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TI-233 as a glutamate channel blocker at the crayfish neuromuscular junction

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1 Effects of TI-233 (4-isopropyl-1-[N_2-(5,6-dimethyl-aminonaphthalene-1-sulphonyl)-L-arginyl]-piperidine) on glutamate-induced responses and nerve-evoked synaptic responses were compared at the crayfish neuromuscular junction.

2 Intracellularly recorded excitatory junctional potentials (e.j.ps) were markedly augmented by TI-233 when they were evoked at long intervals, whereas the unit size of extracellular e.j.ps was hardly affected by TI-233 and, at that stage, the glutamate-induced current was markedly reduced by TI-233.

3 The decay rate of extracellular e.j.ps was slightly increased 3 min after the addition of TI-233 at concentrations higher than 0.05 mM.

4 Repetitive stimulation of the excitatory axon at a high frequency caused a gradual decrease in the amplitudes of extracellular e.j.ps in the presence of TI-233. After prolonged application of TI-233 with repetitive nerve stimulation, the glutamate-induced response became significantly smaller than the control.

5 TI-233 increased the input resistance of the crayfish muscle fibre and facilitated transmitter release at the excitatory neuromuscular junction. These two effects would entirely explain the augmentation of intracellular e.j.ps by TI-233.

6 TI-233 ($> 3\ \mu M$) reduced the amplitude of current responses to trains of glutamate pulses in a dose-dependent manner, but this reduction by TI-233 was time- and activity-dependent. The effect of TI-233 on glutamate-induced responses was voltage-dependent and hyperpolarization increased this effect.

7 Pretreatment of the muscle fibre with concanavalin A did not affect the gradual decline, caused by TI-233, of the successive currents evoked by a train of glutamate pulses.

8 The apparent differences between the glutamate-induced current and nerve-evoked synaptic response revealed by TI-233 can be explained by open-channel block of the glutamate-activated ion-channel, and do not confute the hypothesis that glutamate is the natural transmitter substance at this junction.

Introduction

Glutamate is a putative excitatory transmitter at the crayfish neuromuscular junction, and several criteria for transmitter identification other than the pharmacological criterion have been satisfactorily met at this site (Takeuchi & Takeuchi, 1964; Onodera & Takeuchi, 1975; 1976; 1980; Kawagoe *et al.*, 1981; 1982). Glutamate exists in sufficient quantities in the presynaptic terminals, and stimulation of the excitatory axons causes a release of glutamate in adequate quantities from the presynaptic terminals (Kawagoe *et al.*, 1981; 1982). However, pharmacological identification of the excitatory transmitter has not yet been satisfactorily made at this site because of the lack of selective glutamate antagonists. Some spider venoms have been found to abolish both glutamate responses and excitatory junctional potentials (e.j.ps) at the lobster neuromuscular junction (Abe *et al.*, 1983), but its chemical structure and mechanism of action have not yet been clarified. If glutamate is the excitatory transmitter at this site, the action of glutamate on the sub-synaptic membrane must be identical in every respect with that of the endogenous transmitter when the effects of various drugs are examined. Therefore, the study of glutamate antagonists will provide important information about the physiology of the excitatory synapses, and it is desirable to test the action of various types of glutamate inhibitors on the crayfish neuromuscular

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junction (Shinozaki, 1980). So far, at the crayfish neuromuscular junction, there appear to be some differences between the action of some agents on the nerve-evoked junctional response and their effect on the glutamate response (Shinozaki & Ishida, 1979a, b; Ishida & Shinozaki, 1980; Shinozaki, 1980). Diltiazem and its structural analogue, caroverine, which are potent Ca antagonists, depressed the responses of the crayfish muscle to glutamate without affecting the average unit size of extracellular e.j.ps (Ishida & Shinozaki, 1980; 1983a). In addition to these observations, it was recently found that 4-isopropyl-1-[N_2-(5,6-dimethyl-aminonaphthalene-1-sulphonyl)-L-arginyl]-piperidine (TI-233) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) markedly depressed the glutamate response at the crayfish neuromuscular junction without significantly affecting the average unit size of e.j.ps (Shinozaki *et al.*, 1982; Ishida & Shinozaki, 1983b). TI-233 and W-7 are both known to be potent calmodulin antagonists (Hidaka *et al.*, 1980; Asano *et al.*, 1982). For identification of glutamate as the excitatory transmitter at the crayfish neuromuscular junction, a reasonable explanation for these discrepancies is required. One point worth considering is that exposure time of receptors to transmitter is in general markedly shorter than that to iontophoretically applied glutamate. Hence, an open-channel blocker would affect the response to iontophoretically applied glutamate more severely than the response to the neurally released transmitter. In the present paper the difference between the glutamate response and the nerve-evoked synaptic response revealed by TI-233 is examined in detail at the crayfish neuromuscular junction. Some preliminary observations have been published previously (Shinozaki *et al.*, 1982).

