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# EFFECTS OF A SPIDER TOXIN ON THE GLUTAMINERGIC SYNAPSE OF LOBSTER MUSCLE

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SUMMARY

1. We studied the effect of neurotoxin (JSTX) separated from spider venom on the lobster neuromuscular junction.

2. JSTX selectively suppressed excitatory post-synaptic potentials (e.p.s.p.s) without affecting the inhibitory post-synaptic potentials (i.p.s.p.s).

3. The effect of JSTX was dose-dependent. The threshold dose for suppressing e.p.s.p.s corresponded to a small fraction of the toxin amount in a venom gland. At high concentration, JSTX irreversibly blocked e.p.s.p.s.

4. The reduction in amplitude of extracellularly recorded e.p.s.p.s after JSTX application followed an exponential time course. The rate of suppression increased proportionally with the toxin concentration.

5. JSTX blocked the glutamate potential in the post-synaptic membrane but it failed to affect the aspartate-induced depolarization. Kainic acid potentiated the glutamate-induced depolarization but it was without effect in the presence of JSTX. Depolarization produced by quisqualic acid is suppressed by the toxin.

6. Our results suggest that the spider venom contains specific blockers of glutamate receptors in crustacean neuromuscular junctions.

## INTRODUCTION

In crustacean neuromuscular junction, so far L-glutamate is the only substance which fulfills the criteria of a neurotransmitter, as shown by: (1), the presence of glutamate in the presynaptic axon (Kravitz, Kuffler & Potter, 1963); (2), identical actions of glutamate on excitatory post-synaptic potentials (e.p.s.p.s) in respect of the ionic mechanism and time course (Takeuchi & Takeuchi, 1964; Onodera & Takeuchi, 1975, 1976; Crawford & McBurney, 1976), and (3), release of glutamate upon stimulation of the presynaptic axon (Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970; Kawagoe, Onodera & Takeuchi, 1981). The identification of a specific antagonist for both the action of glutamate and for e.p.s.p.s would provide further evidence that L-glutamate is indeed the neurotransmitter. Although a number of

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substances exhibit antagonistic action against e.p.s.p.s or the glutamate response in crustacean neuromuscular junctions (Lowagie & Gerschenfeld, 1964; Wheal & Kerkut, 1975; Onodera & Takeuchi, 1977; Van Harreveld, 1980), no receptor blockers with a high degree of specificity such as curare, $\alpha$-bungarotoxin in the cholinergic synapse or picrotoxin in the GABA-ergic synapse, have yet been found. In the present study we report a neurotoxin, separated from a spider, which specifically blocks glutamate receptors in the lobster neuromuscular junction. Our preliminary work has been reported elsewhere (Kawai, Niwa & Abe, 1982*a*).

## METHODS

All experiments were performed on the walking leg of the spiny lobster (*Palinurus japonicus*). The excitatory and the inhibitory axons to the stretcher muscle were isolated at the meropodite as has been described previously (Kawai, Mauro & Grundfest, 1972; Kawai & Niwa, 1977, 1980; Niwa & Kawai, 1982; Abe, Kawai & Niwa, 1982) and stimulated using suction electrodes. For recording, the dorsal surface of the stretcher muscle was exposed by cutting a round hole in the exoskeleton and overlying connective tissue so as to make a pool (*ca.* 3 mm in diameter). The volume of the pool was approximately 0·1–0·3 ml. Micro-electrodes filled with 3 M-KCl and 2 M-potassium citrate (10–20 MΩ) were used for intracellular recording and for passage of current, respectively. Extracellular recordings near the synaptic spot were made using 2 M-NaCl electrodes (1–2 MΩ). The extracellular potential recordings were averaged using a signal-average computer. For ionophoretic application of glutamate, micropipettes were filled with 1 M-L-glutamate; the pH was adjusted to 8·0 with NaOH. Bath application of glutamate and its analogues was made by perfusing normal solution at a rate of 5 ml/min from one edge of the pool on the surface of the stretcher muscle; simultaneous sucking out was done at the other edge. Compounds of given concentrations were added to the perfusate. The normal solution consisted of (in mM): 468, NaCl; 10, KCl; 20, $CaCl_2$; 8, $MgCl_2$; and Tris buffer, 2; adjusted to pH 7·4. Na-L-glutamate and Na-L-aspartate were obtained from the Wako Chemical Co., kainic acid and quisqualic acid were from the Sigma Chemical Co.

