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D-Glutamic Acid–Induced Muscle Contraction in the Silkworm, *Bombyx mori*

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Agonists for muscle contraction in silkworms were screened by injecting test solutions into the hemolymph of decapitated silkworm larvae. Kainic acid, a glutamate receptor agonist, and D-glutamic acid induced muscle contractions, and D-aspartic acid was partially effective, whereas NMDA and AMPA, representative mammalian glutamate receptor agonists, did not induce contraction. L-Glutamic acid inhibited the kainic acid or D-glutamic acid–induced contraction. Amino acid analysis revealed that 3% of the total glutamic acid in the silkworm hemolymph is D-glutamic acid. These results suggest that D-glutamic acid acts physiologically as an agonist for muscle contraction in silkworms, and that L-glutamic acid functions as an inhibitor.

Key words: D-glutamic acid, glutamate receptor, kainic acid, muscle contraction, silkworm.

Glutamic acid functions as a neurotransmitter in the mammalian central nervous system (CNS) (*1–3*), where it activates both ionotropic and G-protein–coupled metabotropic receptors, and has important roles in development (*4*, *5*), vision (*6*), olfactory memory (*7*, *8*), and spatial memory and locomotor control (*9*, *10*). In invertebrates, glutamic acid also acts as a neurotransmitter in the CNS (*11*, *12*), where it has both excitatory and inhibitory actions (*13–16*). Glutamic acid is also a neurotransmitter at the insect neuromuscular junction (*17–19*).

Transmission of excitatory stimuli in the brains of vertebrates and in the neuromuscular system of arthropods is mediated by activation of postsynaptic ionotropic glutamate receptors, which are coupled with ion channels (*20*). Ionotropic glutamate receptors are classified into three types based on their predominant sensitivity to one of three glutamate agonists: NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate), or kainic acid (*21*).

Signal transmission at *Drosophila* neuromuscular junctions is primarily mediated by glutamatergic synapses localized on larval body wall muscles, which express at least two ionotropic glutamate receptor subunits, DGluR-IIA (*22*) and DGluR-IIB (*23*). These glutamate receptors have been cloned, and their ability to transfer information about glutamic acid binding and to form ionic channels has been demonstrated by electrophysiologic recordings of *Xenopus* oocytes expressing the cloned receptors. Previous studies with *Drosophila* larval neuromuscular junctions established that neuromuscular transmission is coupled with excitation-contraction (*17*). Virtually nothing, however, is known about neuromuscular transmission in silkworm muscles.

Recent studies revealed how organisms produce, metabolize, and utilize D-amino acids. In particular, free D-serine and D-aspartic acid have some physiologic roles in the mammalian neuronal and endocrine tissues, respectively. These D-amino acids are involved in a variety of biologic activities, and metabolic enzymes stereospecific to these D-isomers have been characterized in mammals (*24*, *25*). D-Glutamic acid is an indispensable component of peptidoglycans in bacterial cell walls (*26*) and of certain antibiotics produced by bacteria and fungi, such as bacitracin and mycobacillin (*27*). Free D-glutamic acid occurs in insect (may beetle) muscle (*28*), some invertebrates (*29*), lower vertebrates (*30*), and rat tissues (*31*), although in most cases the amounts detected are very low. Biologic activity of D-glutamic acid has not been reported so far in these organisms, and the physiologic role of this D-amino acid remains unknown.

In this study, we report the presence of D-glutamic acid in silkworm hemolymph, and demonstrate that kainic acid and D-glutamic acid induce muscle contraction in silkworms. We also demonstrate that L-glutamic acid does not induce contraction, but rather inhibits the kainic acid or D-glutamic acid–induced contraction.

MATERIAL AND METHODS

Animals—Silkworm (*Bombyx mori*, Hu·Yo × Tukuba·Ne) eggs were purchased from Ehime Sanshu (Ehime, Japan). Silkworms were fed with artificial food (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27°C.

Muscle Contraction Assay—Test compounds dissolved in saline were injected into the hemolymph of silkworms with a 1-ml syringe attached to a 27-gauge needle (Terumo, Tokyo, Japan) from the backside at room temperature. Kainic acid was purchased from Merck (Frankfurt, Germany), D-glutamic acid from Sigma-Aldrich (St. Louis, MO), L-glutamic acid was purchased from Nacalai Chemicals (Kyoto, Japan) and Sigma-Aldrich, NMDA from Kanto

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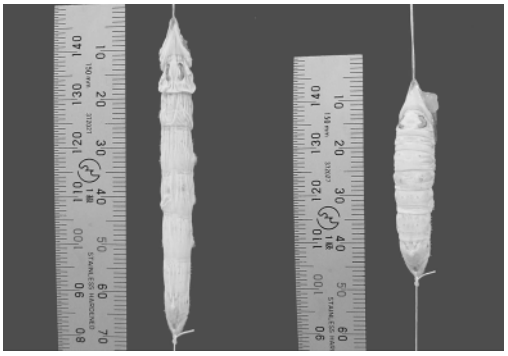

Fig. 1. **Muscle contraction experiment with decapitated silkworm.** The muscle specimen of decapitated silkworm was prepared as described under "MATERIAL AND METHODS." The specimen was extended with 28 g, and stabilized (left panel). Then, 0.05 ml of 2 mM kainic acid solution dissolved in a nutrient buffer was injected into the intra-body of the specimen to induce the contraction (right panel).

