J. Comp. Physiol. 138, 187–199 (1980)

Journal of Comparative Physiology · A

The Sensitivity of Decapod Foregut Muscles to Acetylcholine and Glutamate

Chris Lingle*
Department of Biology, University of Oregon, Eugene, Oregon 97403, USA

Accepted March 13, 1980

Summary. 1. The contractile sensitivity of spiny lobster *(Panulirus interruptus)* foregut muscles to acetylcholine and glutamate was examined. Muscles were of three types: Class I, sensitive only to acetylcholine; Class II, sensitive only to glutamate; and Class III, sensitive to both acetylcholine and glutamate.

2. Foregut muscles of Maine lobsters *(Homarus americanus)*, west coast crabs *(Cancer magister)*, blue crabs *(Callinectes sapidus)*, and rock crabs *(Cancer borealis* and *irroratus)* were tested for sensitivity to acetylcholine and glutamate. In general, homologous muscles in all species showed similar contractile sensitivity to either acetylcholine, glutamate, or both, except that in *Callinectes*, and *C. irroratus* and *borealis* acetylcholine sensitivity in muscles of Class III was reduced or absent.

3. Both glutamate and acetylcholine produce depolarizations of similar magnitude in the same muscle fibers of dually sensitive muscles. Estimated reversal potentials obtained by extrapolation of glutamate and acetylcholine activated depolarizations are in both cases above −20 mV.

4. Acetylcholine receptors are distributed diffusely on fibers of dually sensitive muscles while glutamate receptors are localized to discrete foci.

5. Blockade of synaptic transmission by alteration of divalent cation concentrations does not block depolarizations of acetylcholine or glutamate.

6. Cholinergic blockers do not block synaptic transmission to dually sensitive muscles, nor do they diminish glutamate depolarizations.

7. The results indicate that dually sensitive muscle fibers probably receive glutamatergic innervation while acetylcholine receptors are distributed extrajunctionally.

8. The best transmitter candidate for the LG, MG, and DG neurons of the decapod stomatogastric ganglion is glutamate. Thus, all intrinsic muscles of the decapod foregut receive glutamatergic innervation, while extrinsic muscles receive cholinergic innervation.

Introduction

One step in the differentiation of a cell into a neuron or muscle fiber is the expression and localization of particular types of cell-surface receptors to transmitter substances. On a variety of both muscle (Lea and Usherwood, 1973; Cull-Candy, 1976) and nerve cells (Ascher and Kehoe, 1975; Marder and Paupardin-Tritsch, 1978), in addition to synaptically localized receptors, low levels of receptors may frequently be found on regions of membrane without synaptic contacts. In general, it is not understood whether receptors on extrajunctional regions are functionally significant. Extrasynaptic receptors might simply reflect the incomplete localization of receptors to chemical inputs impinging at other sites on the cell. The results presented here describe some neuromuscular preparations in the decapod crustacean foregut in which glutamatergically innervated muscle fibers display extrajunctional receptors to acetylcholine, a substance most likely not released onto these fibers.

The stomatogastric system of the decapod Crustacea is novel in that two excitatory motor transmitters are thought to be used (Marder, 1974, 1976; Lingle, 1976, 1979; Atwood et al., 1977). Some muscles receive cholinergic excitatory innervation (Marder, 1974, 1976) while other muscles receive a glutamatergic excitatory innervation (Lingle, 1976, 1979). Of the 23 motor neurons that innervate the foregut musculature of the spiny lobster, *Panulirus interruptus*, 9

* Present address: Biology Department, Brandeis University, Waltham, Massachusetts 02254, USA