Spiders (*Nephila clavata*, Joro spider) were collected in central Japan. For purification of the toxin, crude venom from 500 venom glands was homogenized and the supernatant was passed through a Sephadex G-50 column. The effective component was rechromatographed on Sephadex G-15; the purified substance (JSTX) obtained in this manner showed a purity of more than 80%. For convenience in the quantitative study, we took the quantity of JSTX in one venom gland to represent one unit. With respect to concentration, we defined one unit of JSTX/0·1 ml of solution as 1 u. Just before the experiment, we diluted the purified toxin with normal saline to obtain an appropriate concentration. To apply the toxin on the neuromuscular junction, we poured a sufficient amount of toxin solution over the pool so as to make the final concentration of the toxin in the pool equal to that in the toxin solution. To confirm this a methylene blue solution (*ca.* 0·01%) was similarly poured over the pool. Using a spectrophotometer we checked that the dye concentration in the pool was the same as that in the test solution.

## RESULTS

### *Effect of JSTX on e.p.s.p.s*

A typical effect of JSTX on lobster neuromuscular junction is shown in Fig. 1. Upon the application of JSTX (0·02 u.), the amplitude of the e.p.s.p. started to decrease; it declined progressively with time and disappeared completely within about 10 min. In contrast, the i.p.s.p. remained the same as in the control. The resting potential and the input resistance of the post-synaptic membrane were almost the same before and after toxin application (Fig. 1*B*). Thus, the resting conductance channels of the post-synaptic membrane were unaffected by JSTX. The effect of JSTX was almost irreversible, provided a sufficiently high toxin concentration (more than 0·01 u.) was used. Repeated washing for a few hours failed to restore the e.p.s.p. (Fig. 1*A*).

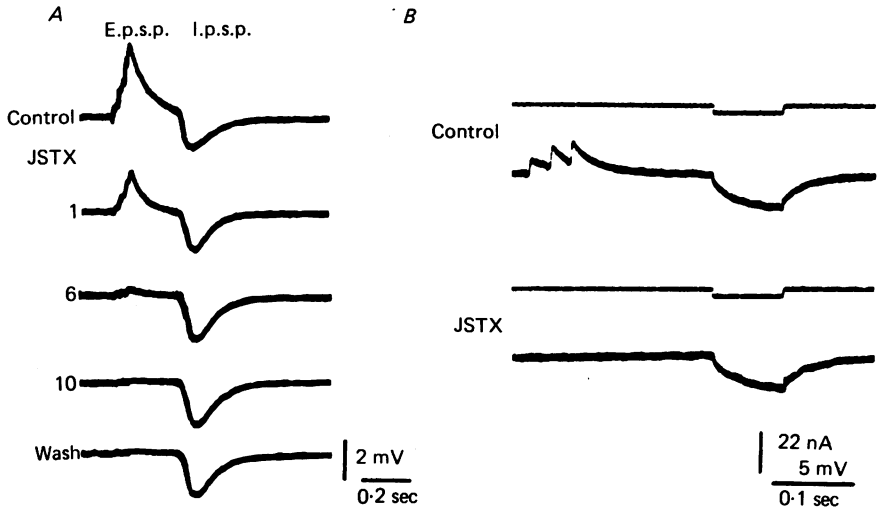


Fig. 1. Effect of spider toxin (JSTX) on the post-synaptic potentials and the membrane input resistance at the lobster neuromuscular junction. *A*, e.p.s.p.s and i.p.s.p.s were evoked successively in the stretcher muscle by repetitive stimulation of the excitatory and inhibitory axon, respectively. The numbers on the left indicate the time in minutes after application of JSTX (0·02 u.). The 'wash' recording was made after rinsing the preparation for 90 min in normal solution. *B*, e.p.s.p.s and electrotonic potentials (the latter produced by intracellularly applied currents). In each record, the upper beam monitors the current. The lower beam shows the post-synaptic potentials in the stretcher muscle. Records were taken before (control) and 15 min after JSTX application.

