The Effects of L-Glutamic Acid, Glutamate Agonists, Acetylcholine and Several Drugs on Excitatory Post-Synaptic Potentials at the Neuromuscular Junction of the Larval Mealworm, *Tenebrio molitor* LINNÉ (Coleoptera: Tenebrionidae)¹

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The effects of L-glutamic acid, quisqualic acid, L-aspartic acid, acetylcholine, curare, atropine, chlordimeform, nereistoxin, fenitrothion and fenitroxon on EPSP at the neuromuscular junction of the larval mealworm, *Tenebrio molitor*, were studied using a micro-electrode technique. Applications of L-glutamic acid, quisqualic acid and L-aspartic acid suppressed 50% of EPSP at concentrations of 4.2×10^{-5} M, 2.3×10^{-5} M and 1.2×10^{-3} M and evoked a depolarization of muscle membrane with this EPSP reduction. However, acetylcholine at 2×10^{-3} M did not suppress EPSP but evoked a depolarization of the muscle membrane. Atropine, curare, nereistoxin and chlordimeform at a concentration of about 2×10^{-3} M suppressed 50% of EPSP. From a pharmacological aspect these results suggest that L-glutamic acid is an excitatory transmitter at the insect neuromuscular junction, because L-glutamic acid and quisqualic acid, its agonist, revealed the effect at forty and eighty-fold lower concentrations than other drugs, respectively.

INTRODUCTION

L-Glutamic acid is considered a highly probable neurotransmitter candidate at the excitatory neuromuscular junction of insects such as locusts (BERÁNEK and MILLER, 1968; USHERWOOD and MACHILI, 1968; ANWYL, 1977; ANDERSON et al., 1978; PATLAK et al., 1979), dipteran insects (NEAL, 1975; JAN and JAN, 1976) and mealworms (YAMAMOTO and WASHIO, 1979, 1980). On the other hand, it has been reported that acetylcholine is the neurotransmitter in mealworms (FUTAMACHI, 1972; BORKOWSKA et al., 1978; JANIZEWSKI et al., 1978). However, there have been few reports on insect neurotransmitters studied pharmacologically. In the present study we examined from a pharmacological viewpoint the effects of L-glutamic acid, acetylcholine and drugs such as quisqualic acid, L-aspartic acid, atropine, curare, nereistoxin, chlordimeform, fenitrothion and fenitroxon on the excitatory post-synaptic potentials (EPSP) using neuromuscular junction preparations of larval mealworms. Quisqualic acid and L-aspartic acid are known as agonists of L-glutamic acid. Atropine and curare are blockers of musclic and nicotinic receptors, respectively, of the cholinergic systems in mammals. There are many insecticides acting on nervous systems. Cartap is a

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derivative of nereistoxin, which is a toxin of the sea annelid (*Lumbrineris*) and blocks synaptic transmission by binding antagonistically to the acetylcholine receptors of synaptic membrane. Chlordimeform blocks synaptic transmission at the neuromuscular junction of insects (YAMAMOTO and FUKAMI, 1976). Fenitrothion and fenitroxon like other organophosphorus insecticides accumulate excess acetylcholine by inhibiting cholinesterase, and this results in the uncontrolled increase of synaptic transmission. Recently, membrane depolarization in the stretcher muscle of a lobster walking leg by paraoxon was reported (ABE et al., 1983). Derivatives of L-glutamic acid would be candidates for selective insecticides because γ -methyl esters of the amino acid blocked the neuromuscular junction of insects (YAMAMOTO and WASHIO, 1979).

MATERIALS AND METHODS

Chemicals. L-Glutamic acid monosodium salt, L-aspartic acid, acetylcholine bromide and nereistoxin oxalate were purchased from Wako Pure Chemical Industries, Ltd. and atropine sulfate and curare (d-tubocurarine chloride) were obtained from E. Merck A. G. and Tokyo Chemical Industries, Ltd., respectively. Quisqualic acid, chlordimeform hydrochloride, fenitrothion and fenitroxon were supplied by Mitsubishi Kasei Institute of Life Sciences, Nihon Nohyaku Co. Ltd. and Sumitomo Chemical Co. Ltd., respectively. All other reagents were of the highest purity available commercially.

