Title: Studies on the Insecticidal Action of Nereistoxin, 4-N, N-dimethylamino-1, 2-dithiolane: III. Antagonism to Acetylcholine in the Contraction of Rectus Abdominis Muscle of Frog

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The sequence of symptoms and that of the burst of discharges in the insects treated with nereistoxin were quite different from those treated with common insecticides (Fig. 1 and 3).

The increase of the respiratory rate in the nereistoxin-poisoned cockroach (Fig. 5) is considered to be due to the nervous excitation, since the compound has no detectable effects on the tissue respiration of the muscle.

It is concluded from the results of the present experiments that nereistoxin acts as a characteristic nervous excitant of which mechanism of action is different from those of common insecticides.

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Reference
4) Yamasaki, T. and Narahashi, T.: Bolyu-Kagaku, 23, 146 (1958)
consequently necessary to refer to its action in vertebrates. According to Nitta, in the experiments with rodents and a frog low dosages of nereistoxin stimulate the pulsation of isolated heart and the contraction of intestine, uterus, bladder and pupil. It has also been demonstrated that the depression of the pulsation rate of the heart evoked by high dosages, and the accelerated secretion of salivary and lachrymal glands by certain dosages are eliminated by the treatment with atropine; and that the relaxation of pupil by a high dosage is avoided by the treatment with eserine.

These facts suggest that at least a part of the effects of nereistoxin takes place in a part of the cholinergic system in vertebrates. This possibility may be presumed from the fact that in a isolated neuro-muscular preparation of frog treated with nereistoxin the contraction of the muscle itself was not affected, but that the contraction evoked by the stimulation of nerve was suppressed. A possible explanation of the mechanism of these phenomena cited above seems to be nereistoxin blocks the nervous conduction or the neuro-muscular junction.

The present study is mainly concerned with the effects of nereistoxin on the acetylcholine receptor in a skeletal muscle of frog.

The knowledge derived from the present study would be applicable to the investigation of the mechanism of insecticidal action of nereistoxin.

Materials and Methods

Chemicals Pure synthesized nereistoxin hydrogen oxalate was used throughout the experiments. Acetylcholine bromide (Ach) was dissolved in distilled water at the concentration of 0.45 M and it was diluted with Ringer solution to a certain concentration.

Experiments with rectus abdominis muscle of frog

In order to investigate the action of nereistoxin on a Ach receptor, the contraction evoked by Ach in the rectus abdominis muscle of the frog, Rana nigromaculatus, was employed. The contraction of the isolated muscle suspended in the frog Ringer solution was recorded as in the method used for Ach assay.

The Ringer solution used in the experiments contained NaCl, 7.0 g; KCl, 0.14 g; CaCl₂, 0.12 g; NaHCO₃, 0.2 g; in 1 l of distilled water.

Nereistoxin was applied to the muscle either simultaneously with or prior to the addition of Ach to investigate the effect of the test compound on the muscle contraction by Ach.

To study the effect on the contraction of the muscle itself, the muscle immersed in the Ringer solution containing nereistoxin was directly stimulated by electrical impulses (10 V, 1 c/s, duration 20 msec).

When eserine salicylate was added to the Ringer solution at the concentration of 0.002%. Extraction of nereistoxin in the poisoned cockroach The adult German cockroach, Blattella germanica, (7–10 days old male) was treated with the acetone solution of nereistoxin at the dosage of 25 μg/g. The solution was topically applied on the ventral side of the abdomen. The treated insect population was divided into groups of 10 individuals. Immediately before homogenization, the number of knocked down insects was recorded and the insects were washed with distilled water to remove nereistoxin that probably might have been remaining on the body surface.

All insects in a single group were homogenized in 2 ml of the chilled frog Ringer solution containing 1% of trichloro acetic acid (TCA-Ringer) with a glass homogenizer.

After the homogenate was transferred into a centrifugal tube, the inside and the pestle of homogenizer was washed twice with small portion of TCA-Ringer and the washings and the homogenate were pooled in the tube. The supernatant was decanted after centrifugation at 2000 G for 15 min. The residue was resuspended in 2 ml of TCA-Ringer, centrifuged and the supernatants were pooled.

