The effect of charatoxin, 4-methylthio-1,2-dithiolane, on the frog sartorius neuromuscular junction[1]

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Summary. The insecticide 4-methylthio-1,2-dithiolane, named charatoxin, blocks the frog muscular twitches elicited through the neuromuscular junction. The activity level and the course of inhibition is comparable to that of 4-dimethylamino-1,2-dithiolane, nereistoxin.

Recently a new dithiolane named charatoxin **I** (fig. 1) has been isolated from the alga *Chara globularis* Thuillier[3]. A chemical synthesis of this compound has been published and it has been shown to possess biological activity[4,5]. An insecticidal effect has been demonstrated for this naturally occurring compound and for a series of synthetic analogues including the open-chain structures **III** and **IV** shown in figure 1[6]. Charatoxin has a fairly close structural similarity with another naturally occurring compound, the nereistoxin, **II** (fig. 1) originally identified from the japanese annelid *Lumbriconereis heteropoda* Marenz[7,8]. Compound **II** also possesses a significant insecticidal effect, and from a series of nereistoxin analogues the commercial insecticide Cartap has been developed[9,10].

Based on results from a variety of experimental systems from vertebrates and invertebrates it has been suggested that the insecticidal action of **II** is due to a blocking of acetylcholine receptors[11,12]. Recently, the effect of **II** on muscle twitch tension in the frog sartorius muscle has been reinvestigated in detail confirming the effect on cholinergic receptors[13].

The present paper describes the effect of substances **I, III,** and **IV** on the frog sartorius neuromuscular preparation and the mode of action of these compounds is discussed in relation to that of **II.**

Materials and methods. Sartorius nerve muscle preparations from *Rana temporaria* were used for the experiments, carried out at room temperature without O_2-bubbling.

Electrical stimulation of the sartorius muscle preparation was given either indirectly via the nerve or directly to the muscle through pairs of platinum electrodes placed either in contact with the nerve or with one thread on either side of the muscle. The stimulation was given as supramaximal rectangular electric pulses of 2 msec duration, 1 or 2 per min.

The isometric twitch tension was recorded by a mechano-electric transducer system (51E01 reactance converter, DISA Denmark) and displayed on a storage oscilloscope or by means of a pen recorder. The frog nerve preparation was a 4-5-cm nerve trunk from the hind leg. Action potentials were electrically elicited once per min from a DISA Ministim through platinum electrodes, placed about 3 cm from the suction electrode which picked up the signal. The amplitude of the action potential was measured from the oscilloscope display through 20-min periods.

For the dissection and the experiments Ringer solution (pH 6.1-6.3) of the following composition (mM) was used: 113 NaCl, 3.3 KCl, 2.7 $CaCl_2$, 2.4 $NaHCO_3$, 0.47 Na_2HPO_4, 0.20 KH_2PO_4.

Charatoxin and the analogues **III** (2-methylthio-1,3-propanedithiol) and **IV** (2-methylthio-1,3-bis-acetylthiopropane) were supplied as ethanolic solutions whereas the test solutions were prepared immediately before use by diluting with Ringer solution. The total concentration of alcohol in the test solutions never exceeded 1.7%, which did not affect the twitches of the muscles.

Nereistoxin was supplied as its oxalate. An adequate amount of $CaCl_2$ was added to the nereistoxin-Ringer to compensate for the precipitated Ca oxalate. Curare was obtained commercially as d-tubocurarine chloride. Each experiment was carried out at least in triplicate.

Results. A somewhat variable sensitivity to toxins was observed between the individual preparations, but when the same preparation was used repeatedly the response variation was very small.

