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RELEASE OF GLUTAMATE FROM THE CRAYFISH NEUROMUSCULAR JUNCTION

BY R. KAWAGOE, K. ONODERA AND A. TAKEUCHI

From the Department of Physiology, School of Medicine, Juntendo University, Hongo, Tokyo 113, Japan

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SUMMARY

1. The superficial abdominal slow flexor muscle was isolated from the crayfish (*Cambarus clarkii*) and placed in a bath solution of 100 μl. The concentration of glutamate in this solution was measured by mass fragmentography using a gas chromatograph-mass spectrometer.

2. The excitatory post-synaptic potential (e.p.s.p.) of the slow flexor muscle and its sensitivity to L-glutamate were similar to those observed in the opener muscle of the dactyl in the walking leg or claw of the crayfish.

3. The background efflux of glutamate during control rest periods was about 20 p-mole/10 min. Nerve stimulation caused a significant increase in the efflux of glutamate. The net release of glutamate above the background was 11·9 p-mole/100 μl. at 10 Hz stimulation and 21·1 p-mole/100 μl. at 20 Hz stimulation.

4. When the amplitude of e.p.s.p. was decreased by streptomycin, thereby reducing the muscle contraction, the net release of glutamate by nerve stimulation was not changed. Streptomycin depressed the e.p.s.p. by its action on the post-synaptic membrane.

5. When the external concentration of Ca was lowered, the amplitude of e.p.s.p. and the net release of glutamate were decreased.

6. It is concluded that L-glutamate is released from the nerve terminals of the crayfish neuromuscular junction.

INTRODUCTION

L-Glutamic acid is the prime candidate for the excitatory transmitter in invertebrate neuromuscular junctions (Gerschenfeld, 1973) and it has also been proposed as an excitatory transmitter in vertebrate central nervous system (Curtis & Johnston, 1974). In the crayfish neuromuscular junction the actions of L-glutamic acid are almost identical with those of the excitatory transmitter (Takeuchi & Takeuchi, 1972; Gerschenfeld, 1973; Onodera & Takeuchi, 1975, 1976, 1980; Takeuchi, 1976); namely: (*a*) the site of action of glutamate is confined to the subsynaptic membrane, (*b*) the excitatory action of L-glutamate is greater than that of related substances, (*c*) applied glutamate mimicks the time course of the excitatory transmitter (for noise analysis, see Crawford & McBurney, 1976), (*d*) the synaptic glutamate receptor is pharmacologically identical with the receptor for the excitatory transmitter and (*e*) the ionic mechanism of glutamate action is the same as that of the neural transmitter.

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One crucial experiment for identifying L-glutamate as an excitatory transmitter is to demonstrate its release by nerve stimulation. The most rigorous controls on the release of glutamate from arthropod neuromuscular junctions were done on the lobster neuromuscular junction (Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970). It was found that the amount of glutamate released by stimulation of excitatory axons at 5 Hz for 15 min was 7×10^{-11} mole which was comparable to the amount of GABA released by inhibitory nerve stimulation. However, nerve stimulation produced an increment of glutamate release which was only about 10% above the background release. This low signal to noise ratio prevented further analyses of the problem, e.g. the effect of Ca on the release.

Essentially similar observations were made on the claw opener of the crayfish (about 45 experiments) (R. Hammerschlag, M. Iimura, K. Onodera & A. Takeuchi, unpublished observations). Although the exicitatory nerve stimulation showed a tendency to increase glutamate release, a definite conclusion was not reached because of a large and fluctuating background release. In those experiments the closer muscle of the claw had to be removed to enable superfusion of the opener muscle; the perfusion fluid was collected from the hole made on the bottom of the shell. These procedures made it difficult to remove all the debris of closer muscle fibres, of afferent nerve fibres and of connective tissue. These tissues contain glutamate which probably leaks out and contributes to the background. In the present experiments, therefore, a simpler system was adopted. Muscle fibres were removed from the animal and placed in a small bath. The bath solution was sampled every 10 min and the concentration of glutamate was measured with mass fragmentography using a gas chromatograph-mass spectrometer (GC-MS) of chemical ionization type. This method is highly sensitive and specific (e.g. Miledi, Molenaar & Polak, 1977). It was found that a significant amount of glutamate was released by nerve stimulation. For a preliminary account of some of this work see Takeuchi, Onodera & Kawagoe (1980).