Methods

The methods used were similar to those described previously (Ishida & Shinozaki, 1980). The opener muscle of the dactyl in the walking leg of the crayfish was used in all experiments. A nerve bundle containing excitatory or inhibitory axons to this muscle was exposed and stimulated with a suction electrode to record e.j.ps. The muscle was perfused with a fresh physiological salt solution at a fixed flow rate of $3\,ml\,min^{-1}$ in a 0.3 ml bath. The solution used was a modified van Harreveld solution containing (mM): NaCl 195, $CaCl_2$ 18, KCl 5.4, Tris-maleate buffer (pH 7.4) 10 and glucose 11. In some cases NaCl was replaced with choline Cl and the concentration of Ca^{2+} was increased to 36 mM. In some experiments the muscle fibre was pretreated with concanavalin A ($1\,mg\,ml^{-1}$) for about 30 min. An intracellular microelectrode filled with 3 M KCl was inserted into the middle portion of a muscle fibre, and an extracellular microelectrode filled with 2 M NaCl was placed on the surface of the muscle at a spot showing the greatest extracellular e.j.p. Glutamate was either ejected iontophoretically by applying a negative pulse to a micropipette (resistance $> 100\,M\Omega$) containing 1 M L-Na-glutamate or administered by bath application. Quisqualic acid was applied in a similar way. The tip of the glutamate or quisqualate micropipette was placed near the most sensitive spot. Iontophoretic application of glutamate or quisqualate by a short pulse produced a transient depolarization with rapid rise time (glutamate- or quisqualate-induced potential). A constant braking current (less than 4 nA) was applied to the ejecting pipette during an experiment. In a number of experiments, the membrane potential of muscle fibres was clamped with an intracellular microelectrode (single electrode voltage-clamp) to record the glutamate-induced current and the extracellular e.j.ps at various membrane potentials. Only a very small change in the membrane potential occurred when the fibre was clamped. The procedures for the voltage-clamp were similar to those described by Onodera & Takeuchi (1976). The potential-recording and current-passing micropipettes were inserted usually within 50 μm from the glutamate sensitive spot. The current micropipette was filled with 2 M K-citrate. In order to measure the input resistance of the muscle fibre, two microelectrodes were inserted separately into the middle portion of a muscle fibre less than 50 μm apart, one for recording voltage changes and the other for passing current pulses (duration 200 ms). The microelectrode for ejecting currents was filled with 2 M K-citrate. Since TI-233 is only slightly soluble even in acidic water (the maximal concentration of TI-233 in a 0.01 N HCl solution is about 5 mM), it was impossible to apply TI-233 iontophoretically through micropipettes. The decay rate and the decay time constant of extracellular e.j.ps were determined by fitting straight lines to the semilogarithmic plots of their decay phases by the method of least squares (Shinozaki & Ishida, 1983c). The decay rate of extracellular e.j.ps was calculated between 80% and 20% of the peak amplitude, and the plots were seen to fall close to a straight line. Experiments were done at a bath temperature of about 22°C.

Drugs

Sodium L-glutamate, mono (Wako), γ-amino-n-butyric acid (GABA; Tokyo Kasei) and concanavalin A (Sigma, Type IV) were used. L-Quisqualic acid was isolated from the plant, *Quisqualis indica L.* TI-233 (4-isopropyl-1-[N_2-(5,6-dimethyl-aminonaphthalene-1-sulphonyl)-L-arginyl]-piperidine) was a generous gift from Dr M. Maruyama (Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo).

Results

TI-233 depresses glutamate- and quisqualate-induced responses

The addition of TI-233 (0.1 mM) to the bath reduced the glutamate-induced depolarization, while the size of e.j.ps was significantly increased (Figure 1a). For the experiment shown in Figure 1b, glutamate was applied every 5 min to the bath for 1 min at a concentration of 0.1 mM. When TI-233 was added at a concentration of 0.01 mM, the peak amplitude of glutamate-induced responses decreased with each successive response, and onset of the decline of depolarization was obviously hastened during the application of glutamate (Figure 1b). In the presence of TI-233 the rate of rise of the glutamate-induced response was reduced in parallel with the decrease in its amplitude. After removal of TI-233 the rise rate of the glutamate-induced response gradually returned to normal and the response was restored very slowly, but the recovery was usually incomplete, particularly when both glutamate and TI-233 were applied at higher concentrations. The potential induced by iontophoretic application of glutamate or quisqualate to a single neuromuscular junction was also depressed by TI-233 in a similar manner. Thus, TI-233 appears to be the most potent glutamate antagonist so far found.

TI-233 augments intracellular e.j.ps evoked at regular intervals

In contrast to glutamate-induced responses, the amplitude of the successive e.j.ps induced by trains of pulses was significantly augmented by TI-233 0.01–0.2 mM. The augmentation appeared soon after the application of TI-233 (Figure 1c), but was not always maintained, and the e.j.p. amplitude gradually decreased. However, there was little change in e.j.p. amplitudes at concentrations lower than 0.01 mM. The restoration of e.j.ps after washing the preparation was significantly more rapid than that of the glutamate-induced response. The threshold concentratoin of TI-233 needed to reduce the glutamate-induced response was about 3 μM which was somewhat lower than that needed to increase the e.j.p. amplitude. At concentrations of TI-233 more than 0.2 mM, the muscle membrane became unstable and it was difficult to measure the synaptic response or the membrane potential with reasonable accuracy. TI-233 caused a slight depolarization of the muscle membrane, amounting to less than 1 mV at a concentration of 0.01 mM and a maximum of about 3 mV at 0.1 mM. This effect may be related to an increase in the input resistance of the fibre. Over a wide range of applied current intensities the electrotonic potential was increased in the presence of TI-233 (up to about 20% above the control amplitude, at a concentration of 0.05 mM). The increase in membrane resistance, however, was not enough to explain the augmentation of intracellularly recorded e.j.ps by TI-233 (Gage & McBurney, 1973).

