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Physiological and Immunocytochemical Determination of the Neurotransmitter at Cricket Neuromuscular Junctions

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ABSTRACT—An attempt was made to determine electrophysiologically and immunocytochemically the neurotransmitter at neuromuscular junctions (NMJs) in cricket abdominal muscles. One mM bath-applied L-glutamate reduced the amplitudes of large and small excitatory junctional potentials (l- and s-EJPs) reversibly by about 75% of the control on the average. It also produced a slow, transient depolarization of about 10 mV. Joro spider toxin, which is an antagonist of L-glutamate, depressed the amplitudes of l- and s-EJPs almost completely and irreversibly at 3.5×10^{-6} M. By using the antibody to glutamate, glutamate-immunoreactive processes whose configuration resembled that of NMJs revealed by nickel staining were obtained. The present results strongly suggest that the neurotransmitter at cricket NMJs is L-glutamate.

INTRODUCTION

Cricket abdominal muscles are innervated doubly and therefore two types of synaptic potentials, i.e., large and small excitatory junctional potentials (l- and s-EJPs), can be recorded from the same muscle fibers (Kawasaki and Kita, 1993, 1995). However, the neurotransmitter generating these synaptic potentials has remained undetermined.

L-glutamate is known to be the neurotransmitter at crustacean and insect neuromuscular junctions (NMJs) (Takeuchi and Onodera, 1973; Anwyl and Usherwood, 1974; Jan and Jan, 1976; Yamamoto and Washio, 1980). Therefore, it seems highly probable that L-glutamate is also a neurotransmitter at cricket NMJs.

It is also known that the neurotransmitter causes desensitization of receptors in the postsynaptic membrane, resulting in a decrease in the amplitude of postsynaptic potentials (Katz and Thesleff, 1957). In our previous experiments (Kawasaki and Kita, unpublished data), L-glutamate, but not D-glutamate, was shown to reduce the amplitudes of l- and s-EJPs.

In the present study, this result was further confirmed, and experiments were performed to determine whether the neurotransmitter at the cricket NMJs is L-glutamate by using an antagonist of L-glutamate and an immunocytochemical method, which reveals glutamate-immunoreactivity. The results of this study indicate it is highly probable that the two types of motor nerve terminals, which generate l- and s-EJPs respectively, release the same neurotransmitter, L-glutamate.

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MATERIALS AND METHODS

Animals

The experiments were carried out using abdominal muscles 202 and 203 of adult male crickets, *Gryllus bimaculatus*, which were kindly supplied by the Laboratory of Animal Physiology, Department of Biology, Okayama University Faculty of Science. Animals were well-fed and stored in an incubator at 30°C until use.

Electrophysiology

l- and s-EJPs were recorded intracellularly with conventional methods, the details of which have been given elsewhere (Kawasaki and Kita, 1993, 1995). In short, the abdominal cavity in which muscles 202 and 203 were left intact with their nerves served as a muscle bath with a capacity of about 0.1 ml. Saline solution flowed continuously through the abdominal cavity with the aid of a peristaltic pump. Oxygen was supplied continuously through an opening made in the trachea to keep the preparation in good condition. To elicit EJPs, the distal cut end of the nerve root innervating the muscle was stimulated with a small suction electrode. l- or s-EJPs themselves have basically the same characteristics in muscles 202 and 203 (Kawasaki and Kita, 1993,1995).

All the experiments were performed at room temperature (22 ± 2°C).

Chemicals

In the desensitization experiments, L- and D-glutamic acids (Wako) were employed. They were dissolved in experimental solutions. As an antagonist of L-glutamate, Joro spider toxin (JSTX-3, Wako) was used. Chemicals and drugs used for immunocytochemistry are described in the following section.