Figure 2 and figure 3 show the results from typical experiments. Figure 2 shows the muscle twitch tensions elicited via the nerve in the frog sartorius nerve muscle preparation plotted against time. It appears that, at concentrations ranging from 0.5×10^{-3}M to 2.0×10^{-3}M, charatoxin and nereistoxin caused partial to complete block of the twitch tension. The blocking of the muscle twitch is seen to be time and concentration dependent for both compounds and the course of inhibition shows the same trend for **I** and **II**. The results presented in figure 2 were obtained by using the same preparation, which means that the dose effect levels for the two toxins can be compared directly.

Figure 3 compares the reversibility of the effect of charatoxin (2.0×10^{-3}M) with that of curare (8.8×10^{-6}M) and nereistoxin (2.0×10^{-3}M) on the muscle twitches elicited by stimulating the muscle directly as well as indirectly (via its nerve). The toxins completely inhibited the indirectly elicited twitches within comparable time spans. The experimental periods were chosen so that time itself certainly had no effect on the twitches. Ringer bathed controls were stimulated for 30-min periods and after these periods the

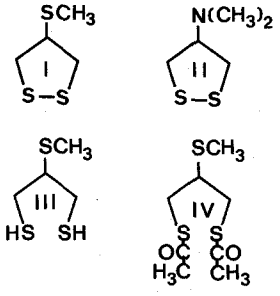

Figure 1. Chemical structures of charatoxin **I**, nereistoxin **II**, and 2 charatoxin analogues, **III, IV.**

Effect of toxins on the nerve action potential amplitude after a 20-min incubation period

Compound	Concentration (mM)	Amplitude
I	2	95 ± 7% (4)
II	2	88 ± 8% (3)
III	2	87 ± 15% (3)
IV	2	7 ± 6% (3)
Curare	8.8×10^{-3}	92 ± 9% (4)
Ringer	–	101 ± 6% (8)

The amplitude values are expressed as percentage (means ± SD) of the initial value. The number in parantheses refers to the number of experiments.

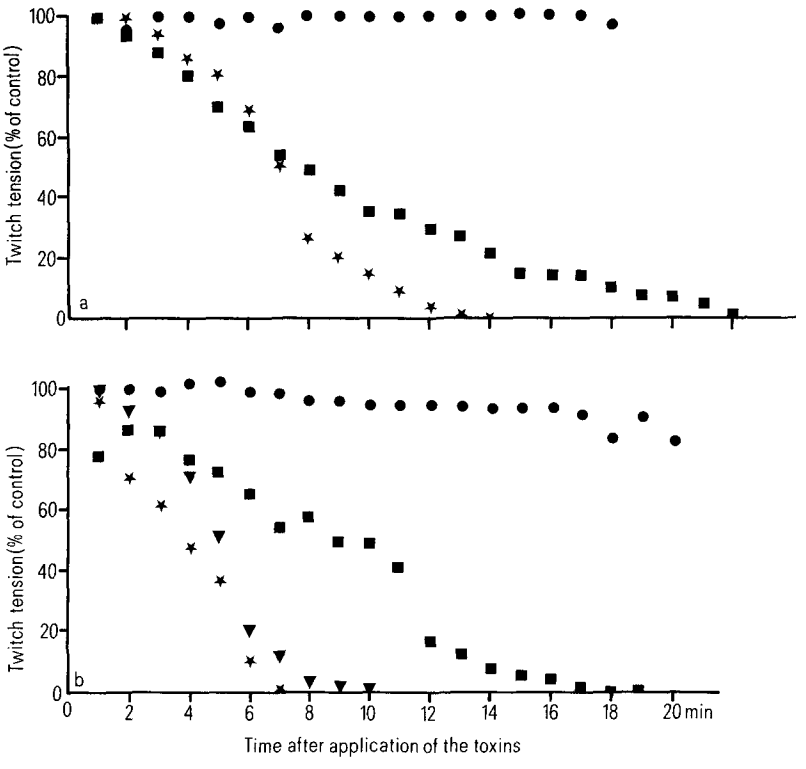

Figure 2. Indirectly elicited isometric twitch tension of the frog sartorius muscle, expressed as percentage of control tension, plotted against time. *a* The effect of nereistoxin, *b* the effect of charatoxin. The concentrations of toxins used were: ●, 0.5×10^{-3}M; ■, 1.0×10^{-3}M; ▼, 1.5×10^{-3}M; and ★, 2.0×10^{-3}M.

twitch tensions were 99±4% (n=4) of the initial twitch tension when stimulation was given via the nerve and 96±9% (n=8) when stimulation was given directly to the muscle.