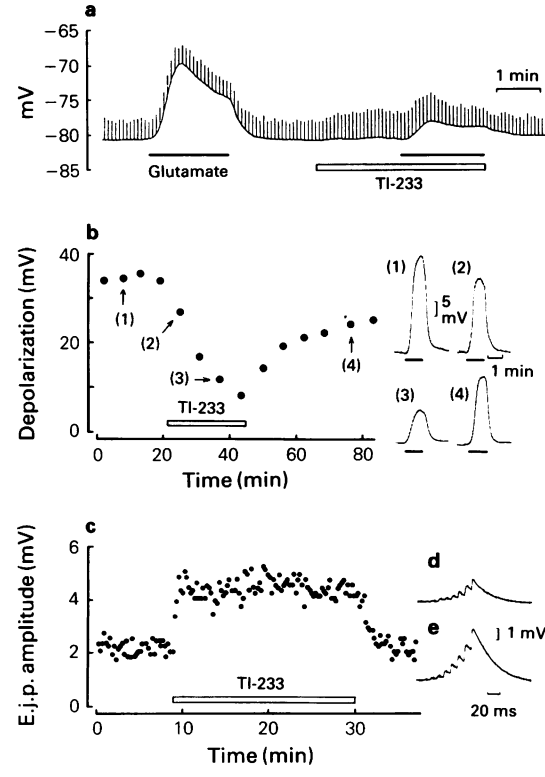


Figure 1 (a) Glutamate-induced depolarization of the muscle fibre and excitatory junctional potentials (e.j.ps) in the absence and presence of TI-233 (0.1 mM). Spikes on the record indicate e.j.ps elicited by a train of 10 stimuli ($100\,s^{-1}$). Glutamate 0.1 mM was added to the medium for a period indicated by black horizontal bars. (b) Effect of TI-233 on amplitude of glutamate responses. Glutamate 0.1 mM was added to the bath every 5 min for a period of 1 min. TI-233 (0.01 mM) was given for 25 min. Ordinate scale: peak amplitudes of intracellularly recorded glutamate-induced responses. Records on the right represent glutamate-induced responses at times indicated on the graph (number in parentheses). The small vertical deflections on the top of depolarization represent successively induced e.j.ps. Glutamate was given for a period indicated by black horizontal bars. Note that the depolarization subsided during the application of glutamate in (2) and (3). (c) The increase in peak amplitudes of e.j.ps caused by TI-233. E.j.ps were induced by trains of 8 pulses (10 ms interval) every 5 s. TI-233 (0.05 mM) was added to the bath for 21 min. Ordinate scale: peak amplitudes of intracellularly recorded e.j.ps. E.j.ps shown on the right side are typical responses recorded before (d) and during the application of TI-233 (e).

Table 1 Quantal analysis of extracellular excitatory junctional potentials (e.j.ps) in the absence and presence of TI-233

	Control			*TI-233*			
Stimulation frequency (Hz)	n	*m*	E_1 (μV)	*Concentration* (mM)	n	*m*	E_1 (μV)
15	419	0.27	125	0.05	144	0.59	126
10	886	0.09	130	0.05	819	0.14	124
15	358	0.30	90	0.1	666	0.37	88
10	1000	0.31	88	0.1	320	0.55	83
10	500	0.23	93	0.1	500	0.39	77
10	1000	0.21	110	0.1	1000	0.55	119
10	900	0.20	124	0.1	900	0.49	122

Measurements were made before and about 3 min after the application of TI-233. *n*, number of stimuli; m, quantal content; E_1, average unit size.

The spontaneous miniature e.j.ps were very infrequent in the present preparation, but in some preparations it was possible to record them. In the presence of TI-233 (0.05 mM), both the mean amplitude and frequency of spontaneous miniature e.j.ps were slightly increased, from 77 ± 4 μV (mean ± s.e.mean, $n = 79$) to 84 ± 5 μV ($n = 105$), and from 1.46 s^{-1} to 1.94 s^{-1}, respectively.

Analysis of the effects of TI-233 on e.j.ps

E.j.ps were set up by repetitive impulses at rates of 10 to 15 s^{-1} at which the quantal content was less than 0.5, the peak amplitude of intracellular e.j.ps being more than 1 mV. The average number of quanta released per impulse was estimated from the number of failures of extracellular e.j.ps (Dudel & Kuffler, 1961). TI-233 was applied at a concentration of 0.05 mM or more. Although it has been suggested that TI-233 inhibits transmitter release from clonal neurones (PC12h cells) by blocking calcium entry (Takahashi *et al.*, 1983), the quantal content of extracellular e.j.ps at the crayfish neuromuscular junction was markedly increased reaching a maximum about 3 min after the application of TI-233, while the average size of the unit potential E_1 was hardly affected. Table 1 shows results of the quantal analysis of extracellular e.j.ps which were recorded about 3 min after the application of TI-233.

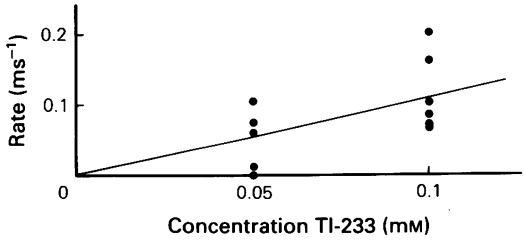


Figure 2 Effect of TI-233 on the decay rate of extracellular e.j.ps. The decay rates of extracellular e.j.ps were determined by fitting straight lines to the semilogarithmic plots of their decay phases by the method of least squares, and the mean values of their decay rates ($n > 50$) were calculated before (α) and after the addition of TI-233 (α') in each preparation. The differences between α' and α were plotted against the concentration of TI-233 and the line was drawn by the method of least squares. The slope of the straight line is 1.09×10^6 $mol^{-1} s^{-1}$.

The decay rate of nerve-evoked synaptic currents at the frog endplate is thought to reflect the average open-time of acetylcholine-activated ion channels (Anderson & Stevens, 1973). When TI-233 was added to the bath at concentrations greater than 0.05 mM, the decay rate was slightly increased at the resting membrane potential about 3 min after the drug application. In Figure 2 the difference between control and test decay rates of extracellular e.j.ps is plotted against the concentration of TI-233. The relationship was approximately linear. The rise time of extracellular e.j.ps did not change. When the membrane was hyperpolarized to − 120 mV, the decay time constant of extracellular e.j.ps was slightly changed from 0.90 ± 0.05 ms (mean ± s.e.mean, $n = 22$) to 0.85 ± 0.04 ms ($n = 29$) in the absence of TI-233 and from 0.87 ± 0.04 ms ($n = 27$) to 0.82 ± 0.03 ms ($n = 46$) in the presence of TI-233 (0.1 mM), indicating that hyperpolarization had little effect on this constant. Neither was the decay phase of extracellular e.j.ps double-exponential nor the peak amplitude reduced in the presence of TI-233. Although it was desirable to examine the effect of high concentrations of TI-233 on extracellular e.j.ps, it was impossible to obtain reasonably accurate results under such conditions, because the membrane potential was not stable at concentrations greater than 0.2 mM and local contractions of the muscle fibres occurred. The experiments confirmed that only the decay rate of