Immunocytochemistry

Whole-mount staining was performed by a modification of the method of Yasuyama *et al.* (1992). After the head, thorax and legs of the cricket were removed, the abdomen was cut open along the midline and pinned on a silicon sheet. The digestive and other internal organs including the adipose tissues around the muscles were also removed. The abdominal cavity was filled with a fixative, 4% paraformaldehyde

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diluted in 0.1 M phosphate buffer (pH 7.4). After 1 hr soaking in the fixative, abdominal muscles 202 and 203 were detached from the abdominal cuticle and immersed in the same fixative overnight at 4°C. Then the muscles were washed four times (1 hr each) in phosphate-buffered saline (PBS: 0.85% NaCl in 0.01 M phosphate buffer, pH 7.2) containing 1% bovine serum albumin (BSA) to block non-specific antibodies, and were incubated in the same solution for 20 hr at 4°C. The specimens were then soaked in 100% methanol with 0.3% H_2O_2 for 30 min at room temperature to reduce background staining that might be attributable to endogenous peroxidase and rinsed in PBS for 30 min. Then they were incubated for 3 hr in PBS containing 1% BSA and 0.8% Triton-X 100 (PBT), which included 2% normal goat serum and 4 drops/ml of avidin D solution (Vector Laboratories blocking kit). All incubations done at room temperature were carried out on a slowly oscillating shaker table to keep the specimens mixed continuously with the solutions. After a subsequent wash for 30 min in PBS, they were transferred to a 1 : 500 dilution of the primary antibody (rabbit anti-glutamate, UCB-Bioproducts) in PBT containing 4 drops/ml of biotin solution (Vector Laboratories blocking kit), and were incubated for 72 hr at 4°C. The blocking kit was used to suppress endogenous avidin-binding activity (Wood and Warnke, 1981). After the specimens were washed four times (1 hr each) and left overnight in PBS at 4°C, they were incubated for 24 hr at 4°C in the biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) diluted 1 : 100 in PBS. Then they were washed four times in the same way and again were incubated for 12 hr at 4°C in PBS containing horseradish peroxidase coupled to biotin-avidin (Vector Laboratories elite ABC kit) to combine the ABC complex with the secondary antibody. The same four washes as previous ones in PBS were followed by treatment with 0.025% 3,3'-diaminobenzidine tetrachloride (DAB, Dojin) in 0.05 M Tris-HCl buffer (pH 7.2) for 1 hr at 4°C. The specimens were then reacted with 0.025% DAB and 0.0025% H_2O_2 in Tris-HCl buffer for 10-15 min at room temperature. The reaction was stopped by washing the specimens three times (15 min each) in PBS. Finally, they were dehydrated through a graded ethanol series and cleared in methyl benzoate.

As the control experiment, the muscles were stained with the primary antiserum preabsorbed with L-glutamate.

RESULTS

Effects of L-glutamate on EJP amplitudes

Figure 1 illustrates the effects of 1 mM L-glutamate on the amplitudes of I- (A) and s-EJPs (B) recorded from muscle 202 at 0.5 and 5 mM $[Ca^{2+}]_o$, respectively. One mM L-glutamate reduced the amplitudes of both EJPs to about 30% of the initial values without changing the resting membrane potential and eliminated spontaneous miniature EJPs. As will be described in the next section and as is shown in Fig. 2, depolarization caused by 1 mM L-glutamate was transient. Therefore, the membrane potential is considered to have returned to the initial level during the later phase of L-glutamate action. As mentioned in the legend of Fig. 1A, muscle contractions often occurred during the initial phase of the action of 1 mM L-glutamate, that might be due to the transient depolarization. The length of the period during which the muscle showed spontaneous, irregular contractions was comparable to the duration of the transient depolarization (see Fig. 2). The recovery from the effect of L-glutamate was rapid and complete in both EJPs on return to the initial solution. Similar results were obtained with I- and s-EJPs in four and six other examples, respectively. Contrary to L-glutamate, D-glutamate had no effect on the amplitudes of I- and s-EJPs,

