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0014-4754/84/030250-03$1.50 + 0.20/0

The effect of glutamate, cysteate and related amino acids on insect muscle

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Summary. Application of Glu, $CySO_2$ or $CySO_3$ to blowfly larvae caused paralysis and increased the membrane potential of larval muscle. The contents of Glu and $CySO_3$ in larval muscle containing motor nerve terminals were markedly decreased after the perfusion of high K^+ saline solution.

It is considered that glutamate (Glu) and aspartate (Asp) function as transmitters in the insect neuromuscular junction and the mammalian brain[1,2]. Among the amino acids, Glu is analogous to Asp and $CySO_3$ (cysteate) in chemical structure and molecular size. Crawford and McBurney[3] analyzed the power spectrum of postsynaptic noise caused by the application of Glu, Asp and $CySO_3$ at the insect neuromuscular junction. It is interesting to note that Tau (taurine), a metabolite of $CySO_3$, has recently been found to be localized mainly in the mammalian liver and brain, and to have a physiological function in the central nervous system[4]. In the insect, it has been shown that a high concentration of Tau is present in insect flight muscle[5]. However, it is not clear whether Tau, Glu and $CySO_3$ function as neurotransmitters in the insect neuromuscular system or not.

In this study, we investigated how Tau, Glu, and $CySO_3$ affect the neuromuscular system of larvae of the blowfly, *Aldrichina grahami*.

Materials and methods. Blowflies, *Aldrichina grahami*, were reared aseptically on semi-synthetic diets at 25 °C as described previously[6]. The amino acids were injected through the spiracular sclerite of the anal side of 4- or 6-day-old larvae. The larval resting time after the injection was measured at 25 °C by phototaxis.

Isolated hemolymph and muscle containing nerve terminals were homogenized in 10 vols of 75% ethanol with a glass-glass homogenizer, and centrifuged at 10,000 g for 10 min. The supernatant was evaporated and delipidized with chloroform/H_2O (2:1). The water layer was adjusted to a pH of 2.0 with HCl. This acidic solution was chromatographed on Dowex 50W-X8 (100–200 mesh, Cl^--form, 1.0 × 5.0 cm). The elution was performed with 3 vols of 0.01 N HCl and 1 vol of H_2O, continuously. These eluates were referred to as the washing fraction. Finally, the column was eluted with 3 vols of 3 M NH_4OH. The ammonium eluate was referred to as the adsorbed fraction. Both the washing and adsorbed fractions were dried with an evaporator and analyzed with a high-performance amino acid analyzer which is equipped with an IEX 215SC column (Toyosoda Co. Ltd., Tokyo, Japan), a visual detector (570 nm) and an integrator (Waters Associates, model 730). The analyzing buffer used was 0.2 M citrate buffer (pH 2.2). Details of the analytical techniques will be published elswhere.

The 4- or 6-day-old larvae were dissected, the viscera and central nervous system removed, and the muscle containing motor nerve terminals and cuticle was perfused with saline solution (172 mM NaCl, 3.3 mM KCl, 1.0 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 20 mM glucose and 10 mM HEPES, pH 7.2) for 30 min before any recordings were made. Intracellular recordings from muscle were made using 3M KCl-filled glass microelectrodes with a resistance of 5–15 mohm. The recordings were monitored using standard procedure.

The isolated muscle preparations were perfused with the normal saline solution mentioned above 30 min before the perfusion of high K^+ saline solution (172 mM KCl, 3.3 mM NaCl, with the other constituents the same as normal saline solution). The perfused muscle preparations were treated by the above-mentioned method.