Insect. Mealworms (*Tenebrio molitor*) were reared on wheat bran at 26°C in the Insectron of our laboratory and the larvae were used throughout this study.

Measurement of EPSP. Preparation of the segmental muscle fibers of the mealworm larvae was made according to the method of YAMAMOTO and WASHIO (1979). The last instar larva was mounted dorsal side up on a sponge platform fixed by wax in the center of a perfusion chamber and split open along its dorsal midline with a perfusion of *Tenebrio*-saline containing 70 mM NaCl, 30 mM KCl, 0.7 mM CaCl₂, 14.3 mM MgCl₂, 445 mM glucose and 5 mM HEPES (modified from YAMAMOTO and WASHIO, 1979). The pH of the saline was adjusted to 7.2 with NaOH. The alimentary canal and associated fat bodies were removed to expose the central nervous system and the uppermost layer of ventral muscle fibers of the body wall. The exposed preparation was perfused by saline or a drug containing saline at a constant flow rate of 1 ml/min. Drugs were dissolved in the saline and the pH was adjusted to an initial value of 7.2 with NaOH. In the case of fenitrothion and fenitroxon ethanol was also added to the medium to give a final concentration of 0.5% because of the insolubility of these substances. In order to evoke EPSP, the peripheral motor nerve was hooked with a pair of Ag-AgCl electrodes and stimulated close to the segmental ganglion with supra-maximal square pulses of 70 μ sec duration and frequency of 0.3 or 0.5 Hz from an ME-6002 electronic stimulator (ME Commercial). Intracellular recordings were made through a glass microelectrode filled with 3M KCl impaled in the muscle fiber. The resistance of this electrode ranged from 5–10 M Ω . The recorded potentials were fed into a Model 8500 electrometer (Dagan), amplified with an AVM-10 amplifier (Nihon Kodan) and observed on a VC-9A oscilloscope (Nihon Kodan) and/or a RECTI-HORIZ-8K pen-oscillograph (San-ei Sokki). Those preparations showing resting potentials of -50—-60 mV and EPSP of 10–30 mV with the standard saline were employed for the experiments. All experiments were carried out at 25 $\pm$ 1°C.

Fig. 1. Pen-oscillographic recording of EPSP and depolarization of muscle membrane. A: L-glutamic acid (5×10^{-5} M), B: quisqualic acid (5×10^{-5} M), C: L-aspartic acid (2×10^{-3} M), D: acetylcholine (2×10^{-3} M), E: curare (3.6×10^{-3} M), F: atropine (10^{-3} M), G: chlordimeform (2×10^{-3} M), H: nereistoxin (2×10^{-3} M), I: fenitrothion (5×10^{-4} M), and J: fenitroxon (2×10^{-4} M). The drugs began to be perfused at the downward arrow and were washed at the upward arrows. Calibration, 10 mV (A-H, J), 20 mV (I); 1 min.

RESULTS

Typical changes of EPSP and membrane potentials at the mealworm neuromuscular junctions recorded on a pen-oscillograph after perfusion of L-glutamic acid, quisqualic acid, L-aspartic acid, acetylcholine, curare, atropine, chlordimeform, nereistoxin, fenitrothion and fenitroxon are shown in Fig. 1. Drug concentrations

Fig. 2. Dose-response curves of the suppression of EPSP. A: quisqualic acid, B: L-glutamic acid, and C: L-aspartic acid.