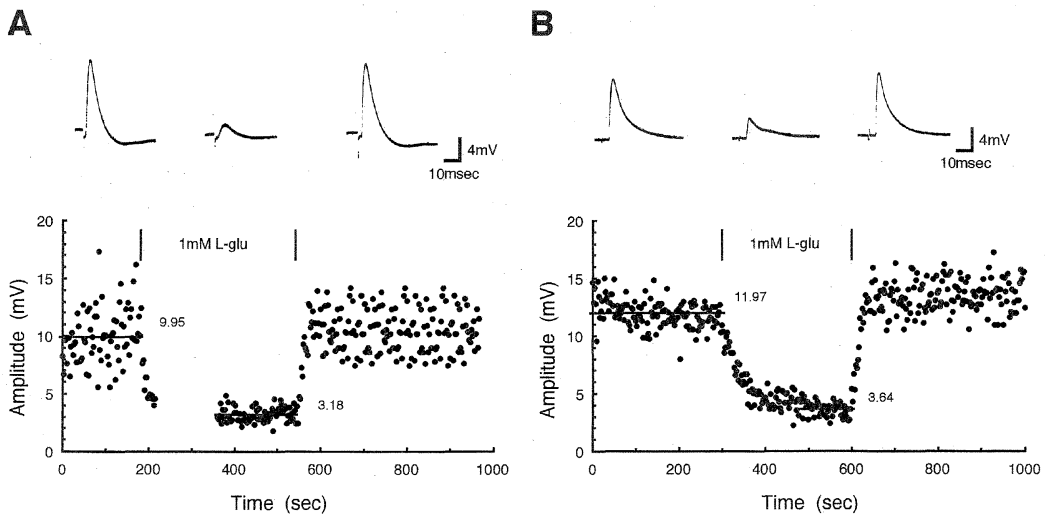

Fig. 1. Desensitization experiments in muscle 202. A: Upper, sample records of I-EJPs recorded from a single muscle fiber at 0.5 mM $[Ca^{2+}]_o$ before, during (indicated by '1 mM L-glu') and after application of 1 mM L-glutamate. The I-EJPs were evoked by stimulating the nerve root once every 2.5 sec. Lower, time course of changes in I-EJP amplitude before, during and after application of 1 mM L-glutamate. The numbers beside the horizontal bars indicate the average amplitude during the periods indicated by the bars. During the early phase of L-glutamate action, spontaneous, irregular muscle contractions, which did not dislodge the microelectrode, often occurred and the measured values were discarded because of the change in the resting membrane potential. B: Upper, sample records of s-EJPs recorded from a single muscle fiber at 5 mM $[Ca^{2+}]_o$ before, during and after application of 1 mM L-glutamate. Lower, time course of changes in s-EJP amplitude before, during and after the application of 1 mM L-glutamate. The numbers beside the horizontal bars indicate the average amplitudes during the period indicated by the bars. In A and B, the bathing solution flowed continuously in the anteroposterior direction at a rate of 1 ml min^{-1} through the abdominal cavity in which abdominal muscles were soaked.

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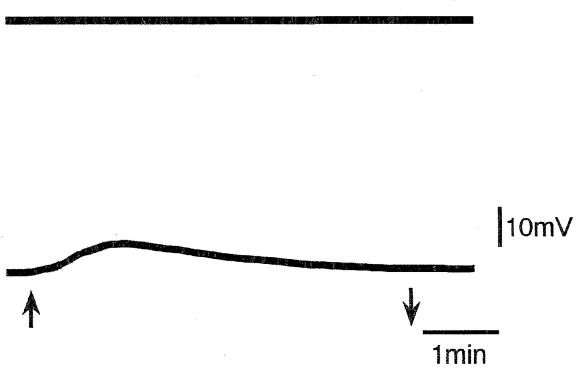

Fig. 2. Depolarization caused by bath-applied 1 mM L-glutamate in a fiber of muscle 202 bathed in flowing normal saline solution. The upward and downward arrows indicate the interval during which the saline solution containing L-glutamate was flowing. The upper trace indicates the reference level and the lower the change in the resting membrane potential.

which was confirmed in five experiments.

