Glutamate is a Fast Excitatory Transmitter at Some Buccal Neuromuscular Synapses in Aplysia

LYLE E. FOX AND PHILIP E. LLOYD
Committee on Neurobiology and Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, Illinois 60637

Fox, Lyle E. and Philip E. Lloyd. Glutamate is a fast excitatory transmitter at some buccal neuromuscular synapses in Aplysia. J. Neurophysiol. 82: 1477–1488, 1999. Studies of the modulation of synaptic transmission in buccal muscle of Aplysia were limited be-cause the conventional fast transmitter used by a number of large buccal motor neurons was unknown. Most of the identified buccal motor neurons are cholinergic because they synthesize acetylcholine (ACh) and their excitatory junction potentials (EJPs) are blocked by the cholinergic antagonist hexamethonium. However, three large identified motor neurons (B3, B6, and B38) do not synthesize ACh and their EJPs are not inhibited by hexamethonium. To identify the fast excitatory transmitter used by these noncholinergic motor neurons, we surveyed putative transmitters for their ability to evoke contractions. Of the noncholinergic transmitters tested, glutamate was the most effective at evoking contractions. The pharmacology of the putative glutamate receptor is different from previously characterized glutamate receptors in that glutamate agonists and antagonists previously used to classify glutamate receptors had little effect in this system. In addition, glutamate itself was the most effective agent tested at reducing EJPs evoked by the noncholinergic motor neurons presumably by desensitizing glutamate receptors. Finally, immunocytoology using an antiserum raised to conjugated glutamate in parallel with intracellular fills indicated that the varicose axons of these motor neurons were glutamate-immunoreactive. Taken together, these results indicate that the fast transmitter used by the noncholinergic neurons is almost certainly glutamate itself. This information should help us understand the role of transmitters and cotransmitters in the generation of feeding behaviors in Aplysia.

INTRODUCTION

Neuronal mechanisms that generate feeding behaviors have been heavily studied in Aplysia. In particular, peripheral modulation at neuromuscular synapses in buccal muscles has been examined extensively (Fox and Lloyd 1998; Weiss et al. 1992, 1993; Whim et al. 1993). These studies have been limited because the identity of the conventional transmitter used by a group of buccal motor neurons was unknown. Here we attempt to identify the conventional fast excitatory transmitter used by these motor neurons.

Many buccal motor neurons have been identified based on a combination of criteria including their size, location, the muscles they innervate, and expression of modulatory peptide cotransmitters (Church and Lloyd 1991, 1994; Cohen et al. 1978). The fast excitatory transmitters used by many of these neurons is acetylcholine (ACh). These neurons contain acetylcholinesterase activity, as determined by the synthesis of ACh from intracellularly injected labeled choline, and their excitatory junction potentials (EJPs) are blocked by the cholinergic antagonist hexamethonium (Church and Lloyd 1994; Cohen et al. 1978). However, three large identified motor neurons (termed B3, B6, and B38) are not cholinergic by these criteria. They do not synthesize ACh from injected choline, and their EJPs are not blocked by hexamethonium (Church et al. 1993; Lloyd and Church 1994; Lotshaw and Lloyd 1990). All three of these neurons express modulatory peptide cotransmitters and innervate different but overlapping regions of intrinsic buccal muscle 3 (I3). B38 expresses the small cardioactive peptides (SCPs) and innervates the anterior region of the I3 muscle (I3a), B3 expresses FMRFamide and innervates the anterior and medial regions of the muscle, and B6 expresses the SCPs and innervates the medial and posterior regions (Church and Lloyd 1991). This muscle also is innervated by two identified excitatory cholinergic motor neurons (termed B9 and B10) that innervate medial and posterior regions. I3 is a nonspiking muscle, and both the cholinergic and noncholinergic neurons evoke graded contractions that result from the summation of EJPs.

In the present paper, we present evidence implicating glutamate as the fast excitatory transmitter used by the identified noncholinergic motor neurons. Three lines of evidence support this conclusion. A survey of transmitters, transmitter agonists, and antagonists indicated that glutamate was one of only a few substances that evoked contractions of isolated I3a muscles. In addition, of the many substances tested, glutamate itself was most effective at reducing the EJPs evoked by the noncholinergic motor neurons presumably by desensitizing glutamate receptors. Finally, immunocytoology using an antiserum raised to conjugated glutamate in parallel with intracellular fills indicated that the varicose axons of these motor neurons were glutamate-immunoreactive.

METHODS

Animals

Aplysia californica (50–150 g) were obtained from Marinus (Long Beach, CA), maintained in circulating artificial sea water (ASW) at 16°C, and fed dried seaweed every 3 days.

Measurement of I3a muscle contractions

AGONIST TEST PROTOCOL. A few bundles of the I3a muscle were isolated in these experiments (to enhance penetration of tested substances), mounted in a small chamber (total volume 300 μl), and
superfused with ASW at a flow rate of 1.5 ml/min. Test substances in ASW (5–10 μl) were injected into the superfusion, just before it entered the chamber, to determine if they evoked contractions. Because the superfusion was aimed at the muscle fibers, the initial concentration of the test substance applied to the muscle was similar to the concentration in the bolus; however, it was immediately diluted. Contractions were measured with an isotonic transducer.

**ANTAGONIST TEST PROTOCOL.** Reproducible submaximal contractions were evoked by periodic bolus injections of ACh or glutamate. Test substances were applied via the superfusion to determine their effects on these contractions. Because the amplitude of the evoked contractions sometimes changed during the experiments, results were quantified by comparing the amplitude of the contractions evoked during treatment with the test substance to the average of the contractions evoked before treatment and after washout. Boluses for both experiments were applied at 5- to 7-min intervals. Most substances tested (Table 1) were obtained from Sigma or RBI.