0340-7594/80/0138/0187/$02.60

(2 PD, 4 GM, 1 VD, and 2 LPG)[1] have been shown to contain choline acetyltransferase and innervate muscles that depolarize and contract to acetylcholine application. Other muscles that do not respond to acetylcholine, but do contract to glutamate, receive innervation from the LP, 8 PY, the IC, or the AM neurons not containing choline acetyltransferase (Marder, 1976). Other aspects of neuromuscular transmission onto muscles innervated by these latter neurons is consistent with a glutamatergic innervation (Lingle, 1979). Apparently conflicting reports between two different decapod species, *Panulirus* and *Callinectes*, have appeared concerning the likely transmitter of the three remaining neurons. On the basis of sensitivity to acetylcholine of muscles innervated by these neurons, Marder, working on *Panulirus*, originally suggested that the three neurons, the LG, MG, and DG, were likely to be cholinergic. However, an analysis of the synaptic vesicles contained within ganglionic terminals of the DG neuron showed the vesicles to be similar to those in presumed non-cholinergic neurons, but not to those in presumed cholinergic neurons (King, 1976). Additionally, in *Callinectes*, Atwood et al. (1977) reported that a muscle innervated by the MG neuron contracted to glutamate, but not acetylcholine. The present study shows that these apparent contradictions are resolved by the demonstration of extrajunctional acetylcholine receptors on the muscles receiving innervation from the DG, LG, and MG neurons in some decapod species, although the synaptic transmitter is likely to be glutamate. Furthermore, this study extends the original findings on *Panulirus* to several other decapod species and shows that each of these species contains foregut muscles of three sorts: 1. those receiving cholinergic innervation, 2. those receiving glutamatergic innervation, and 3. those receiving glutamatergic innervation while showing extrajunctional acetylcholine receptors.

Materials and Methods

Panulirus interruptus were obtained from Pacific Biomarine, Venice, CA; *Homarus americanus* from Maine Coast Seafoods, Service Head, Maine, or, in Boston, from local markets; *Callinectes sapidus* from Gulf Specimen Co., Panacea, Florida, *Cancer magister* from Oregon Institute of Marine Biology, Charleston, Oregon, and *Cancer borealis* and *irroratus* from local Boston markets. Animals were maintained in sea water tanks at appropriate temperatures.

Muscles and attached nerves (identified according to the nomenclature of Maynard and Dando (1974)) were isolated from the foregut, pinned out in Sylgard-lined 1–3 ml perfusion chambers, and superfused continuously with a gravity-fed system at 5–10 ml/min. Inflowing saline was cooled by circulation through a plexiglass heat exchange plate sitting on a Peltier cooling plate. Temperature was monitored by a small thermometer in the muscle chamber. Experiments were done at 8–14 °C for *Homarus* and the *Cancer* species, at 12–16 °C for *Panulirus*, and at 14–18 °C for *Callinectes*. Within a given experiment, temperature varied less than 1 °C. Changes in saline composition or bath applications of drugs were accomplished by switches in the perfusion line.

Saline compositions were the following (in mM): *Panulirus* (Marder, 1976): NaCl, 479; KCl, 12.7; $MgSO_4$, 10; $CaCl_2$, 13.7; Na_2SO_4, 3.9; Tris base, 8.3; maleic acid, 3.6; *Homarus* (Otsuka et al., 1967): NaCl, 466.5; KCl, 15.2; $MgSO_4$, 8.3; $CaCl_2$, 25.9; Tris base, 11; maleic acid, 4.8; *Cancer magister* and *Callinectes* (Auerbach, 1978): NaCl, 466; KCl, 11.3; $MgSO_4$, 19.5; $CaCl_2$, 12.6; Tris base, 11; maleic acid, 4.8; *C. borealis* and *irroratus* (Pantin, 1961): NaCl, 440; KCl, 11; $MgSO_4$, 26; $CaCl_2$, 9.8; Tris base, 11; maleic acid, 4.8. The pH of all solutions was 7.3–7.6. In some experiments SO_4 was omitted from the saline by using $MgCl_2$ and omitting Na_2SO_4 where necessary. No differences in results were observed between salines with or without sulphate.