Depolarization caused by bath-applied L-glutamate

Although recordings of glutamate potentials produced by iontophoretically applied L-glutamate were unsuccessful, depolarization of muscle fibers could be obtained by bath-applied L-glutamate in muscle 202. Fig. 2, which is a representative example of three experiments, shows that 1 mM L-glutamate produced a slow, transient depolarization of less than 10 mV. The amplitude of the depolarization varied from preparation to preparation. As already described in the previous section, the duration of the transient depolarization roughly corresponds to the period during which the plots are missing in Fig. 1A because of muscle contractions. This result provides evidence that L-glutamate is the neurotransmitter at the cricket NMJs.

Effects of antagonist of L-glutamate (Joro spider toxin) on l- and s-EJPs

Figure 3A shows the effect of 3.5×10^{-6} M JSTX-3 on l-EJPs recorded from muscle 202 at 0.5 mM $[Ca^{2+}]_o$. In this representative example, the average EJP amplitude before application of the toxin was 5.12 mV. On application of the toxin, the amplitude began to decline gradually. Twenty min after the application, it fell to a value close to 0 mV and stayed there for the next 10 min. To obtain the average recovery value of EJP amplitudes, we recorded 100 l-EJPs from each of three different muscle fibers at 30, 35 and 40 min, respectively, after the muscle was returned to the control saline. The average amplitude of EJPs 30-40 min after washing of the toxin was only about 40% of the initial value. However, no recovery was seen in four other examples. This contrasted with the effect of L-glutamate.

Figure 3B, which is also a typical example, illustrates the effect of the same concentration of JSTX-3 on the s-EJP amplitude recorded from muscle 203. The control amplitude, which was 4.86 mV, started to decrease slowly when the toxin was introduced to the bath. Thirty min after the addition of the toxin, the amplitude reached a value near 0 mV. The time course of decay was similar to that of the l-EJP amplitude. On return to the control solution, the same measurements as in l-EJPs were carried out and again no recovery was observed. Similar results were obtained in four other examples.

Immunocytochemical observations

Figure 4 shows glutamate-immunoreactive processes in muscles 202 (A) and 203 (B). The configuration of stained regions resembles that of nerve terminals revealed by nickel staining (Kawasaki and Kita, 1995). DAB reaction products seemed to be confined to nerve endings rather than being scattered all over the neuron. No immunoreactivity was observed in preparations stained with the glutamate antiserum