**Motor neuron stimulation experiments**

Animals were immobilized with an injection of isotonic MgCl₂ and the dissection carried out in either low Ca²⁺ (0.5 mM; 0.05× normal), high Mg²⁺ (110 mM; 2× normal) ASW (termed low Ca ASW) or high Ca²⁺ (33 mM; 3× normal), high Mg²⁺ (165 mM; 3× normal) ASW (termed high Ca, Mg ASW). The buccal mass/buccal ganglia complex was removed, bisected along the midline, all nerves severed except ipsilateral buccal nerve 2 (nerve designations from Gardner 1971; muscle nomenclature from Howells 1942; also see Lloyd 1988), except ipsilateral buccal nerve 2 (nerve designations from Gardner 1971; muscle nomenclature from Howells 1942; also see Lloyd 1988), and pinned in a dish with a silicone elastomer (Sylgard, Dow Corning) base. The hemiganglion was desheathed and selectively superfused with either low Ca ASW to suppress synaptic transmission in the ganglion or high Ca, Mg ASW to raise the firing thresholds of neurons in the ganglion. The remainder of the bath containing the I₃ muscle was separated by a barrier (except when a perfusion electrode was used, see following text) through which the intact nerve 2 ran and was superfused with ASW. The test substances were applied in ASW used, see following text) through which the intact nerve 2 ran and was superfused with ASW. The test substances were applied in ASW

**Measurement of I₃ EJPs**

Individual spikes in motor neurons were driven by brief (10–20 ms) depolarizing current pulses. EJPs were recorded with an intracellular
Intracellular electrophysiology recordings, short bursts of two to eight action potentials at 10–12.5 Hz were used to ensure that a burst did not evoke contractions. Intracellular EJPs were quantified by comparing the EJP amplitude during application of the test substance to the average amplitude of EJPs evoked before treatment with the test substance. To minimize posttetanic potentiation (Church et al. 1993; Lotshaw and Lloyd 1990; Whim and Lloyd 1990), intervals (100 s) were used to minimize release of endogenous peptide cotransmitters from the motor neurons and to minimize posttetanic contractions. This procedure confined the contractions to the small area of the muscle covered by the recording chamber and thus markedly reduced movement artifacts in the recordings. The earliest evoked muscle contractions occur after the sixth EJP so the early EJPs in a burst are recorded in the absence of any movement. Stimulation at 16 Hz was used routinely in these experiments. EJPs were recorded by extracellular electrodes placed inside and just outside the wall of the perfusion apparatus. Signals were amplified using a Grass P15D AC amplifier. The test substances were applied in ASW to the inner chamber of the perfusion electrode so the ganglia were not exposed to these substances. Typical application periods were 20 min to ensure adequate penetration into the muscle. Experiments were performed at room temperature (−22°C). In most experiments, long interburst intervals (100 s) were used to minimize release of endogenous peptide cotransmitters from the motor neurons and to minimize posttetanic potentiation (Church et al. 1993; Lotshaw and Lloyd 1990; Whim and Lloyd 1990). Recordings obtained with the perfusion electrode were quantified by comparing the EJP amplitude during treatment with the test substance to the average amplitude of EJPs evoked before treatment and after washout.

Intracellular labeling

The buccal mass/buccal ganglia complex was dissected and pinned in a dish as described in the preceding text except that the ganglia were not desheathed. Neurons were impaled by tapping a single microelectrode [15–30 Ω; filled as described in the preceding text] or via a perfusion electrode (see following text). For intracellular muscle fiber recordings, short bursts of two to eight action potentials at 10–12.5 Hz were used to ensure that a burst did not evoke contractions. Intracellular EJPs were quantified by comparing the EJP amplitude during application of the test substance to the EJP amplitude before its application because it was difficult to maintain intracellular recordings until the effects of the test substances completely reversed. The perfusion electrode consisted of a small chamber (100 μl) and aperture (∼1.5 mm), which was positioned to press firmly down on a portion of the muscle (see Fig. 1 in Church et al. 1993). The inside of the chamber was superfused rapidly with ASW (1.5 ml/min). The remainder of the muscle outside of the recording chamber was superfused with low Ca ASW to suppress synaptic transmission and muscle contractions. This procedure confined the contractions to the small area of the muscle covered by the recording chamber and thus markedly reduced movement artifacts in the recordings. The earliest evoked muscle contractions occur after the sixth EJP so the early EJPs in a burst are recorded in the absence of any movement. Stimulation at 16 Hz was used routinely in these experiments. EJPs were recorded by extracellular electrodes placed inside and just outside the wall of the perfusion apparatus. Signals were amplified using a Grass P15D AC amplifier. The test substances were applied in ASW to the inner chamber of the perfusion electrode so the ganglia were not exposed to these substances. Typical application periods were 20 min to ensure adequate penetration into the muscle. Experiments were performed at room temperature (−22°C). In most experiments, long interburst intervals (100 s) were used to minimize release of endogenous peptide cotransmitters from the motor neurons and to minimize posttetanic potentiation (Church et al. 1993; Lotshaw and Lloyd 1990; Whim and Lloyd 1990). Recordings obtained with the perfusion electrode were quantified by comparing the EJP amplitude during treatment with the test substance to the average amplitude of EJPs evoked before treatment and after washout.