A Grass FT03 force displacement transducer was used for tension studies. Conventional electrophysiological techniques and equipment were used. Microelectrodes were 3 M KCl (10–40 MΩ). Drugs were obtained from the following sources:

L-glutamic acid (monosodium salt), L-aspartic acid (magnesium salt), acetylcholine chloride, tubocurare chloride, atropine sulphate, carbamylcholine chloride, tetramethylammonium hydroxide, picrotoxin, decamethonium bromide, strychnine, nicotine, hexamethonium bromide, D-glutamic acid, L-glutamic acid gamma methyl ester and mecamylamine HCl, Sigma Chemical Company; pempidine tartrate, May & Baker, Ltd; trimethapan camsylat, Hoffman LaRoche; chlorisondamine, Ciba-Geigy; and dihydro-β-erythroidine from P. Adams. Quisqualic acid was the generous gift of H. Shinozaki.

Results

Agonist-Induced Contractures in Stomatogastric Muscles of Panulirus

The first part of this study entailed an examination of the sensitivity of various foregut muscles in *Panulirus* to a variety of neuroactive agents. Substances were tested for ability to produce muscle contracture. Three classes of muscles were found. As in previous studies (Marder, 1976; Atwood et al., 1977) some foregut muscles were found to contract either to acetylcholine or to glutamate. In addition, a third group of muscles was found to contract to both acetylcholine and glutamate.

Muscles of the first class contract only to acetylcholine or the nicotinic cholinergic agonists, carbachol, nicotine, or tetramethylammonium, as found previously (Marder, 1976; Marder and Paupardin-Tritsch, 1980). These muscles are those innervated by the PD, VD, GM and LPG. In addition, previously unexamined muscles innervated by the CD neurons are sensitive only to cholinergic agonists.

Muscles of the second class contract only to glutamate as previously observed (Marder, 1976; Lingle

[1] A description of the anatomy of the decapod foregut musculature and its innervation can be found in Maynard and Dando (1974). The terminology and abbreviations used in the present paper for identification of neurons and muscles follows that of Maynard and Dando (1974)

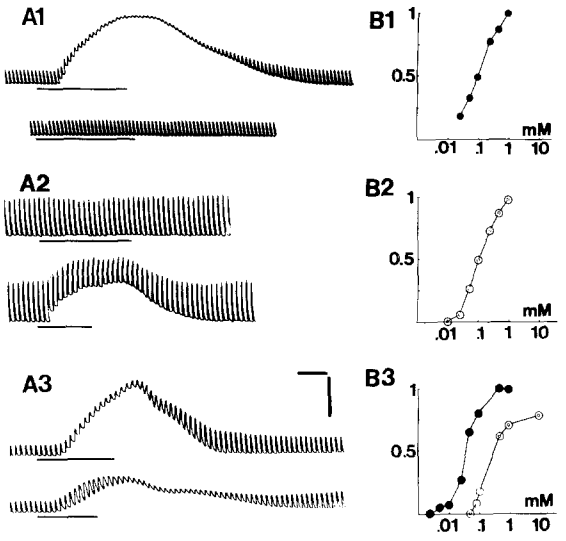

Fig. 1. A Tension records during bath application of 5×10^{-5} M acetylcholine and 10^{-3} M glutamate on selected foregut muscles of *Panulirus*. Nerves to muscles were stimulated with repeated trains of pulses of 30–50 Hz for 300–500 ms. The top trace in each pair corresponds to the application of acetylcholine and the bottom trace, glutamate. Agonist was introduced into the perfusion system during the bar. 10^{-5} g/ml Tensilon, a cholinesterase inhibitor, was applied with acetylcholine. **A**1: muscle cpv 1a, 1b, the dorsal dilator; **A**2: muscle pl; **A**3: muscle gm6b. Calibration: 1 min; 1.1 g in **A**1, **A**2; 5.5 g in **A**3. **B** Dose-response relationship of contractile responses to acetylcholine or glutamate in each muscle. Responses were normalized to the maximal contracture produced during an experiment. Responses to acetylcholine were obtained in the absence of a cholinesterase inhibitor. Filled circles indicate responses to acetylcholine and hollow circles to glutamate. **B**1: muscle cpv 1a, 1b; **B**2: muscle pl; **B**3: muscle gm6b

