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Original Article

Comparative Study on the Actions of Toxin Extracts from Two Different Puffer Fishes on I_{Na} and Respiratory N-M Transmission in the Rat

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Abstract   We performed a comparative study on the effects of toxin extracts prepared from muscle and liver of two different puffer fishes on voltage dependent sodium current (I_{Na}), and compared the results with that of tetrodotoxin (TTX). The amount of toxin contained in the muscle or liver expressed as an amount of equipotent TTX differed in the two species (0.11-57.98 µg TTX/g tissue). In addition, we observed the effects of TTX or toxin extracts on the twitch contraction evoked by direct muscle stimulation of the rat hemidiaphragm or indirect phrenic nerve stimulations, in an attempt to understand the mechanisms involved in the transmission failure in the respiratory muscles, due to the ingestion of TTX bearing puffers, and found that TTX or toxin extracts preferentially affect motor nerve rather than muscle.

Key words：Tetrodotoxin, Puffer toxin, Hippocampal CA1 neuron, Sodium channel, Hemidiaphragm, N-M transmission

Introduction

Tetrodotoxin (TTX) is one of the most potent and oldest known neurotoxin. TTX poisoning incidents due to ingestion of puffer fishes have frequently occurred in Asian countries including Taiwan, Hong Kong, Thailand and especially in Japan, where puffer fishes have been a traditional food for a long time.   The preferred forms of the delicacy are slices of raw fish (“Sashimi”), slightly cooked liver (“Kimo”), and puffer soup (“Fuguchiri”).   Japanese people are aware of the puffer fish toxicity and have devised ways to reduce TTX in liver[1)].   However, TTX poisoning incidents continue to occur in Japan yet.

TTX toxicity is commonly determined by mouse bioassay that is performed by intraperitoneal injection with TTX-associated extracts. After injection, the mice show the characteristic signs and symptoms, like unique scratching of shoulders/mouth by their hind limbs, weakness progressing to paralysis in hind limb, uncoordinated movement, shallowness of breathing, convulsions, and jumping, followed by respiratory failure. Toxicity of an extract is expressed in terms of mouse unit (MU), where 1 MU is defined as the amount of TTX required to kill a 20 g male of DDY or ICR strain in 30 minutes. Minimum detectable limits is about 0.2 µg TTX (1 MU) per an assay[2)].

We reported an electrophysiological bioassay of toxin extracts from puffer fishes. Namely, we observed the effects of toxin extracts from various tissues of wild and cultured puffer fish (*Takifugu rubripes*) quantitatively on voltage-dep-

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endent sodium current (I_{Na}) using single rat hippocampus CA1 neurons which express only TTX-sensitive voltage-dependent sodium channels in high density[3], and compared the results with that of TTX[4]. Assuming that the lethal dose of TTX is 1–10μg/kg[5], it was possible to estimate the maximal edible amount of each tissue. This is important especially for muscle, since muscle contains about 0.2μg TTX/g and estimated maximal edible amount of muscle was 300–3000g, for a man of 50kg body weight in the case of *T. rubripes*[4].

Although, *T. rubripes* is the most popular puffer fish for Japanese dishes, other puffer fishes are also eaten in Japan. However, till now no electrophysiological bioassay has been performed on the different species of the puffer fishes. Thus, it seems of interest and importance to study the toxin levels in the muscles, which is the main edible portion, excised from various species of puffer fishes by investigating the effects on I_{Na} qualitatively from a functional point of view. Therefore we firstly analyzed the effects of toxin extracts from muscle and liver from two different puffer fishes which commonly live in the sea surrounding Japan (Kyushu and Okinawa Islands), by use of CA1 neuron and electrophysiological bioassay technique[4]. Secondly, we observed the effects of toxin extracts on the twitch contraction of the rat hemidiaphragm evoked by phrenic nerve or direct muscle stimulations in the presence of curare d-tc (10^{-6} M) (hereafter referred as indirect and direct stimulations, respectively) in an attempt to study the cause of suffocation, which induces the human death due to the ingestion of puffer fishes.

Materials and methods

Puffers

In the present experiments, we used two different puffer species, namely *Takifugu rubripes* (*T. rubripes*, body wt 1.5–2.5 kg), and Arothron reticularis (*A. reticularis*, body wt 1.5–2.0 kg). Female puffer *T. rubripes* captured in the Sea of

Japan near Fukuoka Prefecture, Japan, and *A. reticularis* captured, Okinawa ocean near Okinawa Prefecture, from November 2007 to March 2008, were used in the present experiments.