Tissue preparation and staining

Immunocytochemistry was carried out using the methods of Longley and Longley (1986) as modified by Pearson and Lloyd (1989). Freshly isolated tissue or tissue in which neurons had been labeled intracellularly was washed with low Ca²⁺ high Mg²⁺ ASW and fixed in 4% paraformaldehyde, 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h at room temperature and then overnight at 4°C. After fixation, the tissue was washed with 30% sucrose in PB for 24 h at 4°C. Buccal nerve 2 was cut so the muscle and ganglion could be processed separately. The biocytin was visualized in the ganglion (as described in the following text) without sectioning to verify that only one neuron was labeled. Frozen sections (7–10 μm) were cut from the muscle in a cryostat and transferred to nylon mesh-bottomed wells containing PB saline (PBS: 0.14 M NaCl, 0.01 M phosphate, pH 7.4) for staining. The sections were washed with PBS (4 × 10 min each) and then cleared overnight with PB containing 0.2% Triton X-100, 0.1% sodium azide (PB-Triton-X) at 4°C. The biocytin was visualized with a 1:250 dilution of either streptavidin-Texas BT (BRL) or avidin-Texas Red (Vector) in PB-Triton-X. After 4 h, the sections were washed with PB-Triton-X (4 × 10 min each), and then nonspecific binding was blocked by incubating the tissue in a blocking solution of PB with 0.5% Triton X-100, 0.1% sodium azide, and 5% normal goat serum (NGS; GIBCO) overnight at 4°C. Primary and secondary antisera were diluted in this blocking solution. Antigli-tamate antiserum (Sigma) was used at a dilution of 1:1000. Sections were incubated with the antisera overnight at 4°C, washed with the blocking solution, and the staining visualized with a fluorescent secondary antisera, BODIPY FL goat anti-rabbit (Molecular Probes) used at a dilution of 1:250. This tissue was mounted on gelatin-coated slides, dried, and coverslipped in a 1:6 PBS:glycerol solution (pH 8.5). The fill was not visible with the fluorescein filter set and the immunofluorescence was not visible with the Texas Red/rhodamine filter set.

RESULTS

Pharmacological studies

Isolated muscle. Conventional transmitters were screened to test if they caused contractions of isolated segments of the I3a muscle. Of the transmitters tested (Table 1), only bolus application of glutamate, ACh, dopamine, and histamine evoked contractions at concentrations of ≤10 mM. We concentrated our studies on glutamate for the following reasons. 1) ACh is clearly not the conventional transmitter used by B3, B6, and B38. These neurons do not contain choline acetyltransferase activity and their EJPs are not inhibited by hexamethonium. The contractions evoked by ACh could be due to the presence of excitatory cholinergic receptors that normally respond to ACh released by the cholinergic motor neurons that innervate I3. 2) It is unlikely that B3, B6, and B38 are dopaminergic. Dopaminergic neurons in the CNS of Aplysia have been identified by glyoxylic acid treatment and the formaldehyde-glutaraldehyde–induced histofluorescence technique (Goldstein and Schwartz 1989; Hawkins 1989; Rathouz and Kirk 1988). Although there appears to be two or three midsize dopaminergic neurons in each buccal ganglion, none of these were large enough or positioned correctly to be the noncholinergic motor neurons. 3) It is unlikely that B3, B6, and B38 are histaminergic. The Aplysia CNS and peripheral tissue were stained with an antiserum directed against histamine (Elste et al. 1990; Sonila et al. 1990). No histamine-like immunoreactivity was observed in any large buccal neurons or in buccal muscle.

The actions of glutamate appear to be selective for I3. Perfusion of buccal muscles that have purely cholinergic motor innervation (termed I5 or ARC and I7-I10) with 0.1–1 mM glutamate for several minutes does not produce contractions (Cohen et al. 1978; Evans et al. 1996). Because 1 mM glutamate was close to the contraction threshold for I3 in our experiments, we compared the response of I3 and I5 muscle fibers isolated from the same animal to boluses of higher concentrations of glutamate. In these experiments, I5 did not respond to the application of glutamate even though concentrations 1,000 times greater than those that evoked contractions in I3 were tested (i.e., ≥1 M). By contrast, ACh (10 μM) readily evoked contractions in I5 muscles. We also determined that the effects of glutamate on I3 were direct. Because I3 is innervated by both cholinergic and noncholinergic motor neurons, it was possible that glutamate indirectly evoked contrac-
Substances that reduce glutamate-evoked contractions

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration*, mM</th>
<th>Trials</th>
<th>Glu Bolus, %†</th>
<th>ACh Bolus, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-glutamate‡</td>
<td>5</td>
<td>3</td>
<td>0 ± 0</td>
<td>83 ± 15</td>
</tr>
<tr>
<td>Homocysteate†</td>
<td>5</td>
<td>2</td>
<td>19 ± 2</td>
<td>83 ± 16</td>
</tr>
<tr>
<td>L-2-amino-4-sulfamoylbutyrate</td>
<td>5</td>
<td>2</td>
<td>37 ± 2</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>DL-α-aminopimelate</td>
<td>5</td>
<td>2</td>
<td>72 ± 3</td>
<td>96 ± 17</td>
</tr>
<tr>
<td>Glu-gly</td>
<td>2</td>
<td>2</td>
<td>55 ± 10</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>Ketamine</td>
<td>1</td>
<td>3</td>
<td>20 ± 7</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Putresine</td>
<td>5</td>
<td>2</td>
<td>76 ± 16</td>
<td>67 ± 25</td>
</tr>
<tr>
<td>Spermine</td>
<td>5</td>
<td>2</td>
<td>74 ± 10</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>1</td>
<td>2</td>
<td>30 ± 12</td>
<td>29 ± 15</td>
</tr>
<tr>
<td>n-Tubocurarine</td>
<td>1</td>
<td>2</td>
<td>85 ± 12</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

A total of 66 substances were tested (see Table 1). Most did not evoke contractions. * Concentration of substance tested as an antagonist in the bath. † Effects of substance or acetylcholine (ACh) on glutamate-evoked (Glu) contractions expressed as percentage control (mean ± SE). ‡ Glutamate and homocysteate cause transient contractions, but once the contraction relaxed, subsequent responses are inhibited.
glu-gly, selectively reduced the amplitude of glutamate-evoked contractions but were less effective than glutamate itself. A series of commonly used glutamate receptor antagonists, including 2-amino-3-phosphonopropionate, 2-amino-5-phosphonovalerate (AP5), amino-7-phosphonoheptanoic acid (AP7), 3-(2-carboxyipiperazin-4-yl)-propyl-1-phosphonate, glutamylamino methanesulfonate, 6-cyano-7-nitroquinolinicline-2,3-dione (CNQX), and 6,7-dinitroquinolinicline-2,3-dione (DNQX) also were screened. None of these antagonists significantly reduced the evoked contractions. Thus the pharmacology of glutamate-evoked contractions in these muscle fibers is different from the published pharmacology of most other vertebrate or invertebrate glutamate receptors.