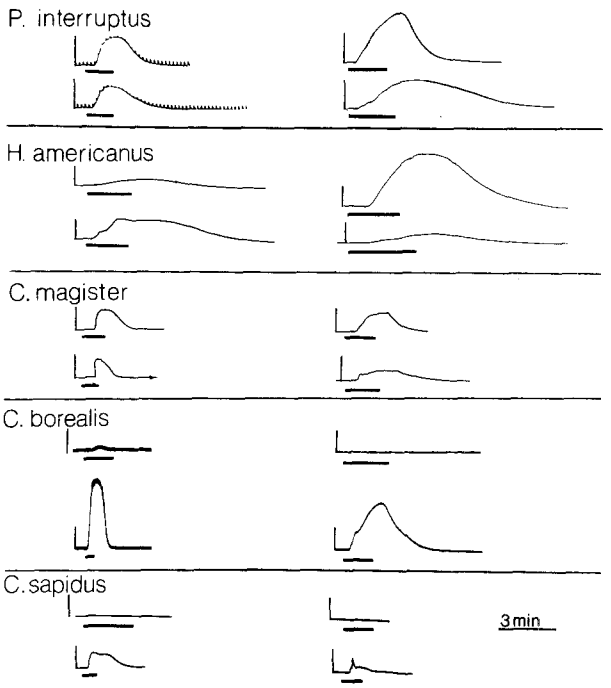

Fig. 2. Tension responses of gm4 (left column) and gm6 (right column) muscles of various decapod species to 5×10^{-5} M acetylcholine and 10^{-3} M glutamate. Top trace in each pair corresponds to acetylcholine application and bottom trace to glutamate application. Agonists were introduced into the perfusion system during the bar. 10^{-5} g/ml Tensilon was used to inhibit cholinesterase activity during acetylcholine application. Horizontal calibration: 3 min. Muscles and vertical calibration bars: *Panulirus:* gm4c-5.5 g, gm6b-5.5 g; *Homarus:* gm4b-top, 2.2 g, bottom, 1.1 g, gm6a-0.55 g; *C. magister:* gm4c-0.55 g, gm6-0.5 g; *C. borealis:* gm4b-0.55 g, gm6-0.55 g; *C. sapidus:* gm4b,c-1.1 g, gm6-0.55 g

1979). The glutamate agonist, quisqualic acid (Shinozaki and Shibuya, 1974), also elicited contracture in these muscles. Muscles in this class are innervated by the PY, LP, IC, or AM neurons.

Muscles of the third class were found to contract to both cholinergic and glutamatergic agents. Muscles which displayed this dual sensitivity have been shown to be innervated by either the LG, DG, or MG neurons. Examples of agonist-induced contractures in one of each of the three types of muscles is shown in Fig. 1A.

The effectiveness of acetylcholine and glutamate in eliciting contracture in muscles representative of each class was also examined (Fig. 1B). The dually sensitive gm6b muscle demonstrated dose-response sensitivities to acetylcholine similar to those of the acetylcholine-sensitive dorsal dilator muscle. Similarly, the dose-response curve to glutamate obtained from muscle gm6b was similar to the sensitivities to glutamate of the glutamate-sensitive pl muscle. Such dose-response curves were constructed in the absence of inhibitors of acetylcholinesterase. The use of 10^{-5} g/ml Tensilon to inhibit acetylcholinesterase increases sensitivity to acetylcholine as much as 10-fold.