METHODS

Preparation

Superficial muscle fibres were isolated from the abdominal slow flexor of the crayfish (*Cambarus clarkii*) together with innervating nerve fibres. This muscle forms a thin sheet, one to four muscle fibres thick, innervated by five excitatory and one inhibitory axons (Kennedy & Takeda, 1965). The preparation was placed in a small bath made of a soft plastic material (Evaflex, Mitsui Polychemical) (Fig. 1). The surface of the plastic was coated with 'Siliconize' (dimethyl polysiloxane, Fuji Systems) to make it water-repellent. The muscle was fixed with fine needles at both tendon ends. Chilled Van Harreveld solution was pumped into the bath at the rate of 0·75 ml./min and removed with a hypodermic needle connected to a suction pump. After washing for 30–60 min, all of the bath solution was removed with a suction pump, and 100 μl. of solution was put into the bath with a pipette (P). After 10 min, the solution was removed with a syringe (S) and stored in a prechilled test tube. Between each sampling the preparation was washed for a minute or two with cold Van Harreveld solution at the rate of 0·75 ml./min. When the composition of the solution was changed, the perfusion system was switched to the new solution and perfused for 1–2 min before the experiment. In order to reduce possible uptake of released glutamate into the surrounding tissues, the bath solution was cooled to 9 °C with a thermoelement (T). Nerve stimulation was applied with a pair of silver wires (E) and the stimulus strength was kept at about twice threshold. The membrane potential was recorded with an intracellular microelectrode and stored on an FM tape recorder (1·25 kHz band width).

The perfusion fluid was a modified Van Harreveld solution (mM): NaCl 207·5; KCl 5·4; $CaCl_2$ 18·8; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 5·7 and pH was adjusted to 7·2.

A different solution (mM): NaCl 207·5; KCl 5·4; $CaCl_2$ 13·5; $MgCl_2$ 5·3; HEPES 5·7 was also used. No essential difference was found between these solutions. When the Ca^{2+} concentration was changed, Ca^{2+} was partially or totally replaced with Mg^{2+}.

Since water tended to condense as a result of cooling the bath solution, the relative humidity in the room was kept at 40% at temperatures of 20 °C.

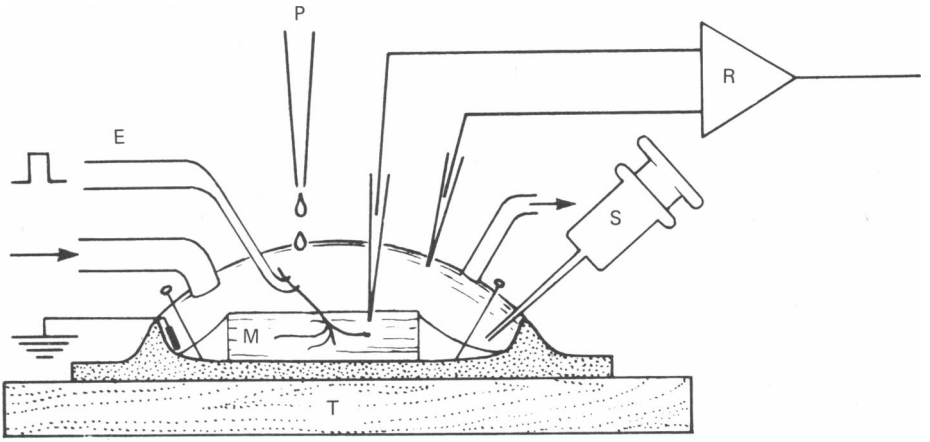

Fig. 1. Experimental setup for collection of samples. An abdominal slow flexor muscle (M) was placed in the bath and washed by introducing the solution from inlet and withdrawn from the outlet. The test solution was added to the bath with a 100 µl. pipette (P) and sampled with a syringe (S). T: thermoelement. E: nerve stimulating electrodes. The e.p.s.p. with recorded intracellularly (R).

Measurement of glutamate

Since details of our procedure for amino acid analyses with GC-MS will be reported elsewhere (K. Murayama, N. Shindo, R. Morishita & K. Ohta, in preparation), we present here just an outline. From each test tube 50 or 75 µl. of the solution was sampled. After adding 50 p-mole of deuterium-labelled L-glutamate (2d-L-glutamic acid, Aldrich Chemicals) as an internal standard, the solution was evaporated to dryness overnight under a vacuum in the presence of phosphorus pentoxide. Pentafluoropropionyl (PFP) glutamate methyl ester was prepared by a modified method of Caprioli, Seifert & Sutherland (1973). The sample was dissolved in 250 µl. methanol and 100 µl. thionyl chloride was added slowly at −80 °C. After incubation at 45 °C for 30 min, the solution was evaporated to dryness by blowing N_2 gas over the surface. The sample was then dissolved in 100 µl. PFP anhydride and allowed to react at room temperature for 15 min and the reagent was evaporated to dryness with N_2 gas. The residue was dissolved in 100 µl. acetone (gas chromatographic grade) and centrifuged at 3000 rev/min for 5 min. One µl. of the supernatant was injected in the GC-MS (Finnigan Type 4000). Also included in each experiment were standard solutions containing 0, 5, 10, 20, 40 and 100 p-mole of L-glutamate plus internal standard. These were dissolved in saline solutions which had not been in contact with muscle fibre. The gas chromatographic column used was a 2 mm (i.d.) × 1 m glass column packed with 3% OV 17 on Chromosorb (60–80 mesh) with the injector temperature at 200 °C and the column temperature at 150 °C. The sample was ionized at 70 eV using CH_4 as the reagent gas.