Buccal muscle in A. kurodai also is innervated by excitatory motor neurons that are not inhibited by hexamethonium. EJPs and contractions evoked by stimulating these noncholinergic neurons were inhibited almost completely by 10 mM glutamic acid diethyl ester (GDEE) (Nagahama and Takata 1990). We examined whether GDEE inhibited ACh- or glutamate-evoked contractions of isolated I3a muscle fibers from A. californica. Four different concentrations (1–10 mM) were tested, and all of them reduced ACh-evoked contractions (to 37 ± 6% of control at 5 mM, n = 2). Indeed, contractions evoked by ACh were inhibited much more than those evoked by glutamate. GDEE also has been shown to be a cholinergic antagonist in vertebrates at concentrations > 2 mM (Watkins and Evans 1981). Because GDEE was not specific for glutamate-evoked contractions in our system, we did not pursue it further.

EXTRACELLULAR EJPS. We next studied the effects of the pharmacological agents on EJPs evoked by stimulation of motor neurons. A perfusion electrode was used to record extracellular EJPs in a small portion of the I3 muscle that was perfused with ASW while the remainder of the muscle was superfused with low Ca ASW to suppress synaptic transmission and muscle contractions (Church et al., 1993). This procedure permitted stable long-term recordings, rapid solution turnover, and simultaneous recordings from a population of fibers thereby reducing sampling bias. Results from this technique are qualitatively similar to those obtained with intracellular electrodes (Fox, and Lloyd 1997). First we verified that EJPs evoked by B3, B6, or B38 were not inhibited by application of hexamethonium to the muscle. By contrast, evoked EJPs were inhibited when glutamate was applied to the muscle. Superfusion with 1 mM glutamate reduced EJPs to 44 ± 3% for B3 (n = 19), to 7 ± 4% for B6 (n = 3), and to 33 ± 12% of control for B38 (n = 3) (Fig. 3). These effects presumably reflect desensitization of the glutamate receptor as there was little effect on cholinergic EJPs evoked by B9 (reduced to 86 ± 6% of control, n = 4). Even at higher concentrations (10 mM), glutamate had little effect on cholinergic neuromuscular synapses in I5, indicating again that glutamate’s action was selective. Most of the glutamate agonists that evoked contractions (d- and l-aspartate, cysteate, CSA, and HCA; Fig. 4) and substances that specifically reduced glutamate-evoked contractions (ASBA and HCA; Fig. 5) were tested for their ability to inhibit B3-evoked EJPs. All of these substances had little effect on B3-evoked EJPs. The effects of ASBA and HCA on EJPs were much smaller than their effects on contractions evoked by glutamate boluses. These differences might be a consequence of the procedures used to test the substances. Bolus application of glutamate might activate extrasynaptic receptors that are not normally activated by glutamate released from the motor neurons. These substances may act on other components of the excitation-contraction coupling mechanism and not directly effect synaptic transmission. Although there were some differences in the effects of the test substances on neuronally evoked EJPs and glutamate evoked contractions, glutamate was the most effective substance tested at reducing both of these responses. In addition, glutamate was selective in that it only inhibited EJPs evoked by noncholinergic motor neurons.

Pharmacological evidence suggests that some central glutamate receptors in mollusks are NMDA-like or kainate/AMPA-like; however, this does not seem to be the case for peripheral glutamate receptors (Bahls et al., 1995; Dale and Kandel 1993; Gapon and Kupfermann 1996; Morez et al., 1993). A number of ionotropic glutamate receptor antagonists (AP5, AP7, kynurenic acid, and DNQX) were tested and found to have little effect on B3-evoked EJPs (Fig. 5). Thus the pharmacology of these neuromuscular synapses does not appear to be NMDA-like or kainate/AMPA-like and is clearly different from the published pharmacology of central glutamate receptors in mollusks. The pharmacology of the synapses between the noncholinergic motor neurons and buccal muscle appears to be similar to the neuroglandular synapses in the salivary glands of Helisoma. These synapses are not inhibited by NMDA or kainate/AMPA receptor antagonists but are blocked almost completely by 1

---

**FIG. 3.** Effects of 1 mM glutamate on extracellularly recorded junction potentials (EJPs) from I3 using a perfusion electrode. Glutamate reduced the amplitude of EJPs evoked by stimulating the noncholinergic neurons (B3, B6, and B38), but had little effect on cholinergic EJPs evoked by B9 stimulation. Effects of the glutamate reversed within 30 min of washout. Top: extracellular recording of the EJPs from I3. Bottom: intracellular recording from the motor neurons (as labeled in the figure). Recordings are from different experiments.
mM D-glutamate (Bahls et al. 1995). We found that even at 10 mM, D-glutamate only slightly reduced B3-evoked EJPs (Fig. 6; to 78 ± 7% of control, n = 3). Therefore the pharmacology of B3-evoked EJPs differs from that found previously for both central and peripheral glutamate receptors in mollusks.