TCA-Ringer has been used by some workers to achieve the stable extraction of Ach from arthropod tissues. To eliminate Ach from the test insect which might interfere with nereistoxin assay, the following procedure was successful.

The supernatant was incubated with 1 ml of a cholinesterase preparation for 20 min. at 37°C. The enzyme preparation was the supernatant of
Results

Effects of nereistoxin on the contraction of frog muscle evoked by Ach. The contraction of uneserinized muscle evoked by a high concentration (1.2 x 10⁻⁸ M) Ach was not affected by nereistoxin which was applied together with or prior to the application of Ach (Fig. 1 a, b). The record in Fig. 1 b shows that a single application of nereistoxin did not evoke any response of the muscle. When the concentration of Ach was low (7 x 10⁻⁷ M), the contraction was considerably affected. Fig. 1 c shows the shortening of the muscle did not reach to the normal level by the application of nereistoxin. The sensitivity of the muscle to Ach was suppressed even after the washing of the muscle with the Ringer solution. Nevertheless, the suppression was eliminated and the sensitivity to Ach was greatly increased by eserinization of the muscle (Fig. 1 c).

In the muscle sensitized to Ach by eserinization, the contraction was affected by nereistoxin.

70 homogenized heads of house fly, Musca domestica, per 1 ml of a Ringer solution containing 0.15 M NaCl and 0.04 M MgCl₂.

After the elimination of Ach, the final volume was brought to 10 ml with the addition of the frog Ringer solution to make up the "test extract" for the pharmacological assay of nereistoxin on the poisoned cockroaches.

Pharmacological assay of nereistoxin in the test extract. As will be described later, the rate of suppressive effect of nereistoxin on the muscle contraction evoked by Ach was proportional to the nereistoxin concentration (Fig. 3). Therefore the amount of nereistoxin in the extract of the poisoned cockroach could be assayed pharmacologically with the rectus abdominis muscle of the frog.

The contraction evoked by 7 x 10⁻⁷ M Ach was recorded at first, then the muscle was thoroughly washed with the Ringer solution until the muscle returned to be relaxed. Then the Ringer solution was replaced with the test solution (extract). After the incubation for 10 min, the muscle was washed with the Ringer solution three times and the contraction by 7 x 10⁻⁷ M Ach was recorded.

Because of the presence of sensitizing substances in the extract, 7 x 10⁻⁷ M Ach was prepared with the extract of the normal cockroaches. To make up a standard test solution of nereistoxin, the homogenate of 10 cockroaches (ca. 0.5 g) to which 12.5 µg of the compound was added was simply extracted as in the preparation of the test extract.

The assay was conducted under the eserinized condition.

The rate of suppressive effect of nereistoxin on the muscle contraction was calculated as follows:

\[
\text{Contraction height after the treatment with test solution} \times 100 \\
\text{Normal contraction height} - \text{Suppression Rate (％)}
\]

The amount of recovered nereistoxin in the extract was estimated from the graph in which the suppression rates were plotted versus the known concentrations of nereistoxin (Fig. 3 b). Temperature. The kymography and the test with German cockroach were conducted in the room at the temperature of 25 ± 1 °C.
of a lower concentration than in the uneserinized preparation. As shown in Fig. 2a, nereistoxin applied together with Ach depressed the shortening of muscle and then relaxed the muscle from contraction. However, the sensitivity of the muscle to Ach recovered gradually after replacing nereistoxin with the fresh Ringer solution.

As in the uneserinized preparation, the contraction of eserinized muscle was suppressed by the exposure to nereistoxin prior to the application of Ach (Fig. 2). The incubation of the muscle

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Effects on nereistoxin on contraction of rectus abdominis muscle of frog evoked by acetylcholine. Ach: acetylcholine, Nt: nereistoxin. Acetylcholine concentration was $7 \times 10^{-7}$M.}
\end{figure}

in $2 \times 10^{-8}$M nereistoxin for 10 min. reduced the shortening evoked by $7 \times 10^{-7}$M Ach approximately by half of the normal shortening (Fig. 2b). Also in this experiment, the reduced sensitivity of the muscle to Ach recovered after the immersion in the fresh Ringer.