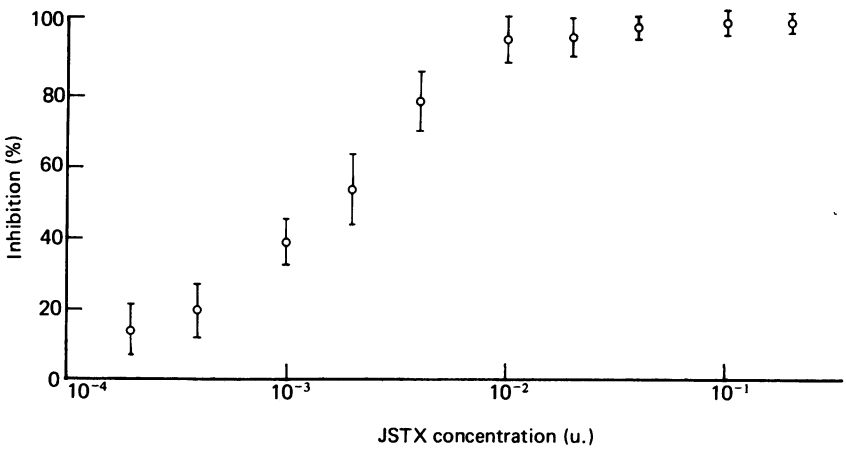


Fig. 2. Dose–response curve for JSTX. Ordinate: percent inhibition of the peak amplitude of e.p.s.p. Abscissa: JSTX concentration on logarithmic scale. Circles: mean value of three to ten experiments. Bars indicate ±S.D.

The dose–response curve of JSTX is shown in Fig. 2 where the percent inhibition of the peak amplitudes of the e.p.s.p.s were plotted against the toxin concentration. Each point was taken 30 min after the e.p.s.p. peak had settled to a steady level at a given toxin concentration. At relatively low JSTX concentrations (below $1 \times 10^{-3}$ u.), the e.p.s.p. returned to the original level after repeated washing of the

preparation. At higher toxin concentrations, however, once the size of the e.p.s.p. decreased to below 20–30 % of the control, recovery of the e.p.s.p. values to the original amplitude was difficult. The threshold concentration of JSTX was around $10^{-4}$ u.; the dose for suppressing the e.p.s.p. to half of the original amplitude was *ca.* $2 \times 10^{-3}$ u.

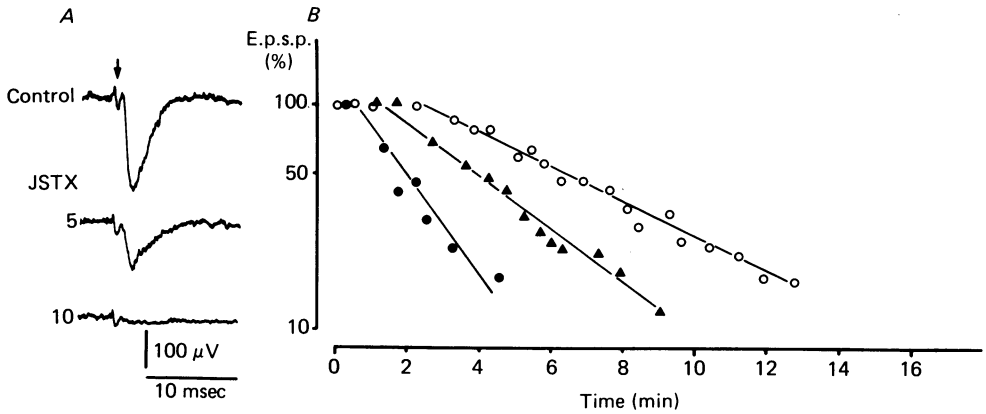


Fig. 3. *A*, effect of JSTX on the extracellularly recorded nerve terminal spike (arrow) and e.p.s.p. Each record is the average of twenty consecutive sweeps. *B*, time course of depression of e.p.s.p. by JSTX. Data were taken from two different synapses. One synapse was treated with JSTX at a concentration of $6 \times 10^{-4}$ u. (open circles) and $3 \times 10^{-3}$ u. (filled circles). The other data were taken from the experiment shown in *A*. The toxin dose was $2 \times 10^{-3}$ u. (triangles). Ordinate: percent of control amplitude of averaged extracellular e.p.s.p. on logarithmic scale. Abscissa: time (in min) after application of JSTX. For further details, see text.