producing clear patterns of changes of EPSP and muscle membrane potentials were used in the above experiments. The amplitude of EPSP decreased rapidly with marked depolarization of muscle membrane by applications of 5×10^{-5} M L-glutamic acid, 5×10^{-5} M quisqualic acid and 2×10^{-3} M L-aspartic acid. These reduced EPSP and muscle membrane potentials were restored almost completely after washing with the drug-free standard saline, so these reactions seemed to be reversible (Fig. 1 A, B, C). In the case of quisqualic acid, increased amplitudes of EPSP were observed during the restoration period, and the same phenomena were observed at higher concentrations of L-glutamic acid and L-aspartic acid. In some preparations less depolarization by L-glutamic and L-aspartic acids were also observed, suggesting a different sensitivity among the muscle fibers. Quisqualic acid did not produce this type of less-depolarization. Acetylcholine did not suppress the amplitude of EPSP but evoked depolarization of muscle membrane at a concentration of 2×10^{-3} M. For further examination of these phenomena, atropine and curare which block acetylcholine receptors were used. It is considered that curare and atropine are blockers of nicotinic and muscarinic receptors, respectively. Curare did not evoke the depolarization of muscle membrane but greatly suppressed EPSP at a concentration of 3.6×10^{-3} M, and the potentials were not restored completely after washing with the drug-free standard saline (Fig. 1E), showing a different pattern from those obtained by L-glutamic acid and its agonists (Fig. 1A, B, C). Atropine evoked the depolarization of muscle membrane and the suppression of EPSP at 10^{-3} M. In this case atropine acting as an agonist though atropine is usually considered as the antagonist (Fig. 1F). When higher concentration of atropine was used, this compound showed an agonistic reaction at first and then an antagonistic reaction as a curare-type with the passage of time.

The effects of some pesticides such as chlordimeform, nereistoxin, fenitrothion and fenitroxon on the nerve-muscle system were also examined. Chlordimeform evoked both a slight depolarization of the muscle membrane and suppression of EPSP at 2×10^{-3} M (Fig. 1G), and clear depolarization at 10^{-2} M. Nereistoxin suppressed EPSP without depolarization of the muscle membrane at a concentration of 2×10^{-3} M (Fig. 1H). Fenitrothion and fenitroxon (5×10^{-4} M and 2×10^{-4} M, respectively, higher concentrations were not available because of their insolubility) showed little increase in the amplitude of EPSP (Fig. 1I, J).

Fig. 3. Dose-response curves of the suppression of EPSP. A: nereistoxin, B: chlordimeform and C: atropine.

Fig. 4. Pen-oscillographic recording of depolarization of muscle membrane for acetylcholine. A: 2×10^{-5} M, B: 2×10^{-3} M, C: 8×10^{-3} M, D: 10^{-2} M. Calibration, 10 mV (A-C). 20 mV (D); 1 min.

Fig. 5. Dose-response curve of depolarization of muscle membrane for acetylcholine.

Table 1. The concentration of ten drugs necessary for 50% suppression of EPSP amplitude (IC_{50}) and their effects on the depolarization of muscle membrane

	IC_{50} (M)	Depolarization of muscle membrane
Quisqualic acid	2.3×10^{-5}	+
L-Glutamic acid	4.2×10^{-5}	+
L-Aspartic acid	1.2×10^{-3}	+
Acetylcholine	—	+
Atropine	1.8×10^{-3}	+
Curare	1.7×10^{-3}	—
Nereistoxin	1.6×10^{-3}	—
Chlordimeform	1.6×10^{-3}	+
Fenitrothion	—	—
Fenitroxon	—	—