The figure shows an experiment in which the preparation was washed after total blocking of the indirectly elicited twitch, and the recovery followed with time. It appears that for charatoxin and nereistoxin almost total recovery was reached within 10 min. For preparations treated with curare it was found that the time for reaching total recovery was generally longer than the experimental period used here.

The experiments illustrated in figure 3 were performed using indirect as well as direct stimulation and from the figure it appears that **I** and the 2 reference compounds show some, but reversible reduction of the directly elicited muscle twitch tension. At 2.0×10^{-3}M the 2 charatoxin analogues **III** and **IV** caused total block of the indirectly elicited muscle twitch tension.

It has been reported that **II** has no effect on the action potential in the insect neuron at doses where partial to complete blocking of the cholinergic transmission was observed[14]. The effect of the toxins on the action potential amplitude in the frog nerve was determined at concentrations that induced complete blocking of the indirectly elicited muscle twitch with the toxins applied for time periods twice as long as those necessary for completing the blocking in the nerve muscle preparation. It appears from the table that only compound **IV** showed a significant inhibition of the nerve signal transmission.

Discussion. The present results show that the effect of **I** on the frog sartorius nerve muscle preparation corresponds to that of **II**. The applied doses of the compounds were relatively high, so unspecific reactions with the proteins in the preparations cannot be completely ruled out. Since the activity level and course of inhibition of **I** compares very well to that of **II** it is suggested that **I** is blocking the cholinergic transmission as has been described for **II**,

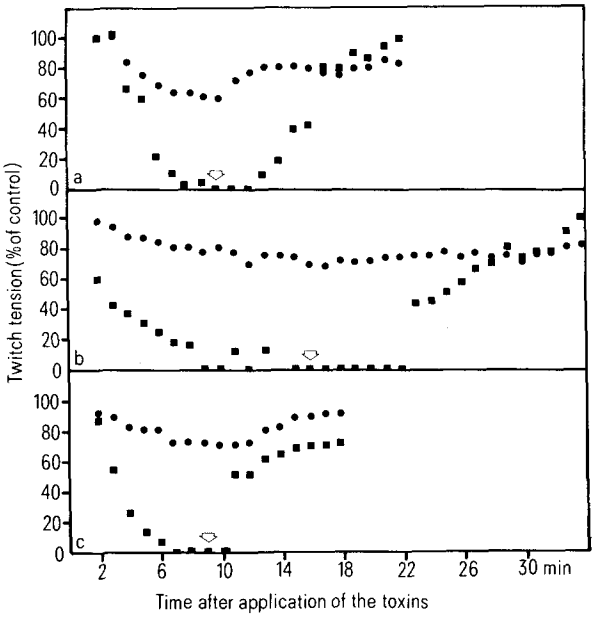

Figure 3. Directly (●) and indirectly (■) elicited isometric twitch tension of the frog sartorius muscle, expressed as percentage of control tension plotted against time. removal of toxins by washing with normal Ringer. The concentrations used were 2.0×10^{-3}M for nereistoxin (a), 8.8×10^{-6}M for curare (b), and 2.0×10^{-3}M for charatoxin (c).

although it does not mean that the molecular targets for the 2 compounds have been proved to be identical.

We have found no indications of effects of **I** on the action potential in the frog nerve, but the experiments show that **I** and **II** in high concentrations to some extent exert a

blocking effect directly on the muscle. This effect is significantly inferior to the inhibitory effect observed when the muscle is stimulated via the nerve. These observations suggest that the target for these compounds is to be found in the nerve muscle junction.