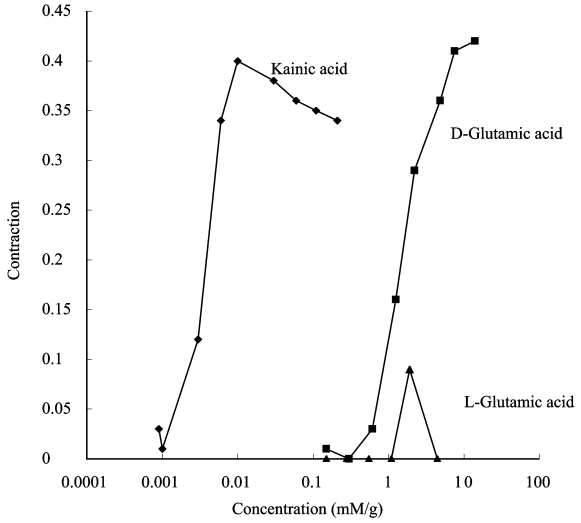

Fig. 2. **Contraction of silkworm muscle caused by kainic acid and D-glutamic acid.** Compounds dissolved in a nutrient buffer were injected into the intra-body of the specimen. Contraction values were calculated from the sizes of the specimen before and after the injection.

Chemicals (Tokyo, Japan), and AMPA from Sigma-Aldrich. 4-Fluoro-7-nitro-2,1,3-benzoxaddiazole (NBD-F) was purchased from Wako Pure Chemicals (Tokyo, Japan).

Muscle specimens of silkworms (5th instar larval stage, 4–6 g) were prepared as described below. The head of the silkworm was cut off with scissors, and peritrophic membranes containing digested food were removed, followed by removal of the silk glands. The specimen was tied with strings to a transducer to measure isotonic contraction with a load of 28 g, and stabilized until autonomous vibration terminated. In all cases, the sample was attached to the transducer within 1 min after decapitation. The time course of contraction was recorded on an analogue recorder (data not shown). Test samples were dissolved in nutrient saline (91 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 5.6 mM glucose) and injected into the body fluid of decapitated silkworms with a 1-ml syringe attached to a 27-gauge needle (Terumo). Concentration values of injected materials were standardized according to the weight of the specimen. The contraction process was very rapid; it reached maximum level within 1 s after injection and continued for more than 10 s thereafter. The contraction ratio was calculated and expressed by measuring maximum length of each individual specimen before (x cm) and after (y cm) the injection by the formula $(x - y)/x$. Representative data of three independent experiments are shown in Figs. 2 and 3.

Measurement of D-Glutamic Acid in Silkworm Hemolymph—The concentration of D-glutamic acid in silkworm hemolymph was determined by the following method. Trichloroacetic acid (final 5% [w/v]) was added to the hemolymph, and the soluble fractions (100 μl) were neutralized by the addition of 1 N NaOH (20 μl), then diluted with 200 mM sodium borate buffer (pH 9.5). The sample (20 μl) was mixed with 10 μl of 50mM NBD-F in dry acetonitrile and incubated at 60°C for 5 min. The reaction forming fluorescent derivatives was terminated by the addition of 470 μl of 1% trifluoroacetic acid. The sample was filtered through a membrane filter with a pore size of 0.45 μm, and a 10-μl aliquot was subjected to high pressure liquid chromatography (HPLC). The HPLC system for determination of D- and L-aspartic acid (*32*) was modified for determination of D- and L-glutamic acid. The system was composed of an octyl silica (C8) column and a chiral Pirkle-type column (OA-3100, Sumitomo Chemicals Center, Osaka, Japan) connected by an automated column switching apparatus. Measurements were repeated more than three times, and a representative result is shown in Fig. 4.

RESULT

Contraction of Silkworm Muscles by Injection of Kainic Acid into the Hemolymph of Living Larvae—We examined the pharmacologic effects of various compounds injected into the hemolymph of living silkworms (data not shown). Kainic acid, a well-known glutamate receptor agonist, induced contraction of the larval bodies. The concentration of kainic acid in hemolymph required to induce the contraction was approximately 10 μM. The silkworms died after the injection of kainic acid (data not shown).