Mass spectra of PFP glutamate methyl ester gave major peaks at *m/e* 322, 290, 262. Among these peaks *m/e* 262 was the strongest and was used for the present experiment. The height and area of the mass fragmentogram at *m/e* 262 that appeared at the retention time of L-glutamate were calculated with a computer (INCOS 2000 Data System). The concentration of glutamate in the samples was calculated from the ratio of the area at *m/e* 262 to that at *m/e* 263 (mass fragmentogram of 2d-glutamate) using the calibration curve obtained with standard solutions (Fig. 2), and the amount of glutamate was indicated as p-mole in 100 µl. solution.

A sample containing only the deuterated derivative yields, besides an intense peak at *m/e* 263, a small peak at *m/e* 262. The calibration curve has been corrected for these values. The peak of

m/e 263 appeared 0·3 sec earlier than m/e 262. The deuterated derivative may run faster through the gas chromatographic column than the non-deuterated compound. The mass fragmentography was a very useful method for the present experiment, because the sensitivity of this method was high enough for our purpose and it was possible to detect 1 p-mole of glutamate in 100 μl. solutions and the salts contained in Van Harreveld solution facilitated the acylation with PFP and no desalting procedure was necessary (K. Murayama *et al.* unpublished).

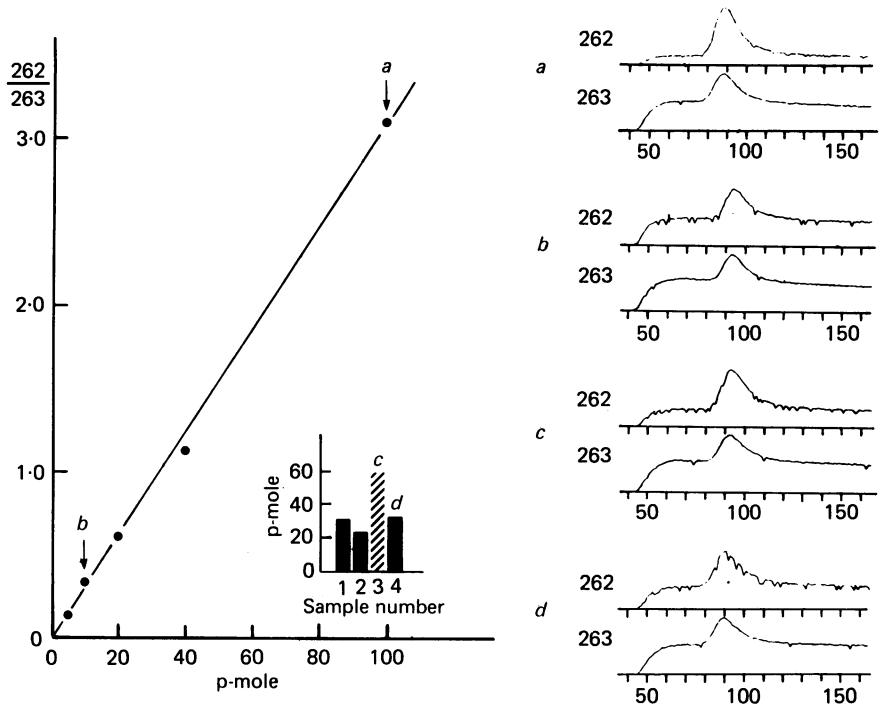

Fig. 2. Calibration curve for the measurement of glutamate. Ordinate: the ratio of the areas under mass fragmentograms for L-glutamate and deuterium-labelled L-glutamate (m/e 262/263). Abscissa: the amount of L-glutamate in 100 μl. solution. Mass fragmentograms for 100 p-mole (*a*) and 10 p-mole L-glutamate (*b*) are shown on the right. (*c*) and (*d*) are the mass fragmentograms of samples collected during stimulation at 20 Hz and a control rest period, respectively. The amount of glutamate in the samples is shown in the inset. The internal standard was 50 p-mole deuterium-labelled L-glutamate (m/e 263). Numbers on the abscissa of mass fragmentograms give the retention time, in sec × 0·3. The sensitivity of the mass fragmentogram changed, keeping the peak amplitude constant.

Drugs used were HEPES (Wako Junyaku), streptomycin sulphate (Meiji Seika), thionyl chloride (Nakarai), PFP anhydride (Pierce) and L-glutamic acid (Ajinomoto). The water was purified with Milli-Q system (Millipore).