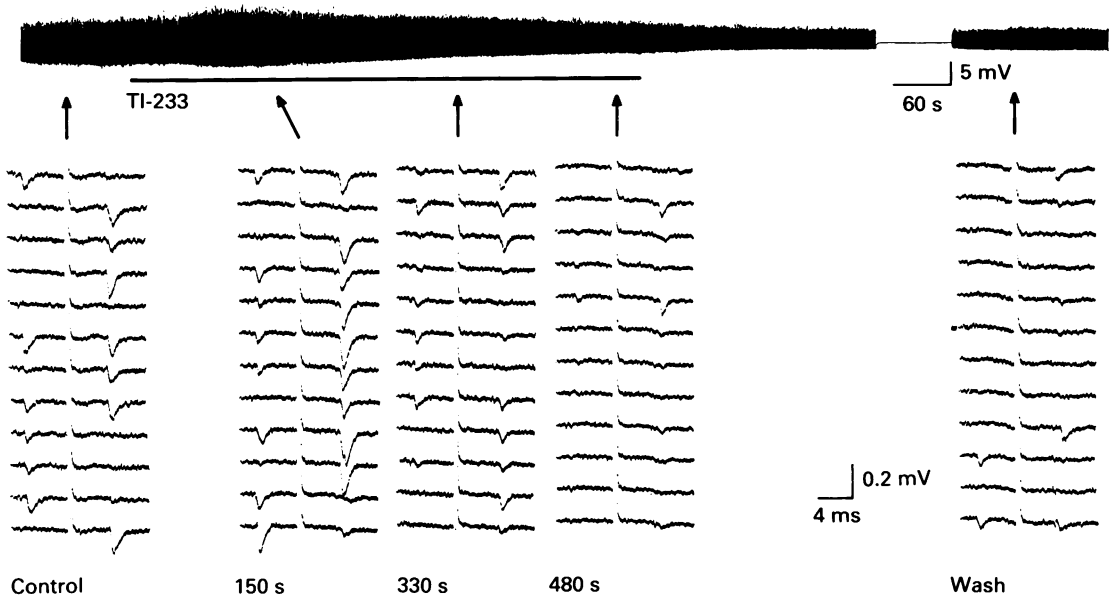


Figure 3 Decrease in e.j.p. amplitude and transmitter release during prolonged application of TI-233. Upper trace, recording (AC-coupled) of intracellular e.j.ps. E.j.ps were evoked every 100 ms by paired pulses with an interval of 10 ms. TI-233 (0.05 mM) was added for the period indicated. Lower traces, extracellularly recorded e.j.ps at times shown by arrows; time after application of TI-233 is indicated below each set of recordings. The lower traces start after the first stimulus artefact.

synaptic currents evoked at relatively low frequency was slightly increased by the drug.

When excitatory axons were stimulated at 25 Hz in the presence of TI-233, the peak amplitude of intracellularly recorded e.j.ps was transiently increased and then gradually declined. This frequency of nerve stimulation was higher than that shown in Figure 1c. Once the amplitude of e.j.ps had been markedly reduced under such conditions, with concentrations of TI-233 above 0.1 mM, the response did not completely recover no matter how long the preparation was washed. The degree of the decline varied from one preparation to another, but it seemed to be related to the frequency of nerve stimulation. In the experiment shown in Figure 3, the excitatory axon was stimulated every 100 ms by a pair of pulses with a 10 ms interval to evoke unfailing transmitter release at almost all junctions. The paired pulse was more effective than a single pulse in ensuring transmitter release. TI-233 was added at a concentration of 0.05 mM for 10 min. The experiment showed that, in the continued presence of TI-233, the amplitude of intracellularly recorded e.j.ps was transiently increased and then gradually declined, and extracellular e.j.ps gradually decreased in size

Table 2 Quantal analysis of extracellular e.j.ps induced every 100 ms by a paired pulse with an interpulse interval of 10 ms

		1st response		*2nd response*	
		m	E_1	*m*	E_1
Control		1.11	84.2	1.63	85.2
TI-233	150 s	1.36	83.2	2.19	78.0
	330 s	1.13	58.4	1.44	52.8
	480 s	0.65	49.8	0.83	49.0
Wash	10 min	0.28	59.0	0.39	61.0

Number of stimuli was 250 and measurements were made before and 150 s, 330 s and 480 s after the application of 0.05 mM TI-233. After washing the preparation the response was not completely restored to the control level. *m*, quantal content; E_1, unit size (μV).