We next examined which amino acids had an effect similar to kainic acid. None of the L-forms of the 20 amino acids found in proteins produced contractions (data not shown). D-glutamic acid induced contraction of larval bodies (data not shown). The concentration of D-glutamic acid necessary for contraction was approximately 1 mM in hemolymph, 100-fold higher than that of kainic acid. In contrast to kainic acid, the effect of D-glutamic acid was transient (data not shown), and silkworm larvae recovered from contraction within 10 min. A second injection of D-glutamic acid following recovery from the first injection produced contraction of the larval bodies equivalent to that produced by the first injection. Therefore, the recovery from the contraction was not due to a loss of sensitivity to D-glutamic acid.

When L-glutamic acid was injected into silkworm hemolymph, locomotor activity was inhibited, and the

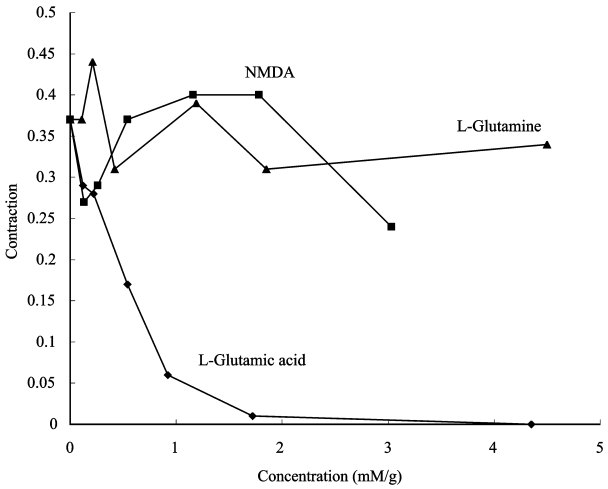

Fig. 3. **Inhibitory effect of L-glutamic acid on the kainic-acid-induced contraction of silkworm muscle.** Compounds dissolved in a nutrient buffer (0.05 ml) with various concentrations were injected into the silkworm specimen, followed by an injection of 0.1 mM kainic acid (0.05 ml). Contraction values were calculated as in Fig. 2.

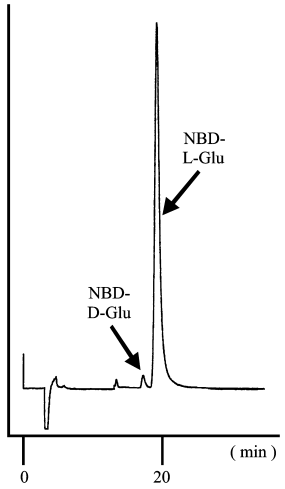

Fig. 4. **Determination of D-glutamic acid in the hemolymph of silkworm larva.** Amino acids in the acid-soluble fraction of hemolymph were reacted with NBD-F, and fluorescent derivatives were analyzed in an HPLC system in which an octyl silica column and a chiral column were linked by an automated column-switching device. An elution profile of NBD-D-glutamic acid and NBD-L-glutamic acid is shown.

larvae appeared to be in an anesthetized state (data not shown). The concentration of L-glutamic acid in hemolymph necessary for the inhibition of locomotor activity was approximately 1 mM. The anesthetic effect of L-glutamic acid lasted less than 10 min and was followed by normal movement of the larvae. Therefore, the relaxation induced by L-glutamic acid was transient, as in the case of contraction induced by D-glutamic acid. When the same amount of L-glutamic acid was again injected into the recovered larvae, it induced a similar reaction to the first injection. Thus, the recovery was not due to a loss of sensitivity to L-glutamic acid.

Muscle Contraction Experiments with Decapitated Silkworms—The *in vivo* injection results strongly suggested that kainic acid and D-glutamic acid induced muscle contractions in silkworms. To further understand this mechanism, we established a method for quantitative measurement of muscle contraction with decapitated silkworms. Kainic acid induced muscle contraction (Fig. 1). The optimum concentration of kainic acid induced a 40% contraction in this system (Fig. 2). Concentrations of kainic acid greater than 10 mM inhibited the contraction (data not shown). D-Glutamic acid also induced contraction, but at a dose more than 100-fold higher than in that kainic acid (Fig. 2). Further contraction was not induced by D-glutamate after the initial contraction caused by kainic acid, and *vice versa*. Concentrations of D-glutamic acid greater than 100 mM inhibited the contraction (data not shown). Because the contraction induced by kainic acid and D-glutamic acid was observed in decapitated silkworms, the reaction does not require an intact brain. The muscle contraction observed in decapitated silkworms was observed after the removal of subesophageal, thoracic, and abdominal ganglia along the body (data not shown), indicating that the target of kainic acid and D-glutamic acid is not in the CNS.