RESULTS

Innervation of the abdominal slow flexor muscle

Properties of the excitatory postsynaptic potential (e.p.s.p.) have been studied extensively in the opener muscle of the walking legs or claw of the crayfish. E.p.s.p.s of the abdominal slow flexor muscle were essentially the same as those in the opener muscle (see Kennedy & Takeda, 1965). Fig. 3*A* shows e.p.s.p.s produced at 20 and 29 Hz, recorded with the intracellular and extracellular micro-electrodes. The e.p.s.p.s showed a marked facilitation as observed in the opener muscle. In Fig. 3*B*

ionophoretic application of L-glutamate (29 nC) to the synaptic region produced a depolarization of 2·2 mV, whereas application of acethylcholine (ACh) (79 nC) to the same spot produced no potential change (note the opposite direction of the injection current). The extracellular e.p.s.p. was desensitized by prolonged application of L-glutamate (40 nA) to the synaptic region, but no desensitization was observed with ACh application (160 nA) (Fig. 3*C*). These observations differed from the report by Futamachi (1972) who showed a depolarization by ACh.

When viewed with Nomarski optics the pattern of innervation was slightly different from that in opener muscle, although the morphology of the nerve terminals seemed essentially the same as that in the opener muscle (Pl. 1). Nerve fibres

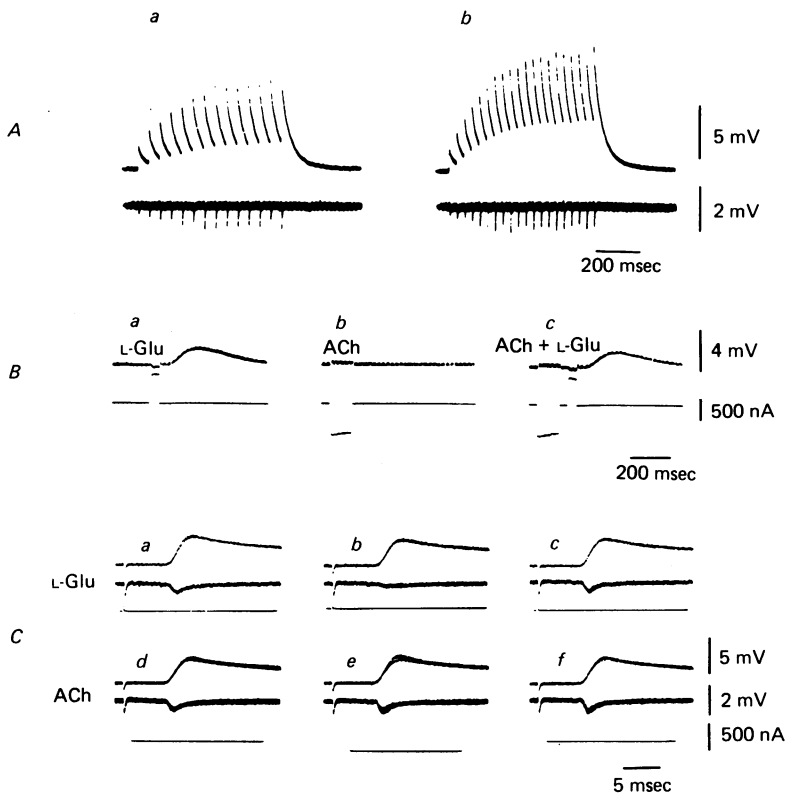

Fig. 3. *A*: e.p.s.p. recorded from the abdominal slow flexor muscle with intracellular (upper record) and extracellular micro-electrodes (lower record). *Aa* at 20 Hz, Ab at 29 Hz stimulation. *B*: ionophoretic application of L-glutamate and ACh from a double barrelled micropipette. *a*, L-glutamate (29 nC); *b*, ACh (79 nC) and *c*, simultaneous application of L-glutamate and ACh. No depolarization was produced by ACh. Injection currents are monitored on the bottom. Direction of injection current was inward for L-glutamate and outward for ACh through the injection pipettes. *C*: desensitization of e.p.s.p. E.p.s.p.s were recorded with intracellular (top trace) and extracellular microelectrodes (middle trace). L-glutamate and ACh were ionophoretically applied to the point where the extracellular recording was made. Injection current was monitored on the bottom trace. *a–f* were recorded from the same synaptic region. *a*, before; *b*, during prolonged application of L-glutamate (40 nA) and *c*, after application. *d*, before; *e*, during application of ACh (160 nA) and *f*, after application. No desensitization was observed by ACh. E.p.s.p. was produced at 2 Hz.

terminated with bouton-like structures on the muscle surface and the glutamate sensitivity was critically localized to the synaptic boutons. The glutamate sensitivity varied from 10 to 40 mV/nC at the edge of a bouton. At a point 8 μm away from the bouton the sensitivity decreased to 1·5 mV/nC. In this experiment collagenase was not used and therefore the spatial discrimination of the ionophoretic method was not as good as in previous experiments on the opener muscle treated with collagenase (Onodera & Takeuchi, 1980). In the opener muscle the spots with high GABA and glutamate sensitivity in the nerve terminal arbolization overlapped, while in the flexor muscles they usually were separated. This observation may be related to the finding that in the abdominal slow flexor muscle presynaptic inhibition was absent (Kennedy & Takeda, 1965).