The effect of the analogues **III** and **IV** on the twitch tensions is of the same order of magnitude as demonstrated for **I** and **II**. It is therefore suggested that the mode of action of **III** might correspond to that of **I** and **II**. The mode of action of **IV** seems to be more complex since this compound is the only one tested that shows an inhibitory effect on the action potential in the frog nerves.

The effects of the open-chain analogues on the in vitro preparations lend no support to the hypothesis that the dithiolane moiety is the active principle and that active analogues are metabolized into dithiolanes[15].

The effect level for charatoxin compares well to that of the structurally related nereistoxin on the sartorius nerve muscle preparation as it appears from figure 2. Since both compounds show insecticidal potency with similar symptoms of intoxication[6] it is indicated that further studies on the insecticidal properties of charatoxin analogues may prove fruitful.

Many questions remain to be answered regarding the precise site of action of charatoxin and its analogues. The results presented here clearly point at the cholinergic transmission as the region of action. But nothing is known about the specificity of the observed inhibition. Further studies on e.g. binding affinity to acetylcholine receptors, and a more detailed electrophysiological investigation of the effect on the membrane potentials should therefore be carried out.

1 The authors are grateful to Dr N. Jacobsen, Cheminova A/S for synthesizing the toxins and the 2 analogues.
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Influence of posttreatment temperature on the toxicity of pyrethroid insecticides to susceptible and resistant larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.)

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Summary. The organophosphorus insecticides chlorpyrifos, leptophos, and phosfolan and the carbamate methomyl was found to be more toxic to larvae of a susceptible strain of *Spodoptera littoralis* (Boisd.) when the posttreatment temperature was increased from 20 to 35 °C. In contrast, the pyrethroids permethrin, fenvalerate, deltamethrin, cypermethrin, and flucythrinate were more toxic at 20 °C than at 35 °C. This effect was more pronounced in the pyrethroid-resistant strains. Evidently, resistance levels were reduced at low temperature. However, the application of piperonyl butoxide or DEF in combinations with the tested pyrethroids on R-strains resulted in reducing the effect of temperature.

Several factors influence insecticide toxicity, one being temperature. It has been shown that the toxicity of natural pyrethrins and DDT correlated negatively with increasing posttreatment temperature[1-3]. On the other hand organophosphates have shown a positive temperature coefficient[2,4,5]. Carbamates were reported to have a slightly negative temperature coefficient[6,7], although methomyl manifested greater toxicity at higher temperatures in some insect species[4,8,9].

Recent developments in pyrethroid chemistry have resulted in synthesis of relatively stable compounds with high toxicity to insects. Some evidence indicates that synthetic pyrethroids have a negative temperature coefficient similar to the natural pyrethrins. This has been reported for the effect of pyrethroids on houseflies[6,10], the cabbage looper[8], crickets[11], and tobacco cutworms[9].

In Egypt, several synthetic pyrethroids have been introduced for the chemical control of cotton pests. The present work describes the effect of posttreatment temperature on the toxicity of some pyrethroids, in comparison with other insecticides, to larvae of a susceptible and 3 pyrethroid-resistant strains of the Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.).

Materials and methods. 4 different strains of *S. littoralis* were used; a laboratory susceptible strain (S-FM), a flucythrinate resistant-strain (R-CB), a deltamethrin-resistant strain (R-DM), and a fenvalerate-resistant strain (R-FN). The S-FM strain was originally obtained from Faiyum Province in 1966 and was reared in the laboratory without any exposure to insecticides. The resistant R-CB, R-DM, and R-FN strains were originally collected from Shrquiya Governorate in 1980. The R-CB strain was subjected to continuous selection for 15 generations, while R-DM and R-FN strains were selected for 22 successive generations. Selection procedure and the history of these strains have already been described[12].