To evaluate the effect of concentration of nereistoxin on the contraction of the muscle by Ach, the muscle was exposed to various concentrations of nereistoxin, and the shortening evoked by $7 \times 10^{-7}$M Ach was recorded. The suppression rate plotted against the nereistoxin concentration is shown in Fig. 3. The data show that nereistoxin applied prior to the application of Ach evoked stronger suppression to the contraction than when applied simultaneously with Ach. For instance, $10^{-8}$M nereistoxin reduced the shortening only about 20% in the simultaneous application, but it reduced 30% and 70% when applied 5 and 10 min. prior to Ach, respectively. It was revealed in Fig. 3b that in the prior application the immersion of longer period evoked stronger depression to the contraction.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig3.png}
\caption{Suppression effect of nereistoxin on the contraction of rectus abdominis muscle of frog evoked by acetylcholine. a: nereistoxin was applied simultaneously with acetylcholine. b: nereistoxin was applied 5 (open circle) and 10 (solid circle) min. prior to the application of acetylcholine. Acetylcholine was $7 \times 10^{-7}$M throughout.}
\end{figure}

Effect of nereistoxin on the contraction of frog muscle evoked by direct stimulation. The contraction of the muscle due to the direct electrical stimulation was not significantly affected by $10^{-8}$M nereistoxin. However, in the same preparation, the contraction evoked by $3.5 \times 10^{-7}$M Ach was considerably reduced still after the muscle was washed with the fresh Ringer solution (Fig. 4).

Amount of nereistoxin in the poisoned cockroach. The consistent relationship between the concentration of nereistoxin and the suppression rate of the frog muscle contraction (Fig. 3) was believed to be applicable to the assay of small amounts of nereistoxin. A longer period of the exposure of the muscle to nereistoxin prior to
it was shown that after 55~60 min., the amount of nereistoxin decreased to 12μg/g, although the knock-down rate was 100%. Along with the revival from the poisoning, the amount of nereistoxin decreased rapidly.

Discussion

In the experiments with the frog rectus abdominis muscle, the following results were obtained; 1) nereistoxin of a concentration which positively suppressed the muscle contraction evoked by a low concentration of Ach did not affect the contraction by a high concentration of Ach (Fig. 1); 2) the contraction which was suppressed by nereistoxin under the uneserinized condition recovered considerably after the increase of actual concentration of Ach acting on the receptor site by eserinization; viz. the action of nereistoxin was antagonized by eserine (Fig. 1); and 3) the contraction evoked by the direct stimulation of the muscle was not significantly affected by nereistoxin of which concentration was sufficient to suppress the contraction by Ach (Fig. 4).

These results demonstrate that the action of nereistoxin suppressing the muscle contraction is the blockade of Ach receptor and that it does not affect the contractile power of the muscle fiber itself. The block by nereistoxin is presumed not to be due to depolarization, since the blocking action is antagonized by eserine and the single

Table 1. Amount of nereistoxin in topically treated* German cockroaches assayed pharmacologically by frog muscle contraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Knock-down rate (%)</th>
<th>Suppression rate (%)</th>
<th>Nereistoxin found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract of untreated insects</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Nereistoxin 6×10⁻⁷M in the extract of normal insects</td>
<td>-</td>
<td>63</td>
<td>7×10⁻⁴M</td>
</tr>
<tr>
<td>Nereistoxin 12.5 μg added to the homogenate of normal insects (0.5g) and extracted.</td>
<td>-</td>
<td>60</td>
<td>25μg/g**</td>
</tr>
<tr>
<td>Extract of treated insects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediately after the treatment</td>
<td>0</td>
<td>10</td>
<td>0.4 μg/g**</td>
</tr>
<tr>
<td>25~30 min</td>
<td>100</td>
<td>60</td>
<td>25 μg/g**</td>
</tr>
<tr>
<td>55~60 min</td>
<td>100</td>
<td>43</td>
<td>12 μg/g**</td>
</tr>
<tr>
<td>3 hr</td>
<td>60</td>
<td>11</td>
<td>0.4 μg/g**</td>
</tr>
<tr>
<td>16 hr</td>
<td>10</td>
<td>9</td>
<td>0.3 μg/g**</td>
</tr>
</tbody>
</table>

* 25μg/g of nereistoxin hydrogen oxalate
** Amount of nereistoxin calculated as the weight per 1 g of insect body.

