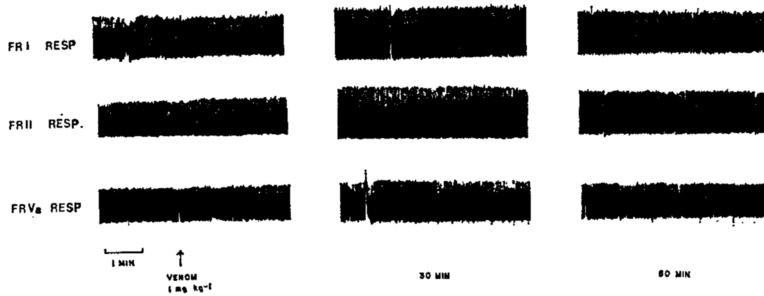

FIG. 4. Effect of venom Fractions I, II (1 mg kg^{-1}) of *Pelamis platurus* on respiration in the rabbit. FR fraction; RESP, respiration. Fraction Vb had no activity.

DISCUSSION

This study indicates that the crude venom, a partially purified toxin, and the pure toxin show essentially identical effects on respiration, blood pressure, heart and skeletal muscle. There were only quantitative differences among them. This suggests that the pharmacological activity of the crude venom of *Pelamis platurus* was not qualitatively influenced by possible contaminants in the venom.

The twitch response of the tibialis anterior muscle to indirect stimuli was depressed to some extent after the venom or toxin *a* was injected, although the response of the muscle to direct stimuli remained unaffected. Moreover, the phrenic nerve stimulation did not cause any response of the diaphragm after injection of the venom or toxin *a*, while the muscle of the diaphragm produced a contraction by direct stimulation. Therefore, it appears that nerve impulse transmission at the neuromuscular junction was interfered with by the venom or toxin *a*, and the extent of the interference was more severe at the neuromuscular junction of the diaphragm than at that of the tibialis anterior muscle. It has been demonstrated that the tibialis anterior muscle is more resistant to neurotoxic Bungarus venom than is the respiratory muscle (Lee & Peng, 1961), and that the concentration of I^{131}-labelled Bungarus venom and bungarotoxins in the diaphragm is higher than in the skeletal muscle (M. quadriceps femoris) after intravenous injection in rabbits (Lee & Tseng, 1966). The difference in the extent of respiratory paralysis and the block of the twitch response of the tibialis anterior muscle may be due to the same mechanisms shown in Bungarus venom or toxins. That is, it appears to be caused by the different distribution pattern of the venom or toxin *a* in the diaphragm and the tibialis anterior muscle and different sensitivity of the muscles to the venom or toxin *a*.

It has been reported that a labelled toxin, erabutoxin *b*, from the venom of the sea snake, *Laticauda semifasciata*, becomes localized at the endplates of the mouse diaphragm (Sato, Abe & Tamiya, 1970). Erabutoxin *a* and *b*, and three sea snake venoms from *Enhydrina schistosa*, *Hydrophis cyanocinctus* and *Lapemis hardwickii*, induce peripheral paralysis and block endplate receptors without affecting the muscle fibres or acetylcholine output at nerve endings in rats and cats (Cheymol, Barme & others, 1967; Cheymol, Tamiya & others, 1972). The character of the blockade is not clear in the case of the sea snake, *P. platurus*, but appears to be similar to that of other sea snakes which belong to the same family, *Hydrophiidae*.

The initial respiratory stimulant effect in the rabbit, which is a constant finding in our experiments, produced by the toxic venom fraction and crude venom of *P. platurus*,

was not observed in the dog by Pickwell & others (1973). This fact could be due to different amounts of the venom administered, or to the species difference of the experimental animals. It is also possible that some variation in potency exists between the venoms collected from different geographical locations or in different seasons. The initial stimulation of respiration by toxin *a* and the respiratory effects of Fractions I, II and V_a may be caused by the effect on the lungs. In the case of cobra venom, similar effect on the lung can be found and this effect is most probably mediated through the release of histamine and 5-hydroxytryptamine (Moran, Uvnos & Westerholm, 1962; Markwardt, Barthel & others, 1966). Since the sea snake venom is neurotoxic like cobra venom, the same mediation possibly occurs.

The initial precipitous fall in systemic arterial pressure of the rabbit, produced by the crude venoms of three snakes, *L. semifasciata* (Tu, 1959), *L. colubrina* (Yang, Lin & Tu, 1963) and *L. laticaudata* (Tu, 1963) was not produced by the crude venom of *P. platurus*. The cause of this phenomenon has not been studied, but may be related to the content of some constituent of the venom, such as phospholipase A (Lee, 1971).

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