Release of glutamate

The glutamate content in 100 μl. solution is shown in Fig. 4. Each column represents the glutamate content of a 10 min collection period, and the filled columns are those during control rest period. The control glutamate efflux varied from preparation to preparation, but it remained relatively constant during the experiment. The average content in the controls was about 20 p-mole/10 min (Table 1). Hatched columns in Fig. 4*B* illustrate the glutamate content when nerve stimulation was applied at 10 Hz (number 3) and 20 Hz (number 6) for 7 min and show that glutamate content increased by about 10 and 25 p-mole, repsectively. Sample records of e.p.s.p.s are shown in the inset. Fig. 4*A* shows an example where nerve stimulation was applied at 25 Hz and the glutamate content increased by about 45 p-mole. Average values of the glutamate content in 100 μl. solution are summarized in Table 1*A*. Although considerable variations occur, the glutamate content increased significantly as a result of nerve stimulation. Table 1*B* shows the average net increase of glutamate release in response to nerve stimulation (difference of the glutamate content during nerve stimulation minus the mean of the background before and after stimulation). The net increase by 20 Hz stimulation was significantly larger than that by 10 Hz stimulation.

Effect of streptomycin

Repetitive nerve stimulation at 20 Hz produced an e.p.s.p.s of about 10 mV. The degree of contraction of muscle fibres, which in many cases was observed under the microscope, varied from preparation to preparation. The amount of glutamate release was not related to the degree of contraction. However, one has to exclude the possibility that contraction of the muscle fibres squeezed L-glutamate out of the extracellular space. This was checked by adding streptomycin to the bath, thereby reducing the amplitude of e.p.s.p. and the muscle contraction. This drug decreases both the amplitude of e.p.s.p. and glutamate potential by an action on the postsynaptic membrane (Onodera & Takeuchi, 1977). The hatched columns in Fig. 5 show the glutamate content when 20 Hz stimulation was applied for 5 min and sample records of e.p.s.p.s are shown in inset. During the period indicated by the bar, 0·5 mM-streptomycin was added to the solution. The amplitude of the e.p.s.p.s was decreased to about 35% of the control and no contraction was observed under the microscope. At the same time, however, the release of glutamate remained unaltered.

When the preparation was soaked in the streptomycin solution for a long period of time, the neuromuscular transmission was often impaired and it recovered only after a prolonged washing. Thus, the third series of stimulation in normal solution produced only a small e.p.s.p. Correspondingly, the release of glutamate was smaller than that of first series of stimulation.

Effect of Ca^{2+} and Mg^{2+}

It has been well established that Ca^{2+} is important for the release of neurotransmitter (Katz, 1969; for crayfish Bracho & Orkand, 1970; Ortiz & Bracho, 1972; Wernig,

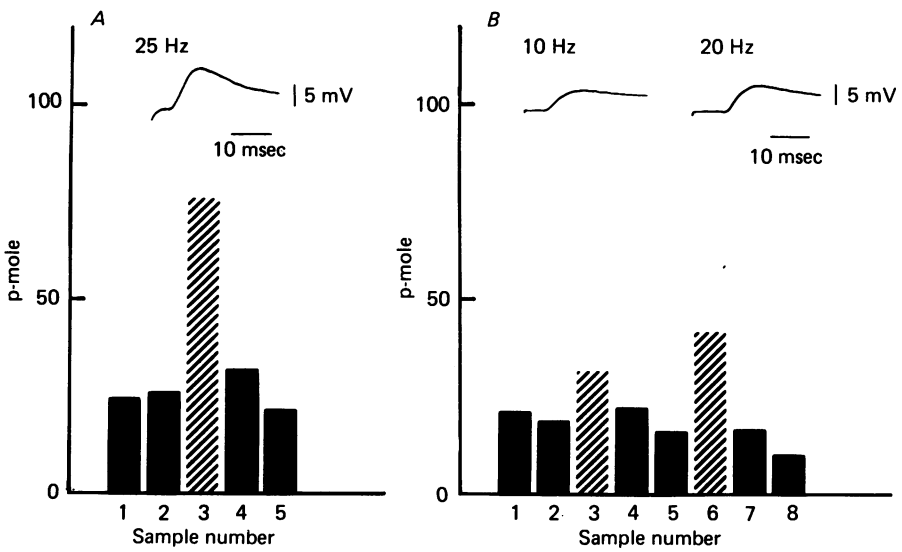

Fig. 4. Examples demonstrating the release of glutamate in response to nerve stimulation. Each column represents glutamate content in 100 μl. solution for a sampling period of 10 min. *A*: nerve stimulation was applied at 25 Hz for 7 min, and the glutamate content in this solution is shown in the hatched column. *B*: stimulation was applied at 10 Hz (number 3) and 20 Hz (number 6) for 7 min. Sample records of e.p.s.p. are shown in inset.