**INTRACELLULAR EJPS.** Although it was technically difficult to do, it was necessary to examine the effects of glutamate on intracellularly recorded EJPs. The reduction in the amplitude of the extracellularly recorded EJPs could have been due to a tonic depolarization of the muscle fibers that would bring the resting potential of the muscle fibers closer to the synaptic reversal potential and thus reduce the amplitude of the EJP. The perfusion electrode would not have detected a slow tonic depolarization as it is an extracellular recording technique. Indeed, application of 1 mM glutamate to I3 reversibly inhibited EJPs evoked by B3, B6, or B38 (reduced to 64 ± 5% for B3, n = 5; to 27 ± 5% for B6, n = 3; to 16 ± 5% of control for B38, n = 3; Fig. 7). The effects of glutamate were measured after the reversal of the transient depolarization. If glutamate functions by desensitization only and if glutamate is also the fast transmitter of the noncholinergic motor neurons, it should reduce the size of EJPs evoked by these neurons without decreasing muscle fiber input resistance. We used the time constant for the decay of EJPs that could be fitted with a single exponential as an indirect measure of changes in input resistance. In glutamate, the time constant of the muscle fibers, measured after the transient depolarization, increased (to 120 ± 10% of control, n = 11), indicating that, if anything, muscle fiber input resistance increased slightly. These results indicate that glutamate desensitizes the receptors for the noncholinergic fast transmitter. Finally, the effects of glutamate were selective because B9-evoked cholinergic EJPs recorded in the same fibers were essentially unchanged (reduced to 95 ± 4%, n = 3; Fig. 7; see following text).

These results were qualitatively similar to the results obtained with the perfusion electrode. However, quantitative comparisons of results obtained by intracellular and extracellular recording techniques must be approached cautiously. Intracellular recordings were necessary to measure the time constants for the decay of EJPs, but they sample individual fibers that could lead to a sampling bias. In addition, the most stable recordings were typically from fibers deep in muscle bundles, and solution exchange was slow. Extracellular recordings sample a large population of fibers near the muscle surface, and thus solution exchange was relatively rapid.

**Immunocytological studies**

Glutamate-like immunoreactivity was visualized using a primary antiserum directed toward glutamate and a fluorescent (BODIPY) secondary antibody. No immunoreactive neuronal somata were observed in the ganglia, and no immunoreactive

---

**Figure 4.** Pooled results of the effects of L-glutamate-like substances on extracellularly recorded EJPs evoked by the stimulation of B3. Glutamate was the most effective substance tested at inhibiting B3-evoked EJPs. Number of experiments are in parenthesis under the substance tested. Pooled results are mean ± SE. L-Glu, L-glutamate; D-Glu, D-glutamate; L-Asp, L-aspartate; D-Asp, D-aspartate; CA, l-cysteate; CSA, L-cysteinesulfinate; HCA, L-homocysteate.

**Figure 5.** Pooled results of the effects of glutamate receptor antagonists on extracellularly recorded EJPs evoked by the stimulation of B3. Glutamate was the most effective substance tested at inhibiting evoked EJPs. Note that antagonists used to categorize glutamate receptors had little effect on EJPs. Number of experiments is in parenthesis under the substance tested. 6,7-Dinitroquinoxaline-2,3-dione (DNQX) concentration (0.1 mM) was 10-fold higher than the concentration that effectively inhibits central glutamatergic synapses (Dale and Kandel 1993). Pooled results are mean ± SE. AP5, 2-amino-5-phosphonooxazole; AP7, amino-7-phosphonoheptanoate; Kyn, kynurenic; ASBA, L2-amino-4-sulfamoybutyrate; AP7, 6,7-Dinitroquinoxaline-2,3-dione (DNQX).

**Figure 6.** Effects of 10 mM D-glutamate on B3-evoked EJPs recorded extracellularly. D-glutamate only slightly reduced the amplitude of evoked EJPs, and its effects reversed within 30 min of washout. Top: extracellular recording of the EJPs from I3a. Bottom: intracellular recording from B3. Pooled data presented in Fig. 4.
large caliber axons were observed in the muscle, nerve, or ganglia. Indeed, preliminary analyses of primary amines present in individual identified neuronal somata by high-performance liquid chromatography indicate that glutamate levels in cholinergic and noncholinergic neurons were similar (unpublished observation). These findings are similar to those observed in lobster where glutamate levels in excitatory motor axons and somata that use glutamate as a fast transmitter were similar to those in inhibitory motor axons and somata that use GABA as a fast transmitter (Kravitz and Potter 1965; Otsuka et al. 1967). In our study, immunoreactivity was associated with fine-diameter varicose axonal fibers in the neuropil of ganglia or in muscle sections. Numerous immunoreactive fibers and varicosities were present in the I3 muscle (Fig. 8). The immunoreactivity appears to be quite specific to glutamate. In blinded observations, staining with the antiglutamate antiserum was eliminated by incubation with 1 mM L-glutamate but was unaffected by incubation with 10 mM aspartate, glutamine, glycine, glucose, cysteate, or homocysteate.

Immunoreactive fibers also were observed in the I5 muscle, which receives only cholinergic excitatory motor input (Cohen et al. 1978). However, the immunoreactive fibers were much sparser than those observed in I3. The immunoreactivity in I5 may be due to the presence of sensory neuron fibers, which are thought to use glutamate as their transmitter. Consistent with this interpretation, glutamate-like immunoreactive fibers in I5 were concentrated in the region where the muscle attaches to the cartilage, an area that is likely to have heavy sensory innervation. Staining in I3 was more uniform and was similar to the pattern observed when immunocytochemistry was carried out using antiserum directed toward peptide cotransmitters expressed in motor neurons (Lloyd et al. 1985; Miller et al. 1992).