*Time course of the decline of e.p.s.p.s*

After exposing the preparation to the toxin, the amplitude of the e.p.s.p. declined progressively until transmission was blocked. Since crustacean muscle fibres are innervated diffusely along the whole muscle membrane (Wiersma, 1961), intracellular recording of the e.p.s.p. did not show the exact relation between toxin concentration and the transmitter action at a single synapse. Therefore, we made extracellular recordings near the synaptic spot (Dudel & Kuffler, 1961). The recorded localized voltage change is proportional to the synaptic current, provided there is little change in the membrane potential. In Fig. 3*A*, the nerve terminal spike, and the subsequent e.p.s.p. were recorded extracellularly in the vicinity of the synapse. After JSTX application, the e.p.s.p. was suppressed but the nerve terminal spike remained unchanged. Fig. 3*B* illustrates the relation between the amplitude of the extracellularly recorded e.p.s.p. on logarithmic scale and the elapsed time after the application of various concentrations of the toxin. The size of the e.p.s.p. decreased approximately exponentially with time. The rate of inactivation is expressed by the following equation:

$$\ln \frac{Y - Y_0}{Y_{max} - Y_0} = -\alpha t \qquad (1)$$

where $Y$ is the amplitude of the averaged e.p.s.p. at time $t$, $Y_0$ is the fully inactivated response, $Y_{max}$ is the control response and $\alpha$ is the observed rate constant. Two curves

in Fig. 3*B* were from an experiment in which the time course of the decline was compared at the same synapse at different JSTX concentrations. After the e.p.s.p. had been suppressed by a low concentration of the toxin ($6 \times 10^{-4}$ u.), the preparation was repeatedly washed for 90 min until the e.p.s.p. returned to the original amplitude. Then the toxin was re-applied at five times the original concentration ($3 \times 10^{-3}$ u.). E.p.s.p.s were depressed more rapidly; the rate constant of the decline changed from 2·3 to 7·2 ($sec^{-1} \times 10^4$).

Fig. 4 shows the relation between the rate constant of the decline and the toxin concentration in various synapses; the relation was almost linear.

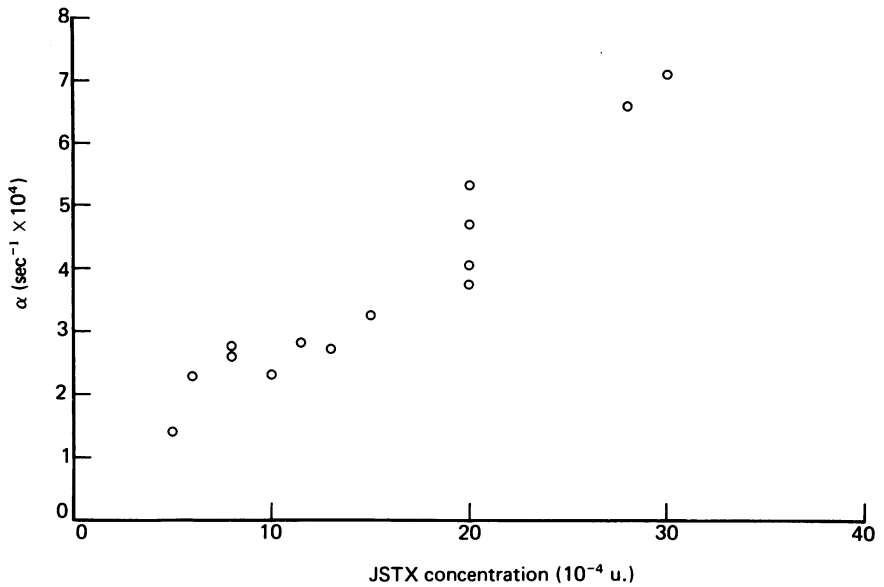


Fig. 4. Relation between the rate constant of reduction of e.p.s.p. ($\alpha$) and the toxin concentration. Calculations were made according to eqn. (1).