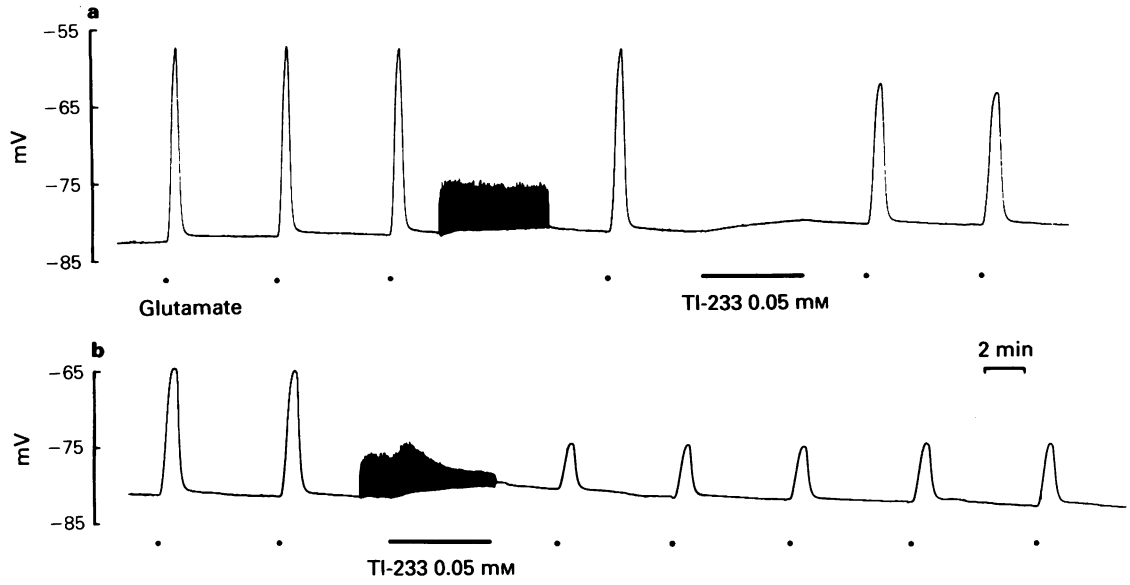


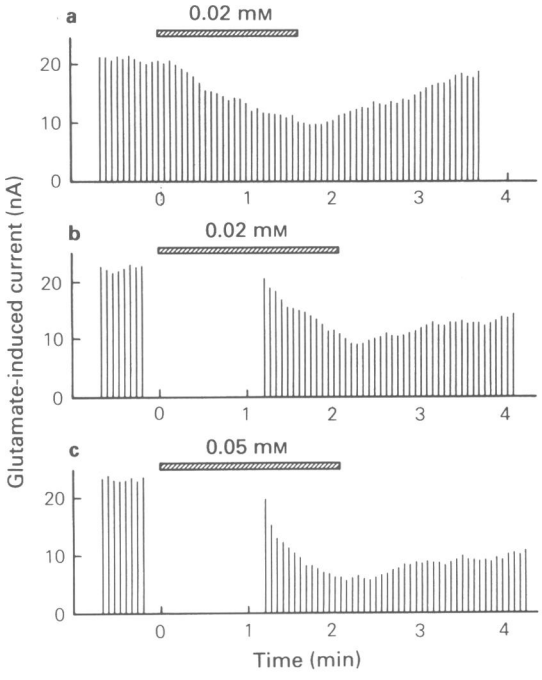
Figure 4 Glutamate-induced responses were significantly reduced after the decrease in e.j.p. amplitude caused by TI-233 (0.05 mM). Every 5 min, 0.1 mM glutamate was added to the bath for 1 min (●). When the amplitude of the glutamate-induced response was sufficiently stable, e.j.ps were elicited by a 5 min period of stimulation with trains of 6 stimuli (pulse interval: 5 ms) given every 400 ms; only a slight decrease in e.j.p. amplitude was observed. The amplitude of the first glutamate-induced response after stopping nerve stimulation was hardly changed. TI-233 was added as indicated. Three minutes after washing out TI-233 the glutamate-induced responses were slightly reduced (see text). (b) When the nerve was stimulated in the presence of TI-233 (0.05 mM), both the e.j.ps and the amplitude of subsequently evoked glutamate-induced responses were markedly reduced.

when the excitatory axon was stimulated by the paired pulse for a long time. The results of the quantal analysis are shown in Table 2. After a transient increase in the quantal content, both it and the unit size were significantly decreased with the lapse of time after the application of TI-233. The response was not restored completely even after washing for more than 1 h.

In the experiment shown in Figure 4, glutamate was repeatedly added to the bathing solution and excitatory axons were stimulated by trains of pulses throughout a period of 5 min. The frequency of stimulation and the number of pulses in each train had been adjusted to evoke intracellular e.j.ps of more than 6 mV. The glutamate-induced response was not affected by these repetitive e.j.ps. Then, TI-233 was added to the bath at a concentration of 0.05 mM for 5 min without applying glutamate. After a 3 min wash, the amplitude of the glutamate-induced response was slightly reduced. In some experiments, when the preparation was washed for a longer period, no such reduction in the glutamate-induced response was observed. Next, TI-233 was re-applied and simultaneously the nerve was stimulated as before. The amplitudes of intracellular e.j.ps, after a transient increase, gradually subsided. Three minutes after the TI-233 bathing solution has been replaced by normal bathing solution, glutamate-induced responses were also markedly reduced. The glutamate-induced response was gradually restored by continuous washing, but the recovery was incomplete. The amplitude of extracellular e.j.ps was also determined, before and after the prolonged simultaneous application of both glutamate (0.1 mM) and TI-233 (0.01 mM). In this case, too, the response was greatly reduced and did not return to the control level; the recovery of extracellular e.j.ps from the effect of the drug was significantly slower when both glutamate and TI-233 were applied than when only glutamate was given.

Analysis of the effects of TI-233 on the glutamate responses

When TI-233 (0.02 mM) was added to the perfusing solution, the glutamate-induced current, evoked every 4 s, was decreased to about 60% of its control amplitude within 70 s (Figure 5a). After a 10 min wash in TI-233-free solution the response was restored to its control amplitude. Next, solution containing TI-233 (0.02 mM) was reintroduced, but the iontophoretic



pulses of glutamate were discontinued until the muscle fibre had been exposed to TI-233 for 70 s (Figure 5b). The amplitude for the first glutamate-induced current after resuming the iontophoretic pulses was almost identical to the control response, but the following successive responses declined rapidly, and after 10 pulses the response amplitude was about 60% of the control. When the concentration of TI-233 was greater than 0.02 mM, the first response after resuming the iontophoretic pulses was slightly reduced (Figure 5c) and this reduction of the response seemed to be dependent on the concentration of TI-233 and, for a given pulse intensity, on the frequency of glutamate pulses. At the end of the experiment shown in Figure 5c, the response was not completely restored to normal. Traces (d and e) in Figure 5 represent the series of glutamate-induced currents interrupted for various periods after their amplitudes had been decreased by TI-233. Only a slight recovery of response amplitude was observed under these conditions.

The rise time of the current evoked by a short pulse (10 ms) of glutamate was shortened at an early stage during application of TI-233 without any change in initial rate of rise or 'slew' rate (Figure 6a), but later on the rising rate also declined. The current evoked by