To determine if the varicose axons of B3, B6, or B38 contained glutamate-like immunoreactivity, double label experiments were carried out. Neurons were identified physiologically, and their cell bodies were injected intracellularly with biocytin and incubated for several days to allow diffusion of biocytin to terminals in the I3 muscle (~30 mm distance). Only a single motor neuron was injected in each preparation. Muscles then were fixed and sectioned, and biocytin was visualized with either avidin- or streptavidin-Texas Red. These experiments had inherent limitations. Large-caliber axons in the muscle filled well but, as expected from the results described in the preceding text, were not immunoreactive, whereas small-caliber varicose axons were immunoreactive but filled less efficiently presumably due to their diffusional distance from the filled cell bodies. Nevertheless, double staining in axonal fibers and varicosities in I3 muscle was detected for B3, B6, and B38, indicating that the terminals of each motor neuron were indeed immunoreactive (Fig. 9). Observation of the ganglia at the end of the incubation period indicated that biocytin was confined to the injected neuron, indicating that there was no significant dye coupling to other neurons.
Individual muscle fibers receive fast excitatory EJPs from both cholinergic and noncholinergic motor neurons

We previously had determined that individual muscle fibers in the anterior region I3 were innervated by excitatory noncholinergic motor neurons (B3 and B38) and an inhibitory cholinergic motor neuron (B47) (Church et al. 1993). Similar results also have been observed in A. kurodai where posterior and medial buccal muscle fibers were innervated by excitatory noncholinergic motor neurons and either an inhibitory or dual component (inhibitory/excitatory) cholinergic motor neuron (Nagahama and Takata 1990). We now report that individual muscle fibers in the medial portion of I3 are innervated by excitatory noncholinergic motor neurons (B3 or B6) and excitatory cholinergic motor neuron (B9) (Lloyd and Church 1994). As expected from previous results, B9-evoked EJPs are inhibited by hexamethonium, whereas those of B3 and B6 are not (Fig. 10). These results are similar to glutamate-like immunoreactivity is present in the axons of B3, B6, and B38 in the I3 muscle. Cell bodies of B3, B6, or B38 were injected intracellularly with biocytin, fixed, and sectioned. Only a single motor neuron was injected in each preparation. Biocytin was visualized with either avidin- or strepavidin-Texas Red (right) and the glutamate-like immunoreactivity was visualized with a fluorescent (BODIPY) secondary antibody (left). Double staining in axonal fibers and varicosities in I3 muscle were detected for each motor neuron, indicating that their axons were indeed immunoreactive. Observation of the ganglia at the end of the incubation period indicated that biocytin was confined to the injected neuron. Scale bars: 10 μm for B6; 50 μm for B3 and B38.

**FIG. 9.** Glutamate-like immunoreactivity is present in the axons of B3, B6, and B38 in the I3 muscle. Cell bodies of B3, B6, or B38 were injected intracellularly with biocytin, fixed, and sectioned. Only a single motor neuron was injected in each preparation. Biocytin was visualized with either avidin- or strepavidin-Texas Red (right) and the glutamate-like immunoreactivity was visualized with a fluorescent (BODIPY) secondary antibody (left). Double staining in axonal fibers and varicosities in I3 muscle were detected for each motor neuron, indicating that their axons were indeed immunoreactive. Observation of the ganglia at the end of the incubation period indicated that biocytin was confined to the injected neuron. Scale bars: 10 μm for B6; 50 μm for B3 and B38.

**Individual muscle fibers receive fast excitatory EJPs from both cholinergic and noncholinergic motor neurons**

We previously had determined that individual muscle fibers in the anterior region I3 were innervated by excitatory noncholinergic motor neurons (B3 and B38) and an inhibitory cholinergic motor neuron (B47) (Church et al. 1993). Similar results also have been observed in A. kurodai where posterior and medial buccal muscle fibers were innervated by excitatory noncholinergic motor neurons and either an inhibitory or dual component (inhibitory/excitatory) cholinergic motor neuron (Nagahama and Takata 1990). We now report that individual muscle fibers in the medial portion of I3 are innervated by excitatory noncholinergic motor neurons (B3 or B6) and excitatory cholinergic motor neuron (B9) (Lloyd and Church 1994). As expected from previous results, B9-evoked EJPs are inhibited by hexamethonium, whereas those of B3 and B6 are not (Fig. 10). These results are similar to glutamate-like immunoreactivity is present in the axons of B3, B6, and B38 in the I3 muscle. Cell bodies of B3, B6, or B38 were injected intracellularly with biocytin, fixed, and sectioned. Only a single motor neuron was injected in each preparation. Biocytin was visualized with either avidin- or strepavidin-Texas Red (right) and the glutamate-like immunoreactivity was visualized with a fluorescent (BODIPY) secondary antibody (left). Double staining in axonal fibers and varicosities in I3 muscle were detected for each motor neuron, indicating that their axons were indeed immunoreactive. Observation of the ganglia at the end of the incubation period indicated that biocytin was confined to the injected neuron. Scale bars: 10 μm for B6; 50 μm for B3 and B38.

**FIG. 10.** Excitatory cholinergic and noncholinergic neurons functionally innervate the same muscle fibers. A: B3- and B9-evoked EJPs were recorded from a single muscle fiber. Neurons were impaled with a single electrode in this experiment, so spikes were elicited with 1 s DC pulses. B: B6- and B9-evoked EJPs were recorded from a single muscle fiber. Hexamethonium (0.25 mM) selectively reduced cholinergic EJPs evoked by B9, whereas the noncholinergic EJPs evoked by B3 or B6 were essentially unchanged. B38 was not examined because its field of innervation does not overlap with B9. Top: intracellular recording of the EJPs from a muscle fiber. Bottom: intracellular recording from the motor neuron (as labeled in the figure). A and B are from different experiments.
observations made in the *Aplysia* gill where individual muscle fibers also receive both cholinergic and noncholinergic EJPs (Carew et al. 1974). However, in both systems it is important to emphasize that we are describing functional innervation; because of electrical coupling between muscle fibers (Lotshaw and Lloyd 1990), the anatomic innervation need not be on the same fiber.