*Effect on the glutamate potential*

The above results suggest that JSTX affects the post-synaptic membrane by reducing the sensitivity to excitatory transmitter action. Fig. 5 shows the action of JSTX on the e.p.s.p. and the glutamate potential induced by the ionophoretic application of glutamate. The toxin suppressed the glutamate potential and the e.p.s.p. in a similar way, although usually, the time course of e.p.s.p. depression was slower than that for the glutamate. Two minutes after toxin application, the peak amplitude of the e.p.s.p. was half of the control value; the glutamate potential was decreased to 17 % of its original value. The time required for block of the glutamate potential and the e.p.s.p. was 3·5 and 11 min, respectively. This difference may be attributable to the sharp localization of the glutamate-sensitive spot (Takeuchi & Takeuchi, 1964), thereby facilitating occupation by the toxin. The e.p.s.p. on the other hand, reflects potential changes from widely distributed synapses and the access of the toxin to the synapses may be less easy.

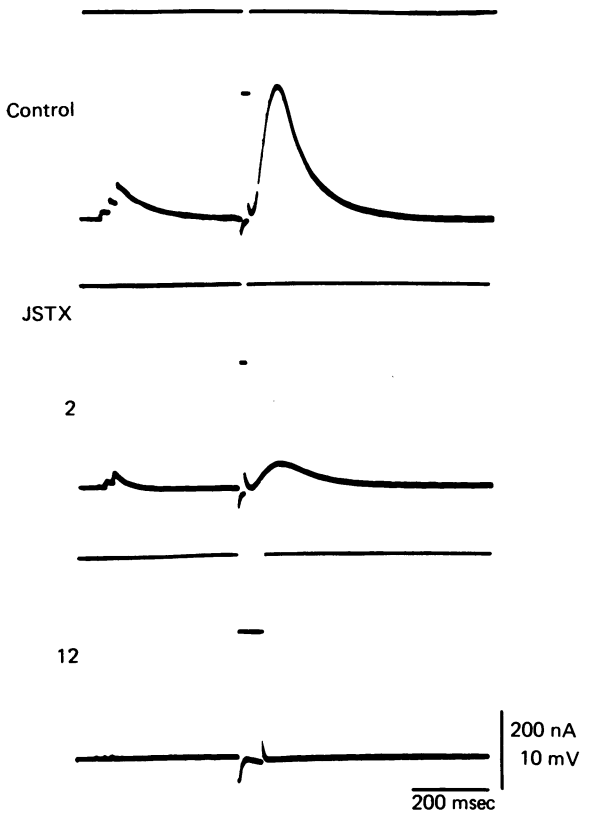


Fig. 5. Effect of JSTX on e.p.s.p.s and glutamate potentials. Upper beam: current of glutamate injection. Lower beam: potential of the post-synaptic membrane. Three consecutive shocks were applied to the excitatory nerve; this was followed by an ionophoretic application of glutamate. JSTX was applied at a concentration of $5 \times 10^{-2}$ u. At 12 min after toxin application, an injection current of longer duration was applied.

*Effects on glutamate analogues*

Next we compared the effect of JSTX on glutamate analogues. The lobster muscle is much less sensitive to aspartic acid than to glutamic acid; ionophoretic application of L-aspartate gave rise to only small depolarization of the lobster muscle membrane. Therefore, we used bath application of aspartate to compare the effect of JSTX. As shown in Fig. 6*A*, 1 mM-L-glutamate solution and 10 mM-L-aspartate produced transient depolarization of the post-synaptic membrane. After JSTX treatment, glutamate at the same dose as in the control produced no appreciable change in the post-synaptic membrane, whereas aspartate gave rise to similar depolarization as in the control solution. Kainic acid (0·1 mM) itself produced no change in the post-synaptic membrane; however, the simultaneous application of glutamate and kainic acid potentiated the glutamate response (Fig. 6*B*). In JSTX-treated preparations, however, kainic acid plus glutamate produced no appreciable change. In contrast to aspartate or kainate, quisqualic acid was similar to glutamate with respect to the toxin action. Depolarization by quisqualic acid ($1 \times 10^{-5}$ M) was blocked by JSTX in a similar way as glutamate (Fig. 6*C*).