TABLE 1. Glutamate released at rest and 10 and 20 Hz stimulation of the nerve

A. Glutamate content of 10 min collection (mean ± S.E., p-mole)

	Stimulation		
Control before	10 Hz	20 Hz	Control after
20·3 ± 1·31 (n = 26)	30·7 ± 3·59* (n = 6)	44·7 ± 4·41* (n = 12)	19·8 ± 1·94 (n = 23)

* $P < 0.01$, compared with control.

B, Net release in response to nerve stimulation (mean ± S.E. p-mole)

10 Hz	20 Hz
11·9 ± 3·0 (n = 6)	21·1 ± 2·38* (n = 12)

* $P < 0.05$, compared with 10 Hz stimulation.

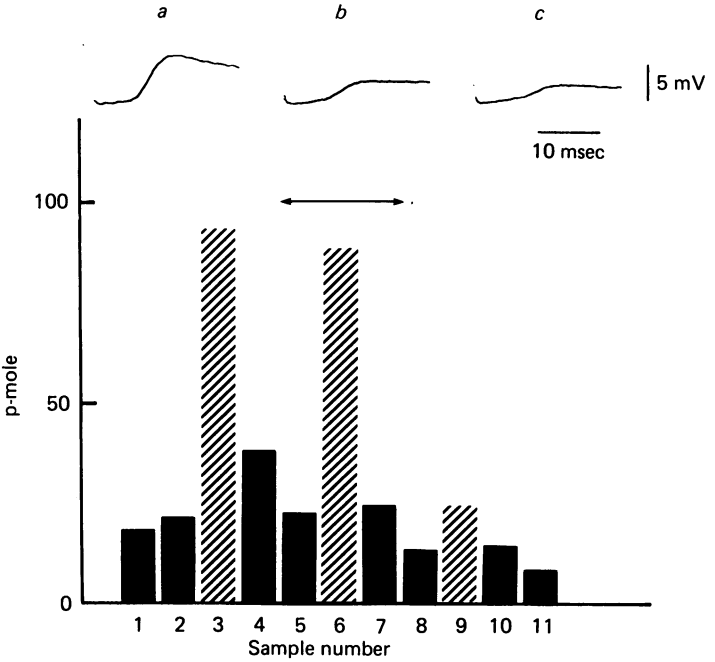

Fig. 5. Effect of streptomycin on the glutamate release and e.p.s.p. During the bar indicated 0·5 mM-streptomycin was added to the solution. Sample records of e.p.s.p.s are shown in inset which were produced at 20 Hz. *a*, before; *b* in streptomycin (0·5 mM) and *c* afterwards.

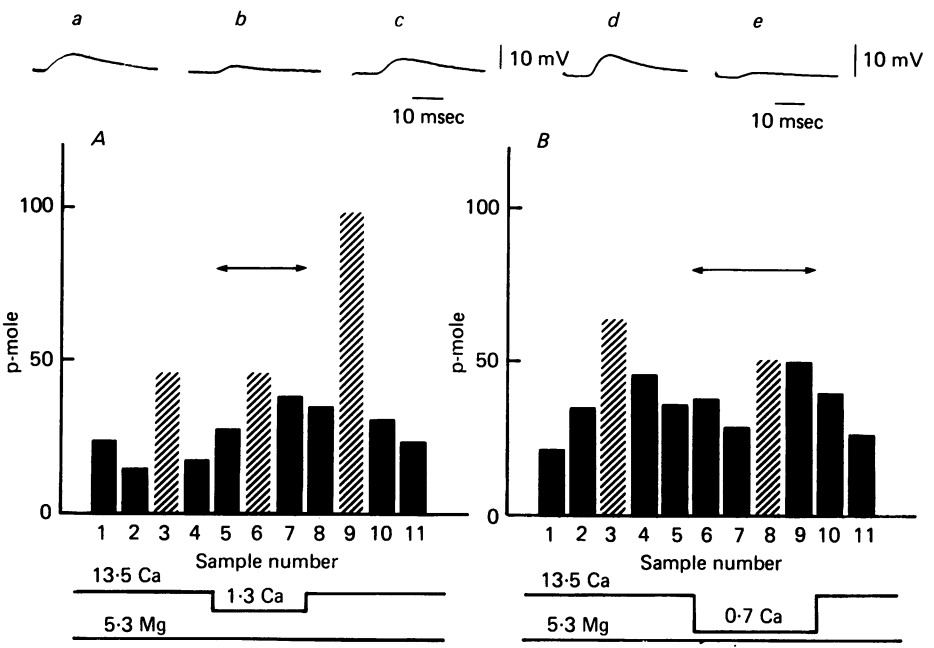

Fig. 6. Effect of Ca^{2+} concentration on the e.p.s.p. and glutamate release. During the bar indicated the Ca concentration was decreased to 1·3 mM (*A*) and 0·7 mM (*B*) in the presence of 5·3 mM-Mg. Sample records of e.p.s.p.s are shown in the inset which were produced at 20 Hz.