65
application of nereistoxin only does not stimulate the frog muscle to contract as a depolarizant, for instance, decamethonium does\textsuperscript{18}.

However, the action of nereistoxin to some visceral muscles of vertebrates\textsuperscript{49} and to the nervous activity of insects\textsuperscript{49} is considered to be stimulative, not being depressive as on the frog muscle.

The different actions of drugs rendering a muscle inexcitable, viz. block by depolarization, block by competition and dual block are explained on the basis of the rate of drug-receptor combination\textsuperscript{19}. Hence, a different type of blocking action is not only specific to the nature of a drug, but also it is observed in the muscle of different species and sometimes even in the different muscles of a same species\textsuperscript{17}. Decamethonium and hexamethonium are the drugs that cause block by depolarization in some muscles, but dual block in others\textsuperscript{18,19,20,21,22}. Therefore, the existence of these opposite actions in a single compound, nereistoxin, would not be contradictory.

If nereistoxin blocks Ach receptor only, it is interesting and worthwhile to demonstrate what the site of action is in insects. Vertebrate skeletal muscles are cholinergic, but many evidences have been presented to show insect peripheral muscles are not cholinergic\textsuperscript{23,24,25,26}. Accordingly, a conceivable mechanism of action of nereistoxin in insects is the block of Ach receptors in the central nervous system which is regarded to be cholinergic\textsuperscript{25,27}.

The pharmacological assay using the frog rectus abdominis muscle was applicable for detecting the approximate amount of nereistoxin. This assay was sensitive enough to assay nereistoxin of concentration in the order of 10\textsuperscript{-7}M (ca. 0.02 \(\mu\)g/ml). The sensitivity was much higher than that of the bioassay with azuki bean weevil, Callosobruchus chinensis, to which the LD-50 of this compound was approximately 7\(\mu\)g/Petri dish: that is, 1 ml of 3\(\times\)10\textsuperscript{-4}M solution per a dish is required to kill 50\% of the weevils\textsuperscript{23}.

If it is assumed that the cockroach did not convert nereistoxin to substances which still suppress the muscle contraction, the results shown in Table 1 explain that intoxication and the recovery from the poisoning were in accordance with the amount of nereistoxin existed internally. These results show that the speed of nereistoxin to penetrate the insect cuticle is fairly rapid and that the revival from the poisoning is due to detoxication or excretion of the toxic substance.

Summary

The effect of nereistoxin on the contraction of isolated rectus abdominis muscle of frog was investigated.

The application of nereistoxin simultaneously with and preliminarily to the application of Ach suppressed the muscle contraction that might be evoked by Ach. A single application of nereistoxin did not stimulate the muscle to contract. The contraction evoked by direct stimulation of the muscle was not affected by nereistoxin.

From these results, it was concluded that the action of nereistoxin suppressing the muscle contraction is due to the blockade of the Ach receptors. Therefore, a conceivable mechanism of insecticidal action of this compound is the block of Ach-receptors.

The proportional relationship between the concentration of nereistoxin and the strength of the suppressive effect to the muscle contraction was applicable to assay small amounts of nereistoxin. This procedure was applied for detecting the amount of this compound in the body of German cockroach. The result showed that the compound penetrated through the cuticle rapidly, and the revival from the poisoning was in accordance with the decrease of nereistoxin in the body.

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References

M. V. Norris らの植物体におけるマラソン残留量の定量法に、アルカリ分解試薬として、前報において用いた塩酸化カリウムを添加する実験を行った。添加による影響は、なお検討が必要であるが、一般に添加する量は、分析操作時間に制約される。この実験結果は、将来マラソンの使用状況を把握するための基礎資料として有用である。