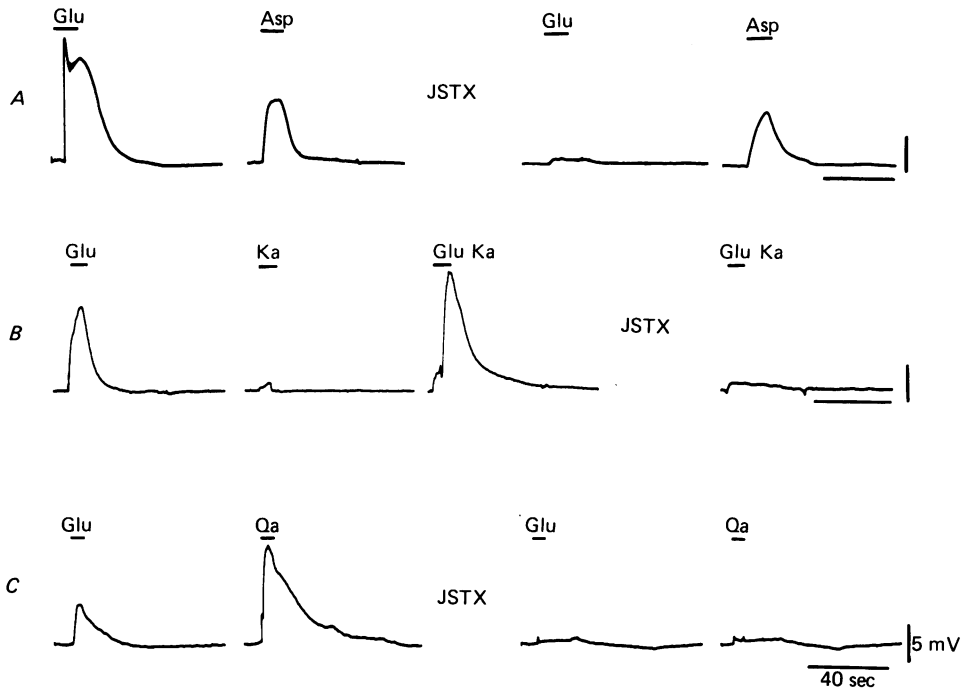


Fig. 6. Effects of JSTX on glutamate-related compounds. Records were obtained from three different muscle fibres (*A–C*). Glutamate and the other agonists were applied at the times indicated by bars. Glu: glutamic acid ($1 \times 10^{-4}$ M), Asp: aspartic acid ($1 \times 10^{-3}$ M), Ka: kainic acid ($1 \times 10^{-5}$ M), Qa: quisqualic acid ($1 \times 10^{-6}$ M). The JSTX concentration was $1 \times 10^{-1}$ u. in all experiments. For further details, see text.

## DISCUSSION

The present experiments revealed that JSTX blocked the glutamate receptor in the lobster neuromuscular junction with a high degree of specificity. To date, several glutamate antagonists have been reported in the crustacean neuromuscular junction (cf. Nistri & Constanti 1979; Cotman, Foster & Lanthorn, 1981; Watkins, 1981). However, many of these either lack specificity or require high concentrations for the antagonistic action. In the present experiments, the JSTX threshold concentration for suppressing the e.p.s.p. was approximately $10^{-4}$ u.; u. is an arbitrarily defined concentration corresponding to the toxin amount in one venom gland per 0·1 ml. volume. The dry weight of one venom gland was 100–200 $\mu$g. If JSTX is the major constituent of the venom and if the molecular weight of the toxin is tentatively assumed to be 500, 1 u. is in the range of $1–3 \times 10^{-4}$ M. The effective concentration for suppressing the e.p.s.p.s to half their original amplitude was *ca.* $2 \times 10^{-3}$ u., i.e. $2–6 \times 10^{-7}$ M. Judging from the profile in the chromatographic elution pattern, the JSTX content constitutes less than 10 % of a venom gland. The effective concentration of JSTX would thus be as low as $10^{-8}–10^{-9}$ M, a low value compared to previously reported glutamate antagonists.