1972; Zucker, 1974). Fig. 6 shows the effect of low-Ca medium on the e.p.s.p. and glutamate release. During the bar the Ca^{2+} concentration was decreased to 10% (Fig. 6*A*) and to 5% of the control solution (Fig. 6*B*). The amplitude of the e.p.s.p. and the net release of glutamate were decreased in low-Ca media.

TABLE 2. Effect of low Ca on net release of glutamate (20 Hz stimulation) (mean ± S.E. p-mole)

Normal before	Low Ca (0·7 mM-Ca, 5·3 mM-Mg)	Normal after
21·6 ± 3·19 ($n = 6$)	8·8 ± 3·49* ($n = 6$)	31·7 ± 2·59 ($n = 3$)

* $P < 0·025$, compared with normal before.

The changes in the amplitude of e.p.s.p. and glutamate release in low-Ca media showed a considerable variation between preparations. When the Ca^{2+} concentration was decreased to 5–10% of the normal solution without Mg^{2+}, the muscle membrane potential became unstable and the e.p.s.p. was not recorded in many occasions. Therefore, in most cases the Ca^{2+} concentration was lowered in the presence of 5·3 mM-Mg. When the e.p.s.p. was not detected in the low-Ca media, the nerve conduction might have been blocked at terminal branches and the experiment was discarded. The average values of the net release of glutamate produced by 20 Hz stimulation are shown in Table 2. The net release in low-Ca media was significantly smaller than that in the normal solution.

When the Ca^{2+} concentration was lowered in the presence of Mg^{2+}, the background release of glutamate showed a tendency to increase. Since spontaneous e.p.s.p.s often appeared repetitively in the low-Ca solution, the increase in the background glutamate release may be attributed to their appearance. The background release of glutamate in the normal solution was 20·8 ± 1·23 p-mol/10 min ($n = 83$) and that in the low-Ca solution containing Mg^{2+} (5·3 and 17·9 mM) recorded from the same muscle fibres was 28·5 ± 2·19 p-mole/10 min ($n = 34$) ($P < 0·005$).

DISCUSSION

Our experiments showed that nerve stimulation increased the glutamate efflux from the crayfish neuromuscular junction. The relatively high signal-to-noise ratio in the present experiments, compared to previous ones (R. Hammerschlag, M. Iimura, K. Onodera & A. Takeuchi, unpublished observation) may be attributed to the simplified system used now. The abdominal slow flexor muscle could be isolated cleanly from the surrounding tissues and the experiments were performed *in vitro*. This muscle is made of a thin layer of muscles fibres suitable for perfusion experiments. The quantum content of the e.p.s.p. of this muscle was larger than that of the opener muscle of the walking leg or claw. In the slow flexor nerve-muscle preparation it was difficult to isolate the inhibitory axon from the excitatory axons. Fortunately, however, there is no presynaptic inhibition in this muscle (Kennedy & Takeda, 1965) and the simultaneous stimulation of the inhibitory axon should not disturb the release of the excitatory transmitter.

It has been proposed by Futamachi (1972) that in the crayfish abdominal slow

flexor muscles the excitatory transmitter is ACh. This conclusion was based on the following observations: (*a*) depolarization is produced by ACh and not by L-glutamate, (*b*) the e.p.s.p. is desensitized by ACh and (*c*) curare depresses and edrophonium enhances the e.p.s.p. However, in the present experiments ACh did not depolarize the muscle and no difference in glutamate sensitivity was found between the opener muscle of the walking leg (Takeuchi & Takeuchi, 1964) and the abdominal slow flexor muscle. The reason for the different results obtained in the present experiments, and those of Futamachi is unknown. It has recently been found that curare blocks both the e.p.s.p. and glutamate action in the neuromuscular junction of *Tenebrio* larvae (Yamamoto & Washio, 1979); but curare seems to act on the ionic channels rather than on the receptors. Furthermore, neither ACh nor choline acetyltransferase have been found in the lobster abdominal slow flexor muscle and its innervating motor nerves (Hildebrand, Townsel & Kravitz, 1974). These observations further weaken the possibility that ACh is the neuromuscular transmitter in the abdominal slow flexor muscle.