Toxin extracted from puffers

Toxin extracts were prepared according to the method described elsewhere[4)6]. Briefly, tissues including liver, ovary, and muscle were excised from puffer fish by a Japanese fugu-licensed chef, transferred into an ice-cold box and transported to the laboratory and kept frozen below − 20 ℃. Frozen samples were divided into pieces of about 20 g, and each piece was homogenized with a small amount of 2% acetic acid-methanol (v/v) under refluxing conditions for 10 min, and centrifuged at 2500 rpm for 15 min at room temperature (21–25 ℃). The supernatant was filtered through a Kiriyama funnel. The filtrate was evaporated under reduced pressure, while the sediments were combined for a second cycle of extraction. Each extraction was carried out 3 times. The residue was diluted with purified water (15 ml), and the solution was extracted with diethyl ether (15 ml) 3 times to remove the hydrophobic component. The water phase was evaporated under reduced pressure and the residue, diluted with 1.5 ml citrate buffer (pH 4.8), was kept frozen below − 20 ℃.

In each experiment, the citrated stock solution contained (mM) ; NaCl 60, choline Cl 100, CsCl 5, CaCl$_2$ 2.5, glucose 10, LaCl$_3$ 0.01, TEA-Cl 5, HEPES 10 and pH was adjusted to 7.4 with Tris-base at room temperature, and the hundred-fold dilution was subjected to further toxicity bioassays (to foil other factors such as electrolyte and hydrophilic components from puffer tissue).

In puffer fish, TTX usually exists as mixtures of its analogs (TTXs)[7], and it is known that 4-epi TTX and 4, 9-anhydro TTX are chemically equivalent to TTX[8]. Recent studies with LC/ MS or LC-FLD showed the presence of 5-deoxy TTX, 11-deoxy TTX, 5, 6, 11-trideoxy TTX or

other TTXs, and saxitoxins (STXs) in marine puffer fish Fugu pardalis collected in Japan[9]. This study also revealed that 5-deoxy TTX, 5,6, 11-trideoxy TTX, 4-Cys TTX and 4,9-anhydro TTX are almost non-toxic. It was also reported that Torafugu pardalis[10], Takifugu poecilonotus, Takifugu vermicularis[11] and Arothron firmameutum[12] contain paralytic shellfish poisoning components, STX and decarbamoyl STX. Therefore, it seems that the effects of toxin extracts on I_{Na} observed in the present experiments are the total sum of biologically active analogs of TTX and STX. However, it is reasonable to assume that TTX may be the main toxin in the toxin extracts, judging from the previous studies[7][13].

Cell preparation

All experiments were performed in accordance with the Guiding Principles for Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and approved by the local animal experiment committee in Kumamoto Health Science University. CA1 pyramidal neurons were isolated from the dorsal site of the rat hippocampus as described previously[3]. Wistar rats (12-18 days postnatal ; KYUDO, Kumamoto, Japan) were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and then decapitated. Hippocampi were removed and cut as fine transverse slices (400 μm) with a vibrating slice cutter (Leica VT1000S) in ice-cold incubation solution (in mM ; NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, glucose 10 and $NaHCO_3$ 24 ; bubbled with 95% O_2 and 5% CO_2). The slices were kept in incubation solution for 1h. Single cells were dissociated from the CA1 region of the slice by using a manufactured vibrating cell isolating setup (S-1 L cell Isolator)[14] in a standard solution (in mM ; NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and HEPES 10 ; pH adjusted to 7.4 with Tris-base at room temperature). Within 30 min, the neurons attached to the bottom of the Petri dish and were used for electrophysiological recordings.

Na$^+$ current recording

Na$^+$ current was recorded in whole-cell patch-recording configuration. Patch pipettes were made of borosilicate glass capillaries of 1.5 mm outside diameter (Model GD-1.5 ; Narisige Scientific Instruments Lab.) with a two-step vertical puller (Model PP830 ; Narisige, Tokyo, Japan). The electrodes were filled with the pipette solution (in mM ; CsF 105, NaF 30, CsCl 5, TEA-Cl 5, EGTA 2 and HEPES 10; pH adjusted to 7.2 with Tris-base) and had a tip resistance of 3-4 M Ω. All experiments were performed at room temperature (about 20-23 ℃). The currents were recorded using an amplifier (CEZ 2300 ; Nihon Kohden, Tokyo, Japan) and filtered at 2 kHz with a low-pass filter (Digidata 1200 ; Axon Instrument, Foster City, CA), and voltage-pulse protocols were generated using a D/A converter (Digidata 1200 ; Axon instruments). The data acquisition software was pCLAMP 8 (Axon Instruments). The neurons were voltage-clamped at a holding potential of $-$ 70 mV throughout the experiments.