Results and discussion. The application of Glu, $CySO_2$ (cysteine sulfinate) or $CySO_3$ to the larval body cavity caused marked paralysis, as shown in the figure. When Glu

Table 1. Amino acid contents of larval muscle after high K^+ stimulation and hemolymph

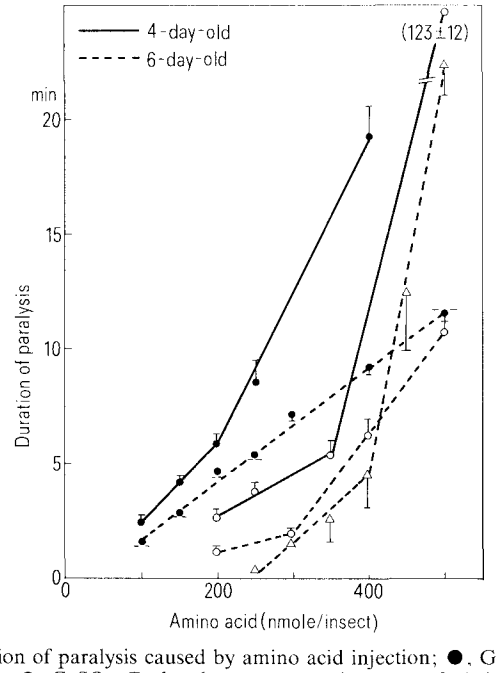
	Muscle (nmole/mg wet weight)				Hemolymph (nmole/insect)	
	4-day-old		6-day-old			
	Control	High-K^+	Control	High-K^+	4-day-old	6-day-old
Taurine	5.87 ± 0.94	6.67 ± 0.94	5.84 ± 0.38	6.51 ± 0.65	25.1 ± 2.4	67.7 ± 2.6
Aspartate	0.21 ± 0.08	0.16 ± 0.06	0.25 ± 0.07	0.16 ± 0.02	22.0 ± 1.1	3.9
Glutamate	0.50 ± 0.03	0.38 ± 0.05	1.58 ± 0.64	0.98 ± 0.27	170.0 ± 9.1	41.6 ± 6.3
Glycine	0.83 ± 0.06	1.24 ± 0.16	0.61 ± 0.05	0.93 ± 0.05	207.5 ± 23.6	49.2 ± 3.6
Alanine	5.58 ± 0.83	8.29 ± 2.08	8.20 ± 1.68	11.59 ± 2.19	307.0 ± 40.3	232.2 ± 12.0
Cysteate	–	–	0.02	–	0	0

Each value represents the mean of 3 determinations ± SD.

was injected into 6-day-old larvae and $CySO_2$ or $CySO_3$ into 4- and 6-day-old larvae, the dose response curves consist of 2 linear components with a depressing effect on larval motor activity. The mammalian brain and the crab peripheral nerve have 2 uptake and 2 binding systems for Glu, $CySO_2$, or $CySO_3$[7,8]. The dose response curves in the figure may reflect similar uptake and binding systems. As the dose response curve was linear in the case of Glu given to 6-day-old larvae, we used its parameter values to calculate the minimum concentration of Glu which could cause paralysis. Hemolymph volume was determined using a micro-suction pump, and the mean volume for a 6-day-old larva was 17 ± 1.2 µl (5 trials ± SD). The minimum of Glu required to cause paralysis of 6-day-old larvae was calculated to be 28 nmoles by the method of least squares. Using these values, we estimated that 1.6 mM of Glu in the hemolymph of a 6-day-old would cause a depression in larval motor activity. The mean concentration of Glu which would cause a neuromuscular block is 1.8 mM in *Luccilia* and 2.7 mM in *Locusta*[9]. It is thus possible that concentrations of Glu in the hemolymph between 1 and 3 mM depress insect motor activity. Paralysis caused by Glu took 20 sec to appear after injection, but paralysis caused by $CySO_2$ or $CySO_3$ appeared as soon as they were injected into the larval body cavity. Irving and Miller[2] reported that the muscle of *Musca domestica* larvae is innervated by both fast and slow excitatory axons. Accordingly, the difference between Glu and $CySO_2$ or $CySO_3$ in the appearance time of paralysis after the injection may be due to a difference of affinity for fast or slow excitatory axons, respectively. No changes in larval behavior were found on injection of 500 nmole of Asp, γ-aminobutyric acid, α-ketoglutarate, Tau, or hypotaurine. When Asp was injected along with Glu, $CySO_2$, or $CySO_3$, it was found to have a synergistic effect on the duration of paralysis; this result agrees with that of Irving et al.[9].