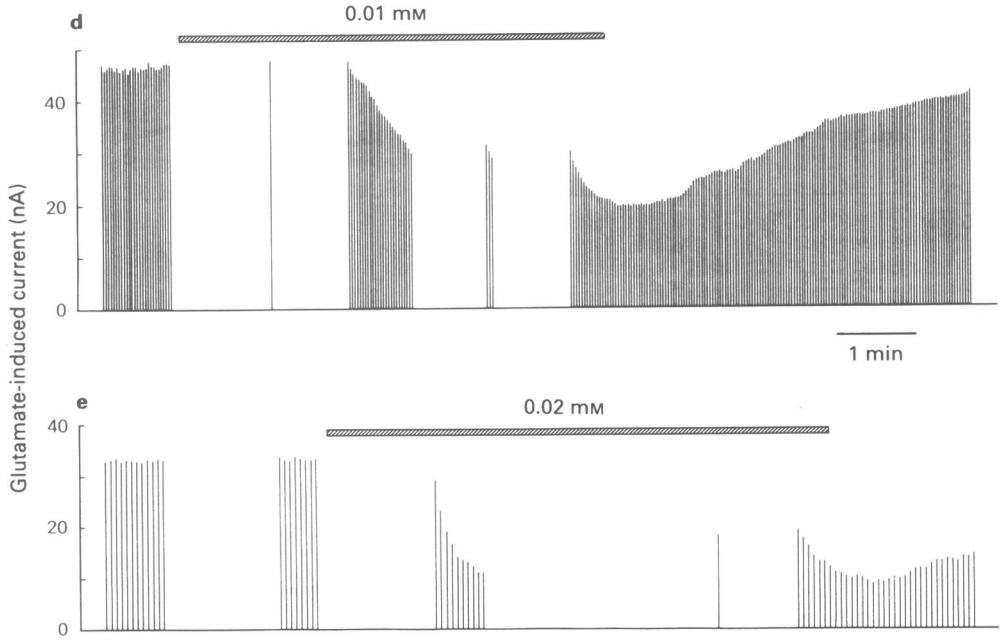


Figure 5 Depression of glutamate-induced currents by TI-233. Glutamate was applied by iontophoresis in pulses at intervals of 4 s. TI-233 was applied as indicated by the hatched bars. (a–c) recordings from the same preparation. (a) TI-233 (0.02 mM) gradually reduced the amplitude of repetitive glutamate currents. (b) After response had recovered, TI-233 (0.02 mM) was re-introduced but glutamate pulses were discontinued for 70 s. (c) As in (b), but a higher concentration of TI-233 was applied and responses declined more rapidly in (c) than in (b). (d and e) Show the slow recovery of the glutamate-induced response from the effects of TI-233, 0.01 mM (d) and 0.02 mM (e). Iontophoretic pulses of glutamate were interrupted for various periods after the response was reduced by TI-233.

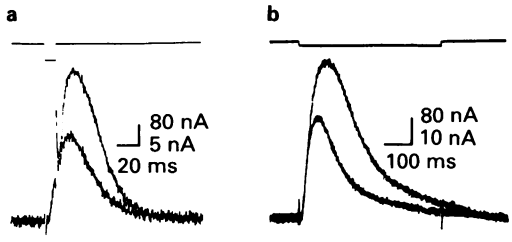


Figure 6 (a) Currents evoked by short pulses (10 ms) of glutamate. Superimposed records, before and during application of TI-233 (0.01 mM). (b) Effect of TI-233 (0.01 mM) on current response to longer pulses of glutamate. Superimposed records as in (a). The smaller responses in (a) and (b) were recorded 2 min after administration of TI-233.

prolonged iontophoretic pulses of glutamate (Figure 6b) slowly subsided to a low plateau level despite the continued presence of glutamate. In the currents evoked by both short and prolonged pulses of glutamate, there was no detectable difference in initial 'slew' rate between the control and test responses.

The above observations might be explained by accelerated desensitization of the receptor (Magazanik & Vyskočil, 1975) or by TI-233 acting as an open-channel blocker (Adams, 1976; Rang, 1982). After treatment of the muscle fibre with concanavalin A, the decline of the repetitive glutamate currents is no longer observed, and a steady depolarization is obtained during prolonged application of glutamate. In the experiment shown in Figure 7, the membrane potential of muscle fibres was clamped at −85 mV, and successive glutamate pulses, given in trains of 10 pulses, were produced. Immediately before applying TI-233 the glutamate pulses were discontinued for 90 s. After resuming the pulses the responses in a train declined gradually, but the first glutamate-induced current was almost as large as the control (Figure 7b); in this experiment the response after

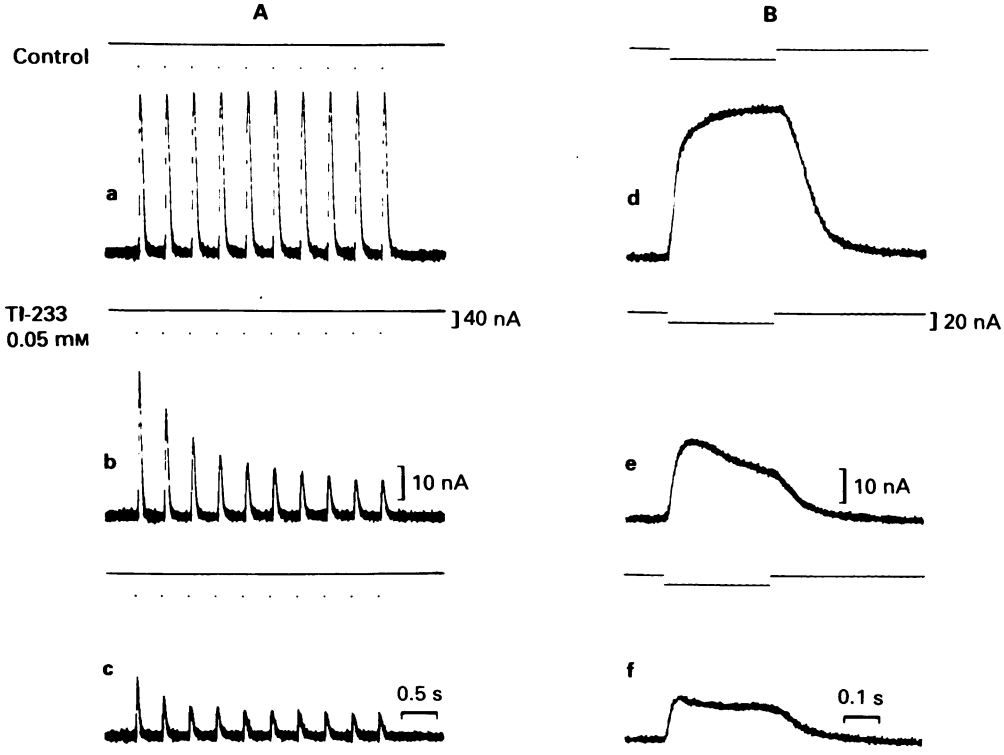


Figure 7 (A) Currents evoked by a train of glutamate pulses (duration 10 ms, interval 380 ms) in a concanavalin A-treated muscle. Glutamate-induced currents were evoked every 15 s. Upper traces: monitored injection currents. Lower traces: glutamate-induced currents. (a) Control; (b) the first series of glutamate-induced responses after a 90 s exposure to TI-233 (0.05 mM), during which time glutamate pulses had been stopped. (c) Current responses to a train of glutamate pulses applied 15 s after recording (b). (B) Currents evoked by long glutamate pulses (duration 300 ms) in the same conditions as in (A). (d) Control; (e) the first response after a 90 s exposure to TI-233 (0.05 mM); (f) 15 s after recording (e).