Phrenic nerve-hemidiaphragm neuromuscular preparations of rat

Experiments were performed on the left hemidiaphragm with attached phrenic nerve excised from Wistar rats (140g-150g). The preparations were kept horizontally in 4 ml organ bath through which an oxygenated modified Krebs solution with or without toxin extracts were perfused at a rate of 16-18 ml/min. Modified Krebs solution contained (mM) ; NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 2.4, glucose 10, $MgSO_4$ 1.3 and HEPES 10 ; pH adjusted to 7.4 with Tris-base at room temperature. The central tendon of the preparation was tied by fine silk thread to a mechano transducer (RCA5734 ; Nihon Kohden, Tokyo) and the other end was fixed through ribs by pins to the bottom of the chamber. The phrenic nerve was stimulated with a bipolar

Ag–AgCl wires using rectangular pulse (10 ms in duration and 2V intensity) and muscle contractions were recorded on a pen recorder (R–03AC Rika Denki, Tokyo) or digitalized with an A/D converter (Digidata 1200 ; Axon Instruments) and stored in the computer for further analysis.   In addition, Electrical field stimulations (EFS) (10 ms in duration and 10V intensity) were also applied to stimulate the muscle directly through a pair of Ag–AgCl plates placed on the surface and bottom of the muscle preparation, so that current pulse would pass transversely across the muscle preparation.

Fig. 1Aa, shows the twitch contractions evoked by direct or indirect muscle stimulations, and the effect of 1μM curare (d–tc) which selectively abolished the nerve–induced twitch contraction. This observation indicates that phrenic nerve or muscle fibers were selectively stimulated by the direct or indirect stimulations under the present experimental condition.   TTX (Sigma 30 nM) also suppressed the twitch contraction evoked by
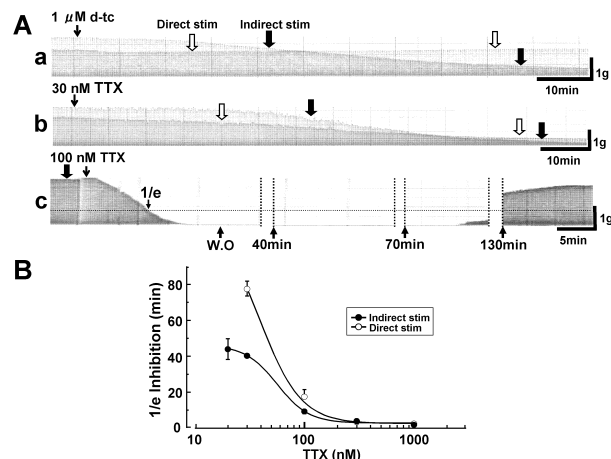

Fig. 1 Effects of 1μM curare (d–tc) or 30 nM TTX on the twitch contractions of rat hemidiaphragm evoked by indirect or direct muscle stimulations.   (A) *a* and *b* ; Small arrows indicate the applications of d–tc or TTX, and large open and filled arrows indicate the direct and indirect muscle stimulations, respectively. *b* and *c* ; Effects of 30 or 100 nM TTX and wash out effects, and the time when the amplitude of twitch contractions fall to 1/e of the initial control amplitude. (B) The relationship between the concentration of TTX and the time when the amplitude of twitch contractions evoked by indirect (●) or direct (○) muscle stimulations, fall to 1/e of the initial control amplitude.
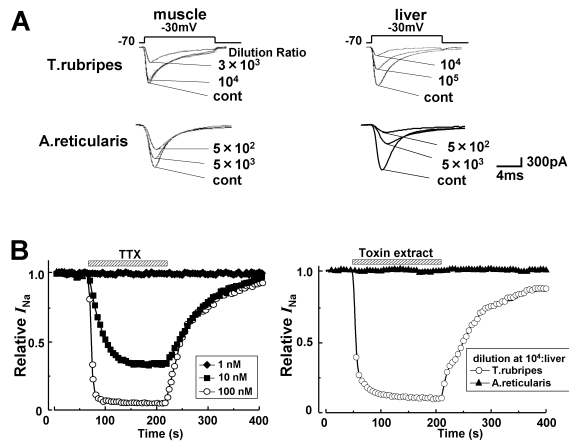
direct or indirect stimulations.   As shown in Fig. 1Ab, however, TTX suppressed twitch contractions evoked by indirect stimulation much faster than those evoked by direct stimulations.   Therefore, we measured the time when the amplitude of twitch contractions fall to 1/e of the initial control value, and tentatively used the value as the time constant for the effects of TTX or toxin extracts (Fig. 1Ac & B).   It should be pointed out that EFS applied to stimulate the muscle directly also excites nerve fibers distributed intramuscularly. Therefore, we applied direct stimulations in the presence of 1 μM d–tc throughout of the present experiments and observed the effect of TTX or toxin extracts.