The average net release of glutamate was about 20 p-mole at 20 Hz stimulation. This corresponds to about 3 f-mole/impulse. Since the number of muscle fibres was forty to sixty, the net release would be 0·05–0·075 f-mole/impulse·muscles fibre. It is difficult to assess the amount of glutamate released at each synapse, because the structure of the terminal nerve distribution is very complicated and perhaps only a part of the released transmitter may be recovered in the fluid. The glutamate sensitivity at the synaptic region was about 10–40 mV/nC and if the transport number of glutamate from the pipette is assumed to be 0·126 (Obata, Takeda & Shinozaki, 1970), 8 mV of depolarization would be produced by 0·25–1·0 f-mole of glutamate. In the lobster opener muscle the release of GABA is 10–40 f-mole/stimulus (Otsuka, Iversen, Hall & Kravitz, 1966). The number of fibres in this muscle is not known, but if it is assumed to be similar to that in the crayfish claw opener (200 muscle fibres), 0·05–0·2 f-mole/impulse·muscle fibre would be released. This value is in the same range of that in the present experiments.

Release of glutamate in the insect neuromuscular junctions has been measured by several groups (Kerkut, Leake, Shapira, Cowan & Walker, 1965; Usherwood, Machili & Leaf, 1968) and these experiments have been reviewed by Kravitz *et al.* (1970). In the lobster neuromuscular junction the amount of released glutamate is of the same order of that obtained with GABA (Kravitz *et al.* 1970). More recently, release of [U-^{14}C]L-glutamate from the opener muscle of the crayfish has been observed upon nerve stimulation (Wang & Boyarsky, 1979); and although the physiological significance of uptake and release of exogenous substance is not completely clear (Minchin & Iversen, 1964), this result supports the present observations.

The effects of low-Ca medium on the neuromuscular transmission were variable from preparation to preparation and relatively large e.p.s.p.s (up to 3–4 mV) were sometimes observed in low-Ca, high-Mg solutions. Further experiments are needed to clarify this point. However, this may be due to the presence of diffusion barriers around the synaptic terminals (Zucker, 1974) or to the contribution of intracellular Ca which is controlled by binding to and being released from the intracellular organnelles (Alnaes & Rahamimoff, 1975). Although in the present experiments the nerve-evoked release of glutamate was not completely blocked by lowering Ca, the

release was significantly decreased, and this result supports the conclusion that glutamate is released from the nerve terminals.

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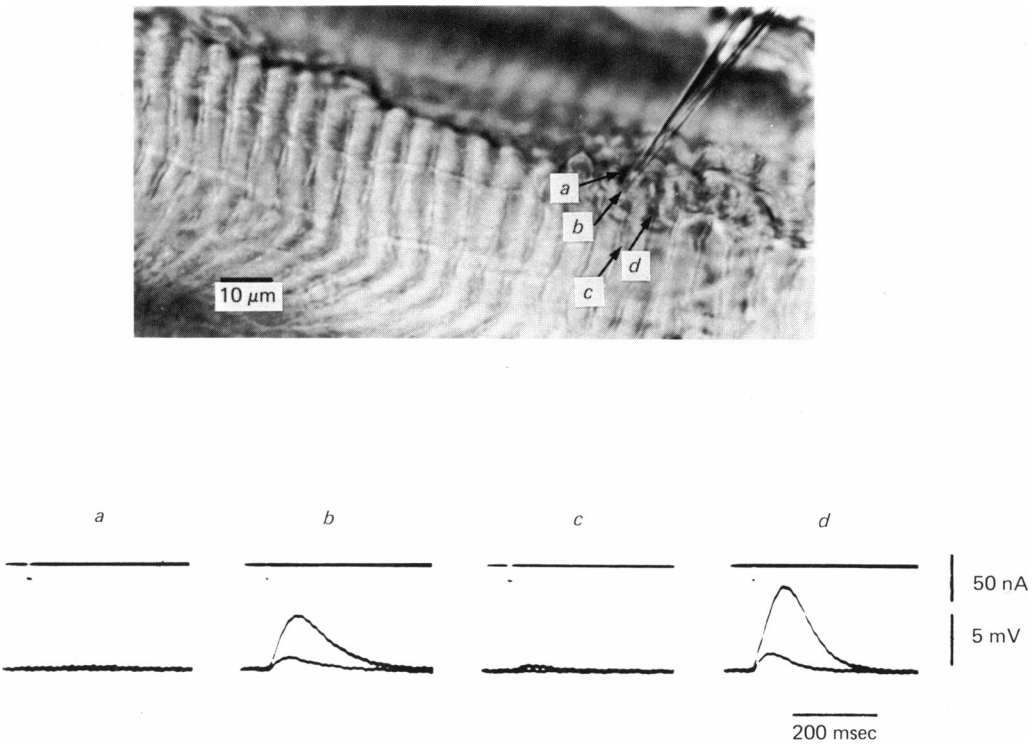
EXPLANATION OF PLATE

Ionophoretic application of L-glutamate to the abdominal slow flexor muscle under visual control with Nomarski optics. The preparation was lightly stained with methylene blue. Arrows indicate the spots where L-glutamate was applied. Sample records of glutamate potential are shown: *b* and *d* were obtained at boutons; *a* was insensitive to L-glutamate; *c* was recorded at a point 8 μm away from the bouton.


a
b
c
d
10 μm
a
b
c
d
50 nA
5 mV
200 msec