Distribution of Glutamate and Acetylcholine Sensitivity in Foregut Muscles of Other Decapod Species

It was natural to wonder whether the phenomenon of dual sensitivity might be of general significance to other decapod species. In order to assess this question, *Homarus americanus, Callinectes sapidus, Cancer magister, Cancer borealis,* and *Cancer irroratus* were examined for ability of acetylcholine and glutamate to evoke contracture in various foregut muscles. The identification of muscle groups was based on the descriptions of Maynard and Dando (1974) for *Homarus* and *Callinectes. Cancer* was observed to be similar to *Callinectes.* Although the neurons innervating particular muscles have not been identified for all species in terms of their function within the pattern generators of the stomatogastric ganglion, it was assumed

Table 1. Sensitivity of decapod stomatogastric muscles to acetylcholine or glutamate

Muscle name	Origin	Motor neuron	CAT[a]	*P. inter.* ACh	*P. inter.* Glu	*H. ameri.* ACh	*H. ameri.* Glu	*C. mag.* ACh	*C. mag.* Glu	*C. bor.* ACh	*C. bor.* Glu	*C. sap.* ACh	*C. sap.* Glu
c.1	e			+	−								
c.2	e			+	−								
c.3	e			+	−								
c.4	e			+	−								
c.5	e			+	−								
c.7	sw	AM		−	+								
g.m.1a	e	GM	+	+	−			+	−	+	−	+	−
g.m.1b	e	GM	+	+	−	+	−	+	−	+	−	+	−
g.m.2a	e	GM	+	+	−								
g.m.2b	e	GM	+	+	−			+	−	+	−	+	−
g.m.3a,c	e	GM, LPG	+	+	−								
g.m.4a	i	DG		+	+	+	+						
g.m.4b	i	DG		+	+	+	+	+	+	(+)	+	−	+
g.m.4c	i	DG		+	+			+	+	(+)	+	−	+
g.m.5a	i	LG(?)		+	+			+	+				
g.m.5b	i	LG		+	+	+	+	+	+			−	+
g.m.6a	i	LG				+	+	+	+	−	+	−	+
g.m.6b	i	LG		+	+			+	+	−	+	−	+
g.m.8a	i	LG						+	+	(+)	+	−	+
g.m.9a	i	MG		+	+			+	+				
g.m.9b	i	MG				+	(+)						
c.v.1	e	VD	+	+	−	+	−	+	−				
c.v.2	i	IC	−	−	+	−	+	−	+				
cp.v.1a	e	PD	+	+	−	+	−	+	−	+	−		
cp.v.1b	e	PD	+	+	−	+	−	+	−	+	−		
cp.v.2a	e	PD	+					+	−				
cp.v.2b	e	PD	+	+	−								
cp.v.4b	i	LP	−	−	+								
cp.v.5	i	LP	−	−	+								
p.1	i	LP	−	−	+	−	+	−	+	−	+		
p.2	i	PY	−	−	+								
p.8	i	PY	−	−	+								

Muscle homologies and probable innervation based on Maynard and Dando (1974) and Govind et al. (1975)

e extrinsic, *i* intrinsic, *sw* stomach wall. Sensitivities of muscles were assessed from ability of application of 1 mM glutamate and 50 μM acetylcholine with 10^{-5} g/ml Tensilon to produce contracture. Responses in parentheses were particularly weak

[a] Presence of choline acetyltransferase (*CAT*) determined by Marder (1974) for identified neurons of *Panulirus interruptus*

that homologous muscles are innervated by similar neurons.

Figure 2 displays representative responses for the dually sensitive gm6 and gm4 muscles from several species. In both *Homarus* and *Cancer magister*, muscles which are homologous to those in *Panulirus* display similar sensitivities to acetylcholine, glutamate, or both. In *Callinectes, C. borealis,* and *C. irroratus,* muscles that are homologous to the dually sensitive muscles of the other three species are primarily sensitive only to glutamate, although in some cases small acetylcholine-evoked contractures may be observed. The lack of effect of acetylcholine on two such muscles, gm8b and gm9, in *Callinectes*, has been previously reported by Atwood et al. (1977). Table 1 summarizes these results.