Statistical analysis

The relative I_{Na} was calculated by the following equation:

Relative $I_{Na} = I / I_0$,

Where I is the peak Na$^+$ current in standard solution contacting TTX (Sigma) and I_0 is the peak Na$^+$ current in standard solution without TTX.   A half–maximal inhibition dose (IC$_{50}$) was calculated for the relative I_{Na} at concentrations between 0.1 and 100 nM standard curve for TTX[4].   The potencies of the toxin extract to inhibit I_{Na} were expressed as an amount of equipotent TTX, comparing the IC$_{50}$ obtained from dilution–inhibition curve and dose–inhibition curve for TTX.   Data were presented as means ± standard error of mean (± SEM).   Student's *t*–test was used, and $P < 0.05$ was considered significant.

Results

Effects of TTX and toxin extracts from various puffer fishes on I_{Na}

Voltage–dependent Na current (I_{Na}) was evoked by a depolarizing step pulse with 10 ms duration in dissociated CA1 neuron from holding potential (V_H) of –70 to –30 mV.   In the presence of TTX (Sigma) at various concentrations, I_{Na} was inhibited within 10 s and recovered in several

Fig. 2 (A) Effects of toxin extracts prepared from muscle or liver of two different puffers (*T. rubripes*, or *A. reticularis*) on I_{Na} recorded from single CA1 neurons. (B) The time course of the effects of various concentrations of TTX or toxin extracts at a dilution of 10^4 prepared from liver of *T. rubripes*, or *A. reticularis* on I_{Na}.

minutes after washing out TTX (Fig. 2). Similar rapid inhibition and slow recovery were observed in the presence of toxin extracts from muscle or liver prepared from the two puffer fishes examined (*T. rubripes*, and *A. reticularis*) at various dilutions (Fig. 2A and B).

Amount of toxin contained in the muscle and liver of various puffers expressed as an amount of equipotent TTX

Fig. 3 shows the dilution-inhibition curve for the toxin extracts from two different puffer fishes. The IC$_{50}$ value of the inhibitory effects of the toxin
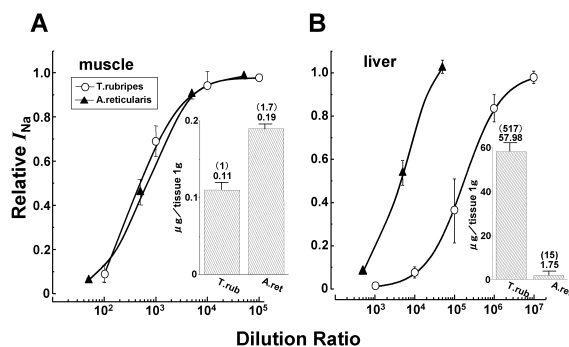

Fig. 3 Dilution-dependent inhibition for toxin extracts prepared from muscle (A) or liver (B) of two puffers (○ ; *T. rubripes*, ▲ ; *A. reticularis*). *Insets* show that the amount of toxin extracts expressed as μgTTX/g tissue in muscle (left panel) or liver (right panel) from *T. rubripes*, and *A. reticularis*.

extracts for the muscle was *A. reticularis* > *T. rubripes*, and for the liver *T. rubripes* >> *A. reticularis*.

The toxin extracts suppressed I_{Na} selectively, and therefore it was possible to estimate the amount of toxin in the muscle and liver prepared from two puffer fishes as an amount of equipotent TTX by comparing IC$_{50}$ of the toxin extracts to that of TTX. *Insets* of Figs 3A and B show the mean value of toxin contained in muscle and liver from the two puffer fishes and expressed as amounts of equipotent TTX. The toxin levels in the liver were 15 and 517 times higher than muscle of *T. rubripes* in the two examined puffer fishes.