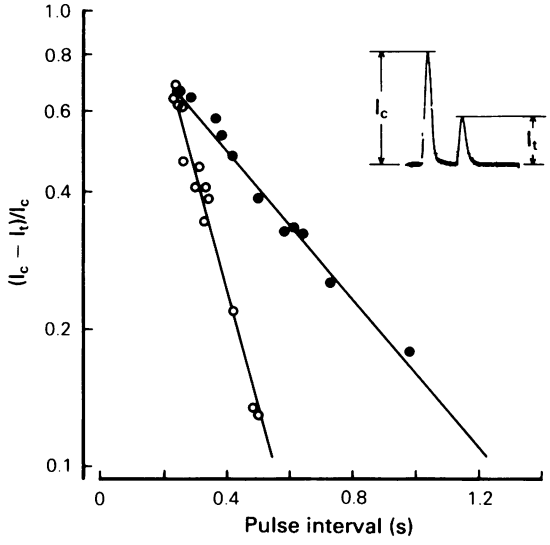


Figure 8 Effect of TI-233 on recovery from the effects of glutamate applied by iontophoresis. Two equiactive glutamate pulses were applied to a glutamate sensitive spot from a single barrel. The fractional inhibition of the 'test' responses by the 'conditioning' one, $(I_c - I_t)/I_c$, is plotted on a logarithmic scale against pulse interval. The straight lines have a slope of $5.9\,s^{-1}$ (control; O) and $1.9\,s^{-1}$ (in the presence of TI-233, 0.03 mM; ●).

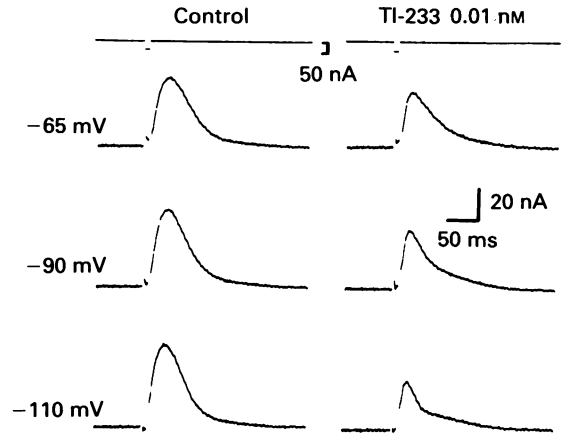


Figure 9 Glutamate-induced currents at various membrane potentials (indicated on the left) before and 3 min after applying TI-233 (0.01 mM). Glutamate pulses were given at 12 s intervals.

washing was not restored completely to the normal level. Also, TI-233 reduced the amplitude of the prolonged glutamate-induced currents in the concanavalin A treated muscle (Figure 7e and f). Although there is no direct evidence that concanavalin A blocks completely and irreversibly the development of desensitization of glutamate receptors, the above results suggest that TI-233 functions as an open-channel blocker rather than an accelerator of desensitization. We cannot, of course, exclude the possibility that TI-233 can antagonize the action of concanavalin A.

A pair of glutamate pulses of relatively small intensity were given so that responses of equal size could be obtained when the pulse interval was long enough, in order to examine the recovery from the effect of the drug (Adams, 1976; Adams & Feltz, 1980; Ishida & Shinozaki, 1980). The 'test' response to the second pulse, I_t, was more markedly reduced in the presence of TI-233 than in the normal bathing solution. However, once the response was markedly reduced by TI-233, complete recovery from the effect of the drug was not observed, and the recovery seemed to vary from the time when the 'conditioning' response to the first pulse, I_c, was slightly reduced and the time when they were markedly reduced. When the 'conditioning' responses were not much affected by TI-233, the fractional inhibition of the 'test' response by the 'conditioning' response was plotted on a logarithmic scale against the pulse interval in order to determine the recovery rate constant (Figure 8). In some experiments the recovery time constants were calculated as 289 ms and 528 ms in the presence of TI-233, when the paired pulse of glutamate was applied every 7 s at various pulse intervals. In this case the plots were almost fitted to a straight line, but I_c gradually declined during the TI-233 experiment. This gradual decline hampered further experiments in all cases. After the 'conditioning' response was markedly reduced by TI-233 to less than about 10% of the control level, it was practically impossible to detect the difference between 'test' and 'conditioning' responses, even when they were induced at pulse intervals of the order of minutes. These observations suggested that there were two types of recovery rate constant one in the order of ms the other in the order of minutes.

Effects of some channel blockers are known to depend on the membrane potential (Adams, 1976; Feltz *et al.*, 1977; Albuquerque & Gage, 1978), hence the influence of potential changes on the inhibitory action of TI-233 was investigated. The pulses of glutamate were repeated at 12 s intervals in the normal bathing solution at various membrane potentials. Next, the membrane potential was clamped at −90 mV and TI-233 (0.01 mM) was added to the bath, while the glutamate-induced current was evoked at the same interval as that in the normal solution. Two minutes after the addition of TI-233, the amplitude of glutamate-induced currents was decreased to about 80% of control. There seemed to be no detectable difference in amplitude of five successive glutamate-induced currents. At this stage, three successive glutamate-induced currents were recorded at various

membrane potentials. As shown in Figure 9, the inhibition of the glutamate-induced current by TI-233 seemed to be more pronounced during hyperpolarization than at -90 mV, and a double-exponential decay of the response was observed together with a reduction in amplitude. In some cases, another small peak superimposed on the response tail in the presence of TI-233. It was apparent that TI-233 caused a distinct change in the time course of the glutamate-induced current depending on the membrane potential, but it was impossible to obtain quantitatively accurate information about the relationship between the membrane potential and the peak amplitude of glutamate-induced currents, because, in the presence of TI-233, a residual effect from the previous response, which varied according to the experimental conditions (see Figure 5), affected the following response variously.