In the presence of JSTX, the amplitude of the extracellularly recorded e.p.s.p. was reduced along a nearly exponential time course. In the simplest form, the action of

JSTX and the receptors can be written as:

$$T + R^k \rightharpoonup \mathrm{TR}' \tag{2}$$

where $T$ is the toxin; $R$, the receptors; TR′ the irreversible toxin–receptor complex. The number of available receptors $r$ can be written:

$$r = r_0 - TR' \tag{3}$$

where $r_0$ is total number of receptors; from eqn. (2), (3) we get:

$$\frac{\mathrm{d}r}{\mathrm{d}t} = -k\,[T]\,[r] \tag{4}$$

$r$ declines exponentially with rate constant

$$\alpha = k\,[T].$$

As shown in Fig. 6, the relation between the rate constant and the toxin concentration is approximately linear, suggesting that toxin–receptor binding obeys first order kinetics. However, for detailed analysis of the kinetics between the receptors and the toxin, the exact molecular weight and the chemico-physical nature of the toxin molecules has to be clarified.

JSTX blocked e.p.s.p.s and the glutamate potential; however, it failed to affect the action of aspartate. Aspartate together with glutamate is a main constituent of amino acids in nervous tissues. In crustacean neuromuscular junctions, high concentrations of aspartate are present in the presynaptic fibre (Kravitz, Kuffler, Potter & Van Gelder, 1963; Sorenson, 1973; McBride, Shank, Freeman & Aprison, 1974). A modification of glutamate action by aspartate in the process of neuromuscular transmission has been suggested (Freeman, 1976; Crawford & McBurney, 1977). Recent data of Kawagoe, Onodera & Takeuchi (1982), however, indicate that aspartate plays little role in the naturally occurring transmission in the crustacean synapse. The different effects of JSTX on aspartate and glutamate in the present experiments also exclude the possibility that aspartate is a neurotransmitter. The presence of kainic acid, a conformationally restricted analogue of glutamic acid, greatly potentiates the glutamate response in the muscle fibres of crayfish (Shinozaki & Shuibuya, 1974*b*), crab (Wheal & Kerkut, 1976), lobster (Constanti & Nistri, 1976) and insect (Daoud & Usherwood, 1975). The effect of kainate can be explained by its action on the extrajunctional glutamate receptors (Onodera & Takeuchi, 1980). The inability of kainate to produce depolarization in JSTX-treated muscle membrane confirms that it is effective only in the presence of glutamate activation. Our results also suggest that, if there are extrajunctional glutamate receptors in the lobster neuromuscular junction, JSTX blocks both the junctional and extrajunctional glutamate receptors. Quisqualic acid is several times more potent than glutamate in the crayfish muscle (Shinozaki & Shibuya, 1974*a*) and rat neurones (Biscoe, Evans, Headley, Martin & Watkins, 1975). The similarity of quisqualate and glutamate has been shown by current-noise analysis of insect muscles (Anderson, Cull-Candy & Miledi, 1978). The suppression of quisqualate-induced depolarization by JSTX suggests that the receptor in the lobster muscle fibres resembles that of the insect.

Regarding the neuro-effective substance in spider venom, only black widow spider venom (BWSV) has been studied extensively (cf. Hurlbut & Ceccarelli, 1979). Crude homogenates of BWSV affect all types of chemical synapses including aminergic synapses (Longenecker, Hurlbut, Mauro & Clark, 1970; Kawai *et al.* 1972; Cull-Candy,

Neal & Usherwood, 1973; Kawagoe *et al.* 1982). In contrast to this venom from *Nephila clavata*, even in the crude state, does not produce massive release of transmitters. Furthermore, the effective component of BWSV, ($\alpha$-latrotoxin) is a polypeptide (*ca.* mol. wt. 130,000) (Frontali, Ceccarelli, Gorio, Mauro, Siekevitz, Tzeng & Hurlbut, 1976), which is quite different from the molecular weight of active substance of JSTX (less than 100,000 as estimated by gel filtration). It is interesting that different species of spiders have entirely different neurotoxins. Since JSTX is also effective in mammalian brain synapses where glutamate is a putative transmitter (Kawai, Niwa & Abe, 1982*b*), it may be promising to utilize this toxin in studying aminergic transmission in the central nervous system and at other synapses.

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