L-Glutamic acid did not induce muscle contraction in the system (Fig. 2); nor did NMDA or AMPA, which are well-known mammalian brain glutamate receptor agonists. GABA, an inhibitory neurotransmitter, was also ineffective (data not shown). We further examined the effect of L- and D-forms of 19 other amino acids, and the results demonstrated that D-aspartic acid was partially effective. None of the other amino acids tested induced muscle contraction (data not shown).

We next examined the action of L-glutamic acid, which was previously found to induce relaxation in living larvae, on muscle contraction in decapitated silkworms. Pretreatment of the muscle specimen with L-glutamic acid inhibited the induction of contraction by kainic acid (Fig. 3) and D-glutamic acid (data not shown). Neither NMDA nor AMPA had an inhibitory effect. Pretreatment of the specimen with L- and D-forms of other amino acids had no effect on the contraction (data not shown). Thus, the inhibitory effect was specific to L-glutamic acid.

Presence of D-Glutamic Acid in the Silkworm Hemolymph—Previous studies of glutamic acid receptors in vertebrates have not indicated a physiologic role of D-glutamic acid as a neurotransmitter. To understand the physiologic role of D-glutamic acid in the induction of muscle contraction, we analyzed the presence of D-glutamic acid in silkworm hemolymph. Amino acids in the acid-soluble fraction of hemolymph were derivatized with NBD-F. The fluorescent derivatives of L- and D-glutamic acid were enantioseparated using a column-switching HPLC system. An elution profile of the fluorescent derivatives of L- and D-glutamic acid in silkworm hemolymph is shown in Fig. 4. The results demonstrated that 3% of the total glutamic acid in the sample was D-glutamic acid. Thus, the concentrations of L- and D-glutamic acid in the hemolymph were 0.63 and 0.019 mM, respectively.

DISCUSSION

This study has demonstrated that kainic acid and D-glutamic acid induce contraction of muscles in silkworm larvae, and that L-glutamic acid inhibits the contraction induced by these agonists. We further demonstrated that a certain concentration of D-glutamic acid is present in the hemolymph of silkworms. The presence of glutamate receptors in fruit fly muscle tissues has been reported, as well as the neuromuscular transmission coupled with excitation (*17*). Our results strongly suggest that kainate-type receptors are responsible for the transfer of signals at the neuromuscular junctions of silkworm muscles, and that D-glutamic acid acts as a physiologic agonist that induces contraction. L-Glutamic acid is suggested to negatively regulate this process. Injection of D-glutamic acid into the body fluid of slugs also induced shrinking of the animal bodies (unpublished results). Therefore, D-glutamic acid might act as an agonist for muscle contraction in other invertebrates.

L-Glutamic acid is the main agonist causing muscle contraction in locust (*33*). At the glutamate receptors of *Bombyx mori*, glutamate agonists (kainic acid and D-glutamic acid) induced muscle contraction, whereas L-glutamic acid did not. It is possible that L-glutamic acid is a very low level agonist for glutamate receptors, in contradiction to previous studies (*20*). It is more likely, however, that D-glutamic acid and L-glutamic acid have opposing actions in the regulation of contraction in silkworms: D-glutamic acid induces contraction and L-glutamate inhibits contraction. Glutamate receptors regulated by D-glutamic acid and inhibitory glutamate receptors (*34*) regulated by L-glutamic acid might work together to regulate silkworm muscle contraction. Another possible model for regulation of the contraction is that a single glutamate receptor molecule has affinity for both D-glutamic acid and L-glutamic acid, resulting in induction and inhibition of muscle contraction in silkworms. In the latter case, our experiments suggest that a kainate-type glutamate receptor has such a capacity in the muscle contraction of silkworms.

At present, it is not known how D-glutamic acid accumulates in the silkworm hemolymph, although other studies report the presence of D-amino acids in insects (*35*) and D-serine in *Bombyx mori* hemolymph (*36*). The content of D-glutamic acid in the diet we fed the silkworms was determined to be 0.6% of the total glutamic acid (data not shown). Therefore, D-glutamic acid might be provided by food that contains mulberry leaf powder. Although plants appear to be able to synthesize D-amino acid derivatives (*37*–*39*), further investigation is required to verify the presence of D-glutamic acid in mulberry leaves as the source from which silkworms incorporate this D-amino acid.

D-Amino acids are synthesized by microorganisms and other living organisms by transformation of L-isomers (*40*), catalyzed mainly by racemases. These D-amino acids are essential for construction of bacterial cell walls (*26*, *41*). Therefore, it is possible that D-glutamic acid is provided by bacteria in the silkworm midgut. Determination of the source of D-glutamic acid, and examination of the effect of elimination of the route, will clarify the importance of D-glutamic acid in the regulatory mechanism of muscle contraction in silkworms.

In conclusion, this is the first paper to suggest a biologic action of D-glutamic acid in silkworms. The work will provide clues to understanding the role of amino acid stereoisomers.

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