Table 1 shows the amino acid content of the hemolymph, and the changes in muscle containing nerve terminals after the perfusion of high K^+ saline solution. Large amounts of Glu, Gly (glycine), and Ala (alanine) are present in the hemolymph, with smaller amounts in that of 6-day-old larvae. In addition, the amount of Tau in the hemolymph of 6-day-old larvae is higher than in that of 4-day-old larvae. The blowfly, *Aldrichina grahami* stops eating 5 days after hatching and pupation occurs 7 days after hatching. It seems possible that the changes in hemolymph Glu, Gly, and Ala depend upon the insect's eating, and the increase in Tau upon the excretion of sulphur from cysteine. Large amounts of Tau and Ala, which increased after the perfusion of high K^+ saline solution, were present in the muscle preparations. Glu and Asp content in the muscle decreased after this perfusion. Since Glu causes paralysis and Asp has a synergistic effect on paralysis caused by injection of Glu, $CySO_2$, or $CySO_3$, it may be that the decrease of Glu and Asp in muscle preparations results from a release of Glu and Asp from the muscle containing nerve terminals by the stimulation of high K^+. Glutamate-pyruvate transaminase is present in insect muscle, and arginine phosphate, which, linked with Gly, is an energy-source in insects[10,11]. Accordingly, the increase of Gly and Ala after the perfusion of high K^+ saline solution could well be due to the results of energy metabolism during muscle contraction. In addition, a small amount of $CySO_3$ is found in the muscle of 6-day-old larvae which disappears after the perfusion of high K^+ saline solution, and the application of $CySO_3$ to the larval body cavity caused a strong paralysis. $CySO_3$ may have a transmission role in the insect neuromuscular system. It is well known that cysteine is metabolized to Tau via $CySO_3$ and is required in the diet of all insects[12]. These data suggest that some of the Tau present in the larval muscle of the blowfly, *Aldrichina grahami*, is from cysteine.

Further, 0.1 mM concentrations of Glu, Asp, $CySO_2$ or $CySO_3$ increased the membrane potential of the muscle. The application of 1 mM Glu, Asp, $CySO_2$ or $CySO_3$ caused the disruption of the glass electrodes by a powerful muscle contraction, making measurement of the correct membrane potential impossible; 10 mM of Gly, Tau, γ-aminobutyric acid, or acetylcholine did not cause any changes of membrane potential, as shown in table 2. Since we have not found any inhibitor of muscle contraction in the larva, like d-tubocurarine in electrophysiological experiments with frog and mammalian muscle, the present results provide circumstantial evidence that an increase in the muscle potential level interferes with the functioning of the neuromuscular system following application of Glu,



Duration of paralysis caused by amino acid injection; ●, Glu; △, $CySO_2$; ○, $CySO_3$; Each value represents the mean of 10 determinations, and the vertical bar represents the SD.

Table 2. Effect of amino acids on membrane potential of larval muscle

Amino acid		Before	After	Δ Potential (mV)
Glutamate	(0.1 mM)	− 60 ± 1.2	− 38 ± 5.4	+ 22
	(1 mM)	− 60 ± 1.7	broken	–
Aspartate	(0.1 mM)	− 60 ± 1.5	− 55 ± 2.6	+ 5
	(1 mM)	− 58 ± 2.0	broken	–
Cysteate	(0.1 mM)	− 61 ± 1.9	− 55 ± 2.1	+ 6
	(1 mM)	− 60 ± 1.3	broken	–
Cysteine sulfinate	(0.1 mM)	− 60 ± 1.2	− 55 ± 2.3	+ 5
	(1 mM)	− 59 ± 1.0	broken	–
Glycine	(1 mM)	− 57 ± 2.5	− 57 ± 1.6	0
	(10 mM)	− 60 ± 2.0	− 60 ± 2.0	0
γ-Aminobutyric acid	(1 mM)	− 60 ± 1.4	− 60 ± 1.5	0
	(10 mM)	− 59 ± 1.2	− 59 ± 1.6	0
Taurine	(1 mM)	− 61 ± 1.1	− 61 ± 0.6	0
	(10 mM)	− 60 ± 0.7	− 60 ± 0.7	0
Hypotaurine	(1 mM)	− 61 ± 0.7	− 61 ± 0.3	0
	(10 mM)	− 59 ± 1.8	− 59 ± 1.1	0
Acetylcholine	(1 mM)	− 60 ± 1.3	− 60 ± 1.4	0
	(10 mM)	− 60 ± 0.3	− 60 ± 0.3	0

The data represents the mean of 7 determinations ± SD.