Basis of Phenomenon of Dual Sensitivity

Several possible schemes can be constructed which might explain the basis of the dual sensitivity observed

in some stomatogastric muscles. First, the muscles in question could receive innervation from more than one excitatory motor neuron, each of which employs a different transmitter. Individual fibers might receive polyneuronal innervation or, alternatively, muscle fibers might be segregated into discrete populations that are homogeneous with respect to sensitivity to either acetylcholine or glutamate. Second, one agent might be acting to evoke the release of the natural transmitter from presynaptic terminals. Third, more than one transmitter might be released by the same neuron, in violation of Dale's principle. Finally, only one of the two substances might be representative of the normal excitatory transmitter to these muscles, while sensitivity to the other agent might be based on some diffuse extra-synaptic sensitivity of unknown function. The following experiments attempt to distinguish among these possibilities.

Nature of Innervation of Dually Sensitive Muscles

Maynard and Dando (1974), and subsequently, Govind et al. (1975) reported that intrinsic gastric mill muscles of *Panulirus argus* and *Callinectes sapidus*, including gm4, gm5, gm6, gm8, and gm9, receive single excitatory motor innervation. Their conclusion was based on three lines of evidence. First, methylene blue stained preparations revealed single excitatory motor axons to each of these muscles. No inhibitory axons were demonstrated either by stain or physiological techniques. Second, stimulation of nerves that contain axons to these muscles revealed only a unitary excitatory junctional potential as stimulus voltages to the nerves were varied. Finally, recordings of potentials in muscle fibers of perfused whole stomachs in which the stomatogastric ganglion maintained its normal motor output revealed only single junctional potentials. I repeated the above experiments on dually sensitive muscles of *Panulirus interruptus* and *Cancer magister* and verified the previous conclusions. In addition, the effect of varying the stimulus voltages applied to the innervating nerves on the amplitude of nerve-evoked contractions in dually sensitive muscles was routinely examined. In all cases only a single amplitude of evoked contraction was observed. Visual inspection indicated that all visible muscle fibers contributed to contractions evoked by stimulation of the nerve. Thus, a single excitatory axon appears to innervate all muscle fibers within a dually sensitive muscle. The dual sensitivity cannot be accounted for by polyneuronal innervation by neurons using different transmitters.