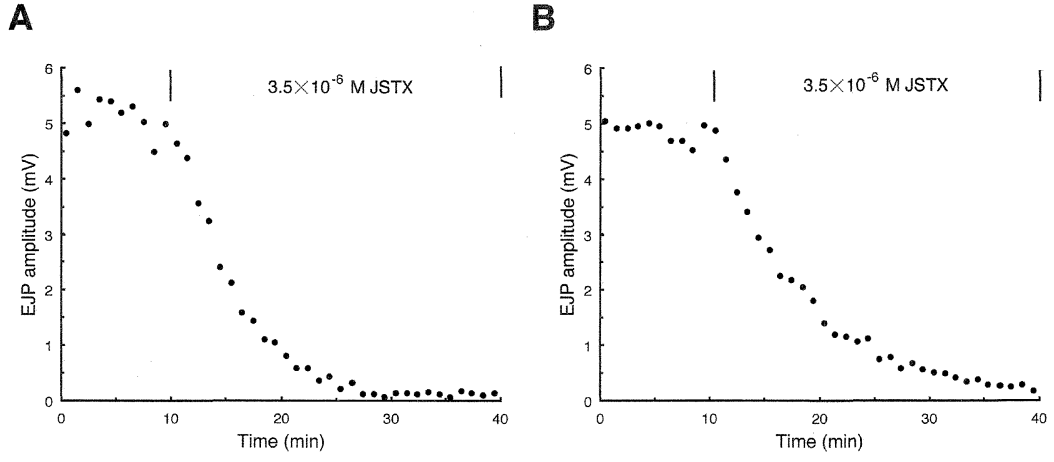

Fig. 3. Effects of 3.5×10^{-6} M JSTX-3 on the amplitudes of EJPs elicited by repetitive stimulation at 0.4 Hz in single fibers of muscles 202 (l-EJPs) and 203 (s-EJPs). A: Effect on l-EJP amplitudes. The toxin was applied to the circulating bath solution during the period indicated by the two vertical bars. Each point represents the average value of 24 amplitudes and the value was plotted at the center of each 1 min period. $[Ca^{2+}]_o$ = 0.5 mM. The control EJP amplitude (the average of the first 10 points) was 5.12 mV. B: Effect on s-EJP amplitudes. Plotting was made similarly to A. The control amplitude was 4.86 mV. $[Ca^{2+}]_o$ = 5 mM.

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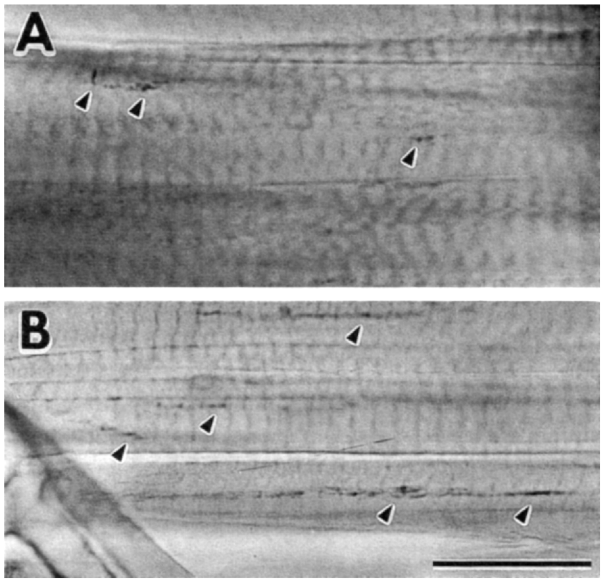

Fig. 4. Light micrographs of whole-mount preparations of muscles 202 (A) and 203 (B) showing glutamate-immunoreactive processes (arrowheads). Scale bar: 50 μm.

preabsorbed with L-glutamate.

DISCUSSION

Although neurotransmitters at NMJs in the crayfish (Takeuchi and Onodera, 1973), locust (Anwyl and Usherwood, 1974) and *Drosophila* (Jan and Jan, 1976) have been reported to be L-glutamate, that at NMJs in cricket abdominal muscles has not yet been determined. This substance is highly likely to also be the neurotransmitter at cricket nerve-muscle synapses.

The neurotransmitter causes desensitization of the postsynaptic receptors of the synapse at which the neurotransmitter is working (Katz and Thesleff, 1957). In the presence of L-glutamate in the bathing solution, the postsynaptic receptors are occupied by this transmitter substance dose-dependently and therefore, the amplitude of EJPs becomes smaller than that of the control ones. We found that the amplitudes of l- and s-EJPs were both reduced reversibly and dose-dependently by L-glutamate: 1 mM L-glutamate depressed the amplitude by about 75% on the average (Kawasaki and Kita, unpublished data). In addition to these results, the present experiments prove confirmatively that L-glutamate is the neurotransmitter at NMJs in cricket abdominal muscles. L-glutamate is known to affect presynaptic receptors that are responsible for autoregulation of neurotransmitter release at lobster nerve-muscle synapses (Miwa *et al.*, 1990), resulting in a change in the amount of neurotransmitter released. Therefore, the possibility that L-glutamate reduces the EJP amplitude by affecting presynaptic receptors cannot be ruled out, although such receptors have not yet been reported at cricket NMJs.