Effects of TTX and toxin extracts on the twitch contractions of the rat hemi-diaphragm evoked by the direct and in direct muscle stimulation

Fig. 4A shows the time course of inhibitory effect of the toxin extract from the liver of *T. rubripes* (Aa) and *A. reticularis* (Ab) on the twitch contractions evoked by direct and indirect muscle stimulations (see method), and the toxin extracts
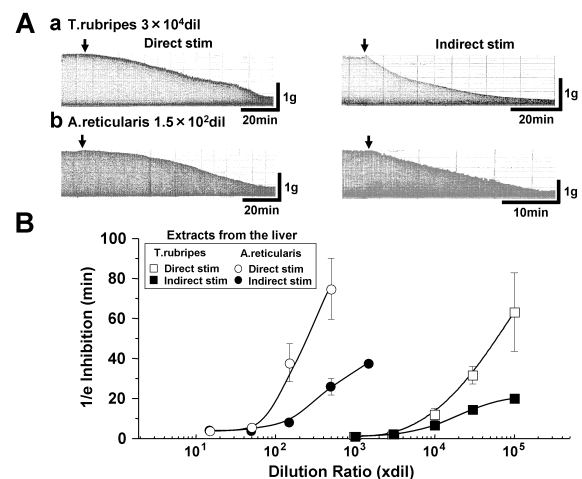

Fig. 4 (A) Effects of toxin extracts from liver of *T. rubripes* (3×10^4 dilution) and *A. reticularis* (1.5×10^2 dilution) on the twitch contractions of rat hemidiaphragm evoked by direct or indirect muscle stimulations. Small arrows indicate the application of toxin extracts. (B) The relationship of the dilution and the time constant (1/e time), where the amplitude of twitch contractions fall to 1/e of the initial control amplitude.

suppressed the twitch contractions evoked by indirect stimulation much earlier than that evoked by direct muscle stimulation. However, at increased concentrations (at dilutions 50 or 3 × 10^3), the toxin extracts suppressed the twitch contractions evoked by direct or indirect stimulations with similar time constants (Fig. 4B).

Discussion

Present study clearly indicates that the toxin level of the two examined puffers in the muscle and liver by use of electrophysiological method is different. For example, in *Takifugu rubripes* (*T. rubripes*), the toxin level in the muscle, which is the main edible portion, was the lowest in the two examined puffers, and only a 1/500 of the value in the liver. On the contrary, in the A. reticularis, the toxin levels in the muscle are relatively high (1.7 times of the value for *T. rubripes*), thereby indicating that *T. rubripes* provide the most safe muscle as food. In the liver, on the other hand, toxin level was higher in *T. rubripes* (517 times of the value for *T. rubripes* muscle), however the value was only 1/33 of that of *T. rubripes* in the *A. reticularis*. A recent study indicated that involvement of a carrier mediated transport system for TTX uptake by liver slices of *T. rubripes*[15]. Therefore, the activity or distribution profile of the transport system for TTX uptake in the liver might be different in the different species. Therefore, it seems reasonable to assume that toxin might be diffusely distributed in the various organs in the puffers in which the transport system in the liver is less developed.

At present, it is well documented that the origin of puffer fish toxin is mainly from the food chain, since the amounts of toxin were dramatically decreased in various tissues excised from cultured puffer fish fed non-toxic diet[4)16)]. Recently, we reported the toxin levels in various origins prepared from the various puffer fishes[4)17)]. According to the statistics provided by Japanese Ministry of Health and Welfare, the numbers of deaths due to puffer poisoning have steadily declined, from more than 10 cases with high mortality every year between 1960 to 1981 to less than 10 cases with low mortality every year since 1982[16]. Nevertheless, food poisoning due to ingestion of TTX-bearing puffers continue to occur frequently due mainly to consumption of homemade dishes which were prepared using wild puffer fish that were not purchased commercially. Over the past 13 years (1995-2007), mortality due to puffer fish poisoning has been 6.4% of announced total puffer poisoning incidents (378 cases) in Japan[16].

In general, the symptoms of TTX poisoning could be loosely divided into 4 stages. Namely, the immediate symptoms is the slight numbness of lips and tongue, which progresses to Stage I ; increasing paresthesia in face and extremities, sensations of lightness and floating, headache, nausea, severe abdominal pain, diarrhea, and/or vomiting. Stage II ; increasing paralysis, motor dysfunction with weakness, hypoventilation and speech difficulties, convulsion, mental impairment and cardiac dysfunction. Stage III ; central nervous system dysfunction, complete paralysis and death (usually occurs between a range of 20 minutes to 8 hours, where a person could still be conscious or in certain case completely lucid before death)[1].