Dose-response experiments of EPSP were conducted with the 6 drugs used above (Figs. 2, 3). Values are means of 3–6 determinations. Dose-response curves for L-glutamic acid, quisqualic acid and L-aspartic acid are shown in Fig. 2. The concentrations producing EPSP suppression were in the order of quisqualic acid < L-glutamic acid < L-aspartic acid. The similarly shaped dose-response curves for nereistoxin, chlordimeform and atropine are shown in Fig. 3. The slopes of the curves for these drugs were rather gentle as compared with those of L-glutamic acid and its agonists. Pen-oscillographic patterns of the change of EPSP obtained by application of acetylcholine are shown in Fig. 4, and the dose-response curve of depolarization potentials is indicated in Fig. 5. The depolarization rates were expressed relative to the amplitude of EPSP with the drug-free saline as 100. Depolarization began to be evoked gradually at 2×10^{-4} M and increased drastically at concentrations higher than 2×10^{-3} M. Data on the 50% suppression concentration (IC_{50}) of the test compounds to EPSP are summarized in Table 1. The IC_{50} values of quisqualic acid and L-glutamic acid were 2.3×10^{-5} M and 4.2×10^{-5} M, respectively, and these were about 80 and 40 times lower than those of other drugs tested.

DISCUSSION

L-Glutamic acid, quisqualic acid and L-aspartic acid evoked the depolarization of muscle membrane at the neuromuscular junction of larval mealworm in the same manner, indicating that they acted as agonists. On the other hand, acetylcholine did not suppress EPSP but did evoke depolarization, suggesting that it is an agonist-like compound. As atropine, curare and nereistoxin which are blockers of acetylcholine suppressed EPSP, cholinergic synapses also seem to be present in neuromuscular junctions of the mealworm. However, this assumption is extremely unlikely because the concentration of acetylcholine which evoked depolarization was very high (2×10^{-3} M). If acetylcholine is a transmitter, the excess amount of it which resulted from the inhibition of acetylcholine esterase by fenitroxon and fenitrothion would greatly increase EPSP. The IC_{50} values of quisqualic acid and L-glutamic acid to EPSP were 2.3×10^{-5} M and 4.2×10^{-5} M, respectively, about 80 and 40 times lower than the values obtained

for other drugs tested. This result suggests that L-glutamic acid is the most probable candidate for the excitatory transmitter at the neuromuscular junction of the mealworm.

USHERWOOD and MACHILI (1968) reported that L-glutamic acid acted on the contraction of locust femur muscle at the lowest concentration among various amino acids and their related compounds. The IC_{50} values of L-glutamic acid and L-aspartic acid to neurally evoked contraction of locust femur muscle were 6.4×10^{-5} M and 1.5×10^{-5} M, respectively (CLEMENT and MAY, 1974). These concentrations were similar to those obtained in this experiment, 4.2×10^{-5} M for L-glutamic acid and 1.2×10^{-3} M for L-aspartic acid. Similarly, quisqualic acid was compared with L-glutamic acid and L-aspartic acid for its effect on the neuromuscular transmission in insects and crustaceae. The relative potencies of these agonists were in the order of quisqualic acid > L-glutamic acid > L-aspartic acid (SHINOZAKI and SHIBUYA, 1974; CLARK et al., 1979; CLEMENT and MAY, 1974; GRATION et al., 1981). This finding agreed with that of our experiment. Measuring muscle contraction and EPSP results in observing synaptic transmission, which is the result of the integrated function of various membrane structures such as neuromembrane, presynaptic membrane, post-synaptic membrane and muscle membrane. Further, the synaptic transmission is related to and greatly affected by ionic concentration, especially that of Ca^{++} . In the future, we shall study the site of drug action, whether the neuronal side or muscle side, and investigate the relationship between drugs and their action on the neuromuscular junction by using a greater variety of drugs.

There are potent insecticides inhibiting neuromuscular transmission such as cartap, chlordimeform and organophosphorus esters, including those having amino acid groups in their molecules like isoxathion. Recently, esters of L-glutamic acid were found to have inhibitory activities on neuromuscular transmission in insects (YAMAMOTO and WASHIO, 1979). The method used in the present study is very simple and convenient. Thus, it might be very useful for screening new selective pesticides and neuro-active substances (MIYAMOTO et al., in preparation).

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