The effect of JSTX-3 on the amplitude of EJPs was examined. JSTX-3, which is effective only on postsynaptic receptors (Miwa *et al.*, 1990), also reduced the amplitude of EJPs almost irreversibly. This is further positive proof that L-glutamate is the neurotransmitter at the cricket NMJs.

An irreversible decrease in the amplitude of EJPs caused by JSTX-3 has also been observed in the crustacean glutamate receptor (Shudo *et al.*, 1987). In that case, the amplitude fell to almost 0 mV 7 min after the application of the toxin, despite a lower concentration (2×10^{-8} M) than that used in our experiment (3.5×10^{-6} M). The crustacean muscle and cricket muscle have postsynaptic L-glutamate receptors in common. However, the sensitivity to JSTX-3 of the receptors in each muscle is considered to be different. It is assumed that postsynaptic non-N-methyl-D-aspartate (NMDA) receptors, which are suppressed by JSTX-3, play an important role at the cricket NMJs. The still remaining junctional potential after the action of JSTX-3 is thought to be due to NMDA receptor activities (Sahara *et al.*, 1991).

Glutamate potentials by the methods of del Castillo and Katz (1955) and of Takeuchi and Takeuchi (1964) could not be obtained in muscle 202, although bath-applied L-glutamate produced a slow, transient depolarization (Fig. 2). There may be some possible reasons for this result, which reflects some morphological characteristics of NMJs in cricket abdominal muscles. Firstly, motor nerve twigs cannot be seen clearly under a light microscope without staining. Secondly, it is possible that the surface fibers are covered with connective tissues, which prevents L-glutamate from reaching the NMJs. However, the glutamate potential could not be recorded even from inner muscle fibers after removing the first and second layer fibers. Thirdly, it is probable that motor nerve terminals are located a bit distant from the surface of the muscle fiber, which may create some diffusion barrier between motor nerve terminals and the micropipette. Estimation of the depth, however, was not possible in preparations stained with nickel (Kawasaki and Kita, 1995). A similar problem has been reported in the dorsal longitudinal flight muscles of *Drosophila* (Koenig and Ikeda, 1983), in which the NMJs are located deep in the invaginations of the muscle fiber membrane. Fourthly, the possibility that a sufficient amount of L-glutamate was not ejected from the micropipette cannot be ruled out. This problem was checked chemically and it was found that a sufficient amount of L-glutamate (1.5×10^{-16} mole/pulse) was ejected iontophoretically from the electrode. At present, the third possibility is considered to be most likely.

In most examinations, the immunocytochemical staining was weak, although different fixatives and soaking times were examined. The soaking times in the primary and the secondary antibody, and in the PBS containing horseradish peroxidase coupled to biotin-avidin were sometimes 2 hr. However, the methods described in the Materials and Methods section produced the best results in the present study. The distribution of stained regions (Fig. 4), which were suggested to be NMJs, was less dense than that of NMJs stained with nickel (Kawasaki and Kita, 1995). The immunocytochemical and nickel staining methods differ in their purposes. The former is used to prove that the neurotransmitter at the cricket NMJs is

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L-glutamate and the latter to show the structure and distribution of NMJs. One reason for our present results is that the antibody diffused only from the surface of the muscle and reached nerve terminals, contrary to the case of nickel staining in which nickel chloride was given from the cut end of the axon and its terminals were all stained. In addition, NMJs may exist a little distant from the surface of the muscle fiber, as suggested in the glutamate potential experiment. To obtain better results, the preparation was treated for 30 min with collagenase (120 units/ml) following the method of Spörhase-Eichmann *et al.* (1992) to accelerate the diffusion of the antibody by making pores through the superficial connective tissue covering the muscle. Despite this modification, a great improvement could not be obtained.

The present electrophysiological and immunocytochemical studies certainly suggest that the neurotransmitter at NMJs in cricket abdominal muscles is L-glutamate.

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