Thus, it seems that severe respiratory failure is the main reason for the mortality of the puffer fish poisoning. The fact that respiratory support plays a key role in the management of patient with puffer fish poisoning supports this view. Therefore, it seems of interest and importance to examine the toxic activities of the tissues of puffer fishes from a functional point of view by investigating the effects on the respiratory neuromuscular transmission. The present study indicates that the toxin extracts from the liver of *T. rubripes* at a relatively low concentration (10^5 dilution) preferentially suppress twitch contraction of the rat hemidiaphragm evoked by indirect stimulations, rather than those evoked by the direct muscle stimulations. However, at an

increased concentration (10^4 dilution) the toxin extracts suppressed the twitch contractions evoked by direct and indirect stimulations with similar time constants (12.0 min or 6.7 min, respectively, see Fig. 4B). Similarly, TTX at relatively low concentrations (20–30 nM) suppressed the twitch contractions evoked by indirect stimulations rather than the direct stimulations. At an increased concentration (100 nM), however, TTX suppressed twitch contractions evoked by direct and indirect stimulations with similar time constants (14.6 min or 9.5 min, respectively). These results would indicate that at an early stage of poisoning due to the ingestion of puffers, namely at a relatively low toxin levels in the blood, motor nerves would be much sensitive rather than the muscle, as in case of sensory nerve impairment which appears at the beginning of intoxication as perioral numbness. However, as the toxin levels increase in the blood during the course of intoxication, the toxin would affect the motor nerve and muscle to a similar extent.

Previously, we reported that the threshold concentration for the electrophysiological bioassay by use of single hippocampal CA1 neurons to detect TTX is 1nM, and IC$_{50}$ value to suppress the peak I_{Na} is 14.1 nM[4]. In the present experiments, however, the threshold concentration for TTX to suppress twitch contraction of rat hemidiaphragm induced by indirect or direct stimulations were 20 nM~30 nM, and IC$_{50}$ value were 56.5 nM or 56.8 nM, thereby indicating that the phrenic–diaphragm neuromuscular transmission is much less sensitive to TTX comparing to that on the isolated single CA1 neurons. The precise reasons for this discrepancy are unknown at present, however, it might be due to the difference in the time lag for the diffusion of TTX into the nerve or muscle, since the Na–channel distributed in the central and peripheral nervous system is identical[18]. In the present study, we observed the effects of TTX and toxin extracts from puffers on the I_{Na} and respiratory neuromuscular transmission, and found that the sensitivity to TTX is in the order, I_{Na} recorded from CA1 neuron $>>$ motor nerve $>$ muscle.

Acknowledgments

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（和文抄録）

2種類の異なるフグより抽出した毒素成分がラット CA1 ニューロンの Na 電流と横隔膜での神経伝達に与える影響の比較研究

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　異なる2種類のフグ（トラフグ，ワモンフグ）の筋肉と肝臓から抽出した毒素成分をラット CA1 ニューロンの電位依存性 I_{Na} への毒素抑制効果で検討し，テトラドトキシン（TTX）と比較した．TTX と同等と考えられる筋肉や肝臓に含まれる組織1ｇ当たりの毒素含有量は，2種のフグで異なり，トラフグの筋肉が最も少なく，トラフグの肝臓が最も高かった．（トラフグ筋肉 0.01 ＜ワモンフグ筋肉 0.19 ＜ワモンフグ肝臓 1.75 ＜＜トラフグ肝臓 57.98μgTTX/g tissue）．また，フグの食中毒は，フグに含まれる TTX を摂取することで呼吸筋の麻痺により死亡に至る．このメカニズムを理解するために，ラット摘出横隔膜神経標本を用いて，片側横隔膜への直接電気刺激と横隔神経への間接電気刺激を与え，2種類のフグの肝臓から抽出した毒素成分と TTX による横隔膜の収縮抑制効果を検討した．その結果，抽出した毒素成分は，一定濃度の希釈倍率（トラフグ 50 倍，ワモンフグ 3000 倍）までは，間接刺激および直接刺激の筋収縮抑制効果には差はみられなかったが，濃度が低くなるにつれ，横隔膜への直接刺激のほうが横隔神経への間接刺激よりも筋収縮抑制時間が長くなり，筋肉より先に運動神経に影響を及ぼしていることがわかった．