$CySO_2$ or $CySO_3$. It has been reported that Asp is a possible transmitter in the mammalian visual cortex[4] and in the muscle of *Musca domestica*[2]. Unfortunately, we could not provide further evidence that Asp plays a role as a transmitter in the larval neuromuscular system.

Consequently, we suggest that Glu, $CySO_2$ and $CySO_3$ may be transmitters in the neuromuscular system of the larva of the blowfly, *Aldrichina grahami*. Further investigation is needed using a specific inhibitor to determine the action of these amino acids on the neuromuscular system.

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0014-4754/84/030252-03$1.50 + 0.20/0

Effect of fighting on the hemogram in an insect *Schizodactylus monstrosus* Drury (Orthoptera, Schizodactylidae)

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Summary. Changes were seen in total hemocyte count/mm^3 blood, in different categories of hemocytes and in blood volume during inter-male fighting in *Schizodactylus monstrosus*. Total hemocyte count showed a steady increase up to the end of fighting though the trend was evident 10 min after the beginning of the fighting. The number of granular hemocyte and spherule cells increased during the first 10 min of fighting, whereas the number of adipohemocytes began to increase after 10 min. A slight increase in blood volume was noted during the early periods of fighting.

In insects, the regulation of metabolic processes during energy stresses has been reviewed by Bailey[2]. Attention has mostly been paid to the variation of sugar, lipid and free amino acid levels in the hemolymph, fat body and muscles in relation to different energy stress situations[3-5]. Alterations in the hemogram as the index of various physiological conditions have also been studied[6-9]. Storage of different nutrients like carbohydrate, lipid and amino acids by the hemocytes and their role in maintaining the normal nutrient balance during different stress conditions are well documented[10-12].

Schizodactylus monstrosus Drury (Orthoptera, Schizodactylidae), a nocturnal carnivorous insect, shows intraspecific aggressiveness. In spite of extensive studies of different aspects of insect hemocytes, no attempt has yet been taken to report any variation in the hemogram during intraspecific fighting. The present investigation attempts to report the variation in total number of circulating hemocytes/mm^3 blood, the number of different circulating cell types and blood volume during different time intervals of fighting. The existence of sessile hemocytes in this insect has already been reported[13]. The present communication also deals with the role of sessile hemocytes during fighting stress.

After collection, insects were kept separately in moist sand jars (90% relative humidity). Cockroach nymphs were supplied as food on alternate days. 7-day-old adult males (obtained by rearing the last instar nymphs in the laboratory) were used in this experiment.

Fighting was initiated in the laboratory between 2 adults fed under similar conditions (8 h after feeding). After initiation it continued actively up to 20–23 min, after which the aggressiveness gradually ceased. Thus, to show the sequence of changes in the hemogram, all estimations were made every 5 min up to 20 min from the commence of fight.

Hemolymph samples were collected by amputating one of

Table 1. Total hemocyte count (cells/mm^3 blood) in control and heat-treated *S. monstrosus* in relation to different time intervals of fighting. Values are mean ± SE of 7 replications

	Time intervals of fighting				
	0 min	5 min	10 min	15 min	20 min
Control	16,400 ± 320	17,000 ± 280	20,200 ± 289	28,050 ± 312	30,975 ± 276
Heat-treated	22,800 ± 316*	23,900 ± 281*	23,100 ± 311	29,080 ± 328	31,650 ± 319
% differences	39.02	40.58	14.35	3.67	2.17

* $p < 0.1$ in comparison to control.