**Discussion**

**Evidence implicating glutamate as the fast transmitter of the noncholinergic motor neurons**

Overall, the pharmacological evidence is consistent with glutamate being the fast excitatory transmitter used by the noncholinergic motor neurons. Except for ACh, glutamate itself was the most potent substance tested at producing contractions. The actions of glutamate were selective for I3. Buccal muscle I5, which has a purely cholinergic motor innervation, did not contract in response to the application of glutamate at any concentration. Glutamate was also the most effective of all substances tested at reducing the amplitude of EJPs evoked by noncholinergic motor neurons apparently through desensitization of receptors. These results were obtained even though many other substances were tested, some with structures very similar to glutamate. Glutamate had no effect on EJPs evoked by cholinergic neurons recorded in the same fibers nor did it reduce the EJP relaxation time constant. These results indicate that glutamate did not decrease the input resistance of the muscle fibers.

Receptors activated by AMPA and kainate in vertebrate CNS and in arthropod neuromuscular junctions desensitize rapidly with a time constant of <50 ms (Dudel et al. 1988, 1990; Trussell and Fischbach 1989), whereas NMDA-activated receptors in the vertebrate CNS desensitize with a slower time constant of 1–10 s (Zorumski et al. 1989). Desensitization of glutamate receptors in I3 was very slow compared with that observed for glutamate receptors in the vertebrate CNS and in arthropods. However, much of the delay is due to the time required for solution exchange and diffusion of glutamate to receptors deep in the fiber bundles. The magnitude of the desensitization appeared to differ between the three noncholinergic neurons. B3-evoked EJPs, recorded with either intracellular or extracellular electrodes, were reduced less than EJPs evoked by stimulating the other neurons. Although this difference could be due to B3’s terminals being deeper in the bundle and less accessible to bath applied glutamate, it is also possible that it represents a real physiological difference between receptors at these neuromuscular synapses. Different subtypes of the glutamate receptor may be expressed at B3’s synapses. The family of ionotropic glutamate receptors is large and diverse, currently including at least 22 cloned subunits (Hollmann and Heinemann 1994). We cannot distinguish between these possibilities based on our results.

Perhaps the most convincing evidence implicating glutamate as the fast excitatory transmitter was the immunocytochemistry using an antiserum raised to conjugated glutamate. First, we were able to identify the immunoreactive varicose axons as being those of B3, B6, or B38 by filling their cell bodies with biocytin. Second, the immunoreactivity was reduced substantially by 1 mM glutamate while even 10-fold higher concentrations of several closely related compounds did not detectably affect the immunoreactivity, suggesting that it was highly selective for glutamate. We were not able to find neuronal cell bodies that contained glutamate-like immunoreactivity in the *Aplysia* CNS. Recently this has been accomplished in the pedal-pleural ganglia using semithin sections to improve the penetration of the antiserum and reduce the background staining (Levenson et al. 1997). Even then only weak to moderate staining was observed in the cell bodies compared with the intense staining of axonal processes in the neuropil. The observation that glutamate-like immunoreactivity was weak in cell bodies and large-caliber axons is consistent with biochemical measurements made in lobster that indicate that glutamate levels are similar in neuronal cell bodies and large-caliber axons of motor neurons that use glutamate and motor neurons that use GABA as their fast transmitters (Kravitz et al. 1963; Otsuka et al. 1967). This may reflect the selective expression of high-affinity uptake sites and the localization of vesicles containing glutamate to synaptic regions (Nicholls 1993).

Taken together, the evidence that glutamate is the fast excitatory transmitter at these neuromuscular synapses is quite convincing. However, other conventional transmitters such as ACh, dopamine, and histamine evoked contractions of the isolated I3 muscle. We do not believe that any of these are the conventional transmitter used by the noncholinergic motor neurons. ACh caused contractions in the isolated muscle, but B3, B6, or B38 do not contain choline acetyltransferase activity and their EJPs are not blocked by hexamethonium, a cholinergic antagonist in *Aplysia* (Church et al. 1993; Lloyd and Church 1994; Lotshaw and Lloyd 1990). We assume that the contractions evoked by ACh were due to the presence of excitatory cholinergic receptors for cholinergic motor neurons (e.g., B9) that functionally innervate the same I3 muscle fibers as the noncholinergic motor neurons. Dopaminergic neurons in the CNS of *Aplysia* have been identified by glyoxylic acid and formaldehyde-glutaraldehyde induced histofluorescence techniques (Goldstein and Schwartz 1989; Hawkins 1989; Rathouz and Kirk 1988). The dopamine positive neurons are all much smaller than any of the identified noncholinergic motor neurons. Also, histamine-like immunoreactivity also has been localized to the CNS and peripheral tissue in *Aplysia* (Elste et al. 1990; Somila et al. 1990). Four histaminergic neurons were detected in the buccal ganglia and were identified as premotor sensory neurons (Evans et al. 1999). Because none of the identified motor neurons that innervate I3 appear to use dopamine or histamine as transmitters, we do not know why these substances evoke contractions. There are several possible explanations. 1) There could be unidentified dopaminergic or histaminergic neurons that innervate buccal muscle from other ganglia. For example, some motor neurons for buccal muscles are located in the cerebral ganglia (Chiel et al. 1986; Jahan-Parwar and Fredman 1983). 2) Dopamine or histamine might have indirectly evoked contractions by stimulating the release of the conventional transmitters from the terminals of identified motor neurons to I3. 3) Dopamine or histamine could be released from modulatory neurons that innervate the muscle or from neuroendocrine organs and act as hormones. Bath application of SCP or serotonin, which are purely modulatory transmitters, can evoke contractions of isolated I3 muscle (Fox and Lloyd 1997). Although dopamine and histamine do not appear to be the fast transmitters used by the noncholinergic...
motor neurons, our results suggest that they may be transmitters in this system and may warrant further investigation.