The glutamate receptor on the crayfish opener muscle can be activated even in Na^+-free salt solution, and Onodera & Takeuchi (1976) have shown that glutamate causes a slight permeability increase to Ca^{2+}, though mainly to Na^+. When glutamate was iontophoretically applied in Na^+-free, 36 mM Ca^{2+} solution, it still produced a small but detectable inward current, its amplitude being less than 20–30% of the responses recorded in the normal medium. After addition of TI-233 (0.01 mM) to the bathing solution, the glutamate current evoked every 5 s gradually decreased in a similar manner to that in the normal solution. Furthermore, changing the Ca^{2+} concentration in the normal solution to between 5 and 36 mM had no detectable influence on the effects of TI-233.

Discussion

Comparison of the effect of a drug on the glutamate-induced response at the crayfish neuromuscular junction with that on e.j.ps is of great interest in relation to the glutamate transmitter hypothesis. Glutamate antagonists are of particular value for this purpose. In the present study, TI-233 depressed the glutamate-induced responses in a stimulus frequency-dependent manner, whereas it had no marked effect on the unit size of e.j.ps evoked at low frequencies, but e.j.ps continuously evoked at high frequencies gradually declined under the influence of the drug. In some respects, the mode of action of TI-233 on the glutamate response seems to resemble that of δ-philanthotoxin (Clark *et al.*, 1982) or that of SKF-525A on the acetylcholine response (Magazanik *et al.*, 1982). Chlorisondamine and trimethaphan are powerful glutamate inhibitors of similar potency to TI-233, but they depressed both glutamate responses and e.j.ps in a similar manner (Lingle *et al.*, 1981; Shinozaki *et al.*, 1982; Shinozaki & Ishida, 1983a,b). Chlorisondamine is presumed to be an open-channel blocker with a large unblocking rate constant at the crayfish neuromuscular junction; it lengthens the current evoked by both short and prolonged iontophoretic pulses of glutamate, but the decay of nerve-evoked synaptic current is accelerated by chlorisondamine and the decay of extracellular e.j.ps is frequently double-exponential at relatively low concentrations of the drug (Shinozaki & Ishida, 1983b). It is possible that the different effects of TI-233 on e.j.ps and glutamate-induced currents result from the different exposure time of receptors to released transmitter compared to that of iontophoretically applied glutamate. As expected for open-channel blockers, the effect on the glutamate-induced current is usually greater than that on the nerve-evoked response. While the agonist is present, receptors might be gradually converted into the 'channel blocked' state resulting from preceding channel openings. If the unblocking rate constant which limits the speed of recovery is extremely small, the number of free receptors available would eventually become very small (Clark *et al.*, 1982). The unit size for continuously evoked e.j.ps should be gradually reduced when the muscle fibre is immersed in the TI-233 solution for a prolonged period, because, unlike chlorisondamine, the unblocking rate constant of TI-233 is presumed to be very small.

TI-233 was shown to have some properties attributable to open-channel blocking of the glutamate-activated ion-channel, and two kinds of the unblocking rate constant were predicted, though these rate constants could not be determined exactly in the present study. This is partly because recovery from the block of the response was too slow. The synaptically-evoked current rises to a peak in about 0.4 ms and decays with a time constant of about 1 ms. If the blocking rate constant is very small (see Figure 2), receptor activation terminates before the channel blocker acts, therefore, there is no difference in amplitude between the control and test responses. This is proved by the observation in Figure 6, in which there is no detectable difference in initial 'slew' rate between the control and test glutamate currents. When e.j.ps are evoked at high frequencies in the presence of TI-233, the unblocking rate constant is very small, hence the available free receptors must decrease in number with an increase in the number of receptors in the 'channel blocked' state. At the same time, this may suggest that the amount of iontophoretically applied glutamate, needed to induce a proper magnitude of response, is very much larger than the amount of transmitter released from the nerve terminal. Thus, the present observations do not contradict the hypothesis that glutamate is the natural transmitter at the crayfish neuromuscular junction, although the possibility that there are two types of glutamate receptors with different sensitivities to the drug (Onodera & Takeuchi, 1980) is still not definitely excluded.

Prolonged application of glutamate to the crayfish neuromuscular junction induces receptor desensitization, and as a result, both e.j.ps and glutamate-induced responses are reduced (Takeuchi & Takeuchi, 1964). This has been an effective way of showing that glutamate and the transmitter act on the same receptor. Some substances have been found to reduce the nerve-evoked response as well as the glutamate-induced response at the crustacean neuromuscular junction (Onodera & Takeuchi, 1977; Abe *et al.*, 1983), but in these cases the possibility that these substances block the different receptors (neuroreceptor and glutamate receptor) could not always be excluded. In the present work, it was confirmed that glutamate and the excitatory transmitter act on the same receptors. The activation-induced reduction in amplitudes of e.j.ps and glutamate-induced currents is consistent with the hypothesis that TI-233 blocks glutamate-gated open channels. The observation that the amplitude of the first glutamate-induced current after resumption of pulses in the presence of TI-233 was almost identical to the control response suggests that the forward (blocking) rate constant is small, but its value appears to be of the same order for all blocking agents, in the range of $10^6\,mol^{-1}\,s^{-1}$ (Adams, 1976; Peper *et al.*, 1982; see Figure 2). This suggests that the molecular structure of the blocking drugs does not greatly influence the binding to the open-channel, which may be governed mainly by diffusion of the molecules to a site inside the channel (Adams, 1976; Dreyer, 1982). So far as we know, there is no significant difference in magnitudes of these rate constants for vecuronium, pancuronium (Shinozaki & Ishida, 1984) and tuberostemonine (Shinozaki & Ishida, 1985), which possess an open-channel blocking action, between cholinergic and glutamatergic systems. However, in the case of TI-233, the molecular structure seemed to be important, because modification of the chemical structure of TI-233, for example, replacement of the dansyl moiety of the drug by the 3-methyl-8-quinoline-sulphonyl group resulted in a considerable loss of in the potency of the drug as both a glutamate and calmodulin antagonist (unpublished observations).

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