Why are two fast excitatory transmitters used at buccal neuromuscular synapses?

Although the EJPs evoked by the cholinergic and the noncholinergic motor neurons were quite variable among preparations, we observed no consistent differences in the amplitude and kinetics of EJPs evoked from either of the two neuronal groups. Of course it is possible that the fast transmitter used by a motor neuron is a consequence of developmental or evolutionary factors and not its function. Alternatively, two fast excitatory transmitters might be important for the optimization of behaviors or the transition between different behaviors. Feeding is highly modulated. The sources of the modulation include: peptides in the motor neurons, modulatory neurons that innervate the muscle, and sensory feedback by proprioceptive neurons. If either the cholinergic or noncholinergic synaptic transmission is targeted selectively by a modulator, it could be important behaviorally. For example, noncholinergic motor neuron B3 and cholinergic motor neuron B9 functionally innervate the same fibers, but FMRFamide potentiates B3-evoked EJPs and contractions while it inhibits B9-evoked EJPs and contractions (Keating and Lloyd 1997). Selective effects of modulators could allow the animal to optimize the feeding movements to the physical characteristics of the food being ingested. It is important to emphasize that this innervation may not represent anatomic synapses on the same muscle fibers because the muscle fibers are coupled electrically.

Comparisons with other systems

Across phyla, ACh and glutamate appear to be by far the most common, and perhaps the only, transmitters used at fast excitatory neuromuscular synapses (Gerschenfeld 1973; Johnson and Stretton 1985; Kehoe and Marder 1976; Segerberg and Stretton 1993). Of course, the fast excitatory transmitter at vertebrate neuromuscular junctions is ACh (Dale et al. 1936). In crustaceans and insects, the predominant fast excitatory transmitter at neuromuscular synapses is very likely to be glutamate, although ACh appears to be used in a limited number of cases (Johansen et al. 1989; Kawagoe et al. 1982; Kravitz et al. 1970; Marder 1976; Shupliakov et al. 1995). Finally, at least in buccal motor neurons in Aplysia, ACh and glutamate are the fast transmitters.

The evidence implicating glutamate as a transmitter in mollusks is constantly growing. In the central ganglia, an excitatory amino acid recently has been implicated as the fast excitatory transmitter used by sensory neurons in Aplysia. One group concluded that homocysteic acid was the likely transmitter (Trudeau and Castellucci 1993), whereas another group suggested it was likely glutamate itself (Dale and Kandel 1993). If our conclusion is correct and the fibers containing glutamate-like immunoreactivity in the cholinergic I5 muscle are indeed those of sensory neurons, then our finding that glutamate but not homocysteate blocked staining suggests that glutamate is the more likely transmitter at least for buccal sensory neurons. The staining of the pedal-pleural ganglia sensory neurons with an antiserum directed against glutamate also supports this conclusion (Levenson et al. 1997). Glutamate also appears to be an important transmitter in molluscan feeding systems. It has been identified as the fast transmitter for retraction phase interneurons in Lymnaea (Brierley et al. 1997) and it is the fast transmitter for the radula mechanosensory neuron B21 in Aplysia (Klein et al. 1998). However, the pharmacology of the synapses formed by these neurons is different from what we observed in that the postsynaptic actions of glutamate are blocked by kainate/AMPA antagonists (i.e., CNQX or DNQX).

Buccal muscle in A. kurodai also is innervated by excitatory noncholinergic motor neurons that have an unusual pharmacology (Nagahama and Takata 1990). The pharmacology of these neurons cannot be directly compared with the glutamate receptors in the CNS of A. californica because different antagonists were used in these studies. It is also difficult to compare them to the buccal motor neurons in A. californica. Although GDEE inhibited EJPs and contractions evoked by stimulating A. kurodai noncholinergic neurons, in A. californica it was not specific in that it inhibited ACh-evoked contractions more effectively than glutamate-evoked contractions.

A peripheral synapse between neuron B4 and salivary cells of Helisoma has a similar pharmacological profile to what we observed (Bahls et al. 1995). None of the classic subtype-specific glutamate agonists or antagonists were effective in either system. Indeed, the pharmacology of the two systems differ in only two respects: d-glutamate is an antagonist in Helisoma, whereas it was essentially without effect on the Aplysia buccal muscle, and l-aspartate was not an agonist in Helisoma, whereas it was the second most effective glutamate-like agonist in Aplysia. However, it is not clear if these differences in pharmacology indicate that they use different glutamate receptors. It is possible that the differences in the pharmacology are due to the fact that the salt concentration in Aplysia blood and physiological solutions is ∼10-fold higher than in Helisoma blood.

In conclusion, our studies of peripheral modulation have been limited because the identity of the conventional transmitter used by some buccal motor neurons has been unknown. Here we have shown that the fast transmitter used by identified noncholinergic motor neurons (B3, B6, and B38) is an excitatory amino acid and very likely glutamate itself. This information should help us understand the mechanisms that underlie feeding in Aplysia by allowing us to continue to dissect physiologically the role of transmitters and cotransmitters at the synapses of identified motor neurons.

This work was supported by National Research Service Awards 1-F31-MH10656 to L. E. Fox and IBN-9728453 to P. E. Lloyd.

Address for reprint requests: P. E. Lloyd, Committee on Neurobiology, University of Chicago, 947 E. 58th St., Chicago, IL 60637.

Received 6 April 1999; accepted in final form 24 May 1999.

REFERENCES


Carew, T. J., Pinski, H., Rubinson, K., and Kandel, E. R. Physiological and biochemical properties of neuromuscular transmission between identifi-