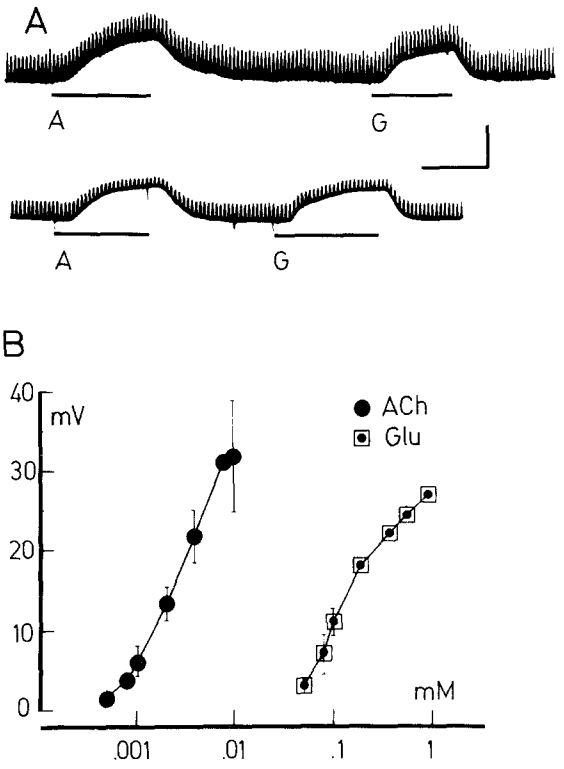

Fig. 3. A Intracellular records from muscle gm6b of *Panulirus* during application of acetylcholine or glutamate. Trains of EJPs at 6 Hz for 500 ms were elicited at repeated intervals. Top trace: A- 0.8 μM acetylcholine with 10^{-5} g/ml Tensilon; G- 50 μM glutamate. Vertical calibration: 5 mV. Bottom: A- 2 μM acetylcholine with Tensilon; G- 100 μM glutamate. Vertical calibration: 10 mV. Horizontal calibration 2 min. **B** Dose-response relationship of depolarizing effects of acetylcholine and glutamate gm6b muscle fibers. Acetylcholine was applied in the presence of Tensilon, a cholinesterase inhibitor. Data is taken from 5 fibers of 3 muscles. Error bars indicate standard deviation of 3–5 applications at a concentration

Effects of Acetylcholine and Glutamate on Membrane Potential and Conductance

The effects of acetylcholine and glutamate on membrane potential of individual muscle fibers were examined in various species. For most experiments muscle gm6b of *Panulirus* was studied as a representative of dually sensitive muscles. Muscles from other species were occasionally examined to substantiate the generality of the findings.

Both acetylcholine and glutamate were found to depolarize the same muscle fibers. Figure 3A demonstrates an experiment in which two different concentrations of both acetylcholine and glutamate were applied while recording from one fiber of muscle gm6b of *Panulirus*. The dose-response relationship of the depolarizing effects of the bath-applied agonists on several gm6b fibers are shown in Fig. 3B. Tensilon was used to inhibit acetylcholinesterase during acetylcholine application. Both acetylcholine and glutamate

were capable of depolarizing the muscle fibers more than 25 mV.

In *Cancer borealis* and *irroratus* in which acetylcholine is not so effective in producing contracture, high concentrations of cholinergic agonist (0.5 mM carbachol or large iontophoretic acetylcholine pulses) are required to produce depolarization, if at all. Although some receptors do appear to be present on muscles of these species, the number of these acetylcholine receptors may be insufficient to produce enough depolarization to activate contracture.

It was possible that the nature of the conductance changes activated by either acetylcholine or glutamate might exclude one of these substances from a role as excitatory transmitter to these muscles. Two approaches were used to assess this question, first, examination of effects of bath-applied glutamate or acetylcholine on membrane resistance, and second, estimation of reversal potentials of iontophoretic acetylcholine and glutamate responses.

Effects of glutamate and acetylcholine on the membrane resistance of dually sensitive muscles were examined. Complete current-voltage relationships were obtained before, during, and after the addition of the appropriate agonist to the saline bathing a muscle. Both agonists produced substantial depolarizations with only slight resistance changes, when resistances were compared at similar membrane potentials. For depolarizations of up to 10 mV, resistance decreases were generally less than 5%. In some cases small membrane resistance increases of a few percent were observed. Examples of the effects of glutamate and acetylcholine on muscle gm6b of *Panulirus* are shown in Fig. 4. Similar results were obtained from gm4 and gm5b of *Panulirus* and gm5, gm6, and gm8a of *Cancer magister*. In order to account for the magnitude of the depolarizations produced by glutamate and acetylcholine in light of the small resistance changes, a substantial portion of the conductance changes must involve an increase in conductance to an ion with an equilibrium potential far from the resting potential, most likely sodium or calcium. However, the results do not exclude the possibility that during bath application of these substances an additional conductance decrease to an ion with an equilibrium potential close to the resting potential may be activated. Although such a conductance decrease could not account for the depolarizations observed, they would mask the conductance increases primarily responsible for the depolarization, as observed for glutamate action on *Homarus* stretcher muscle preparations (Colton and Freeman, 1975).

In order to further characterize the nature of the conductance changes activated by acetylcholine and glutamate, an attempt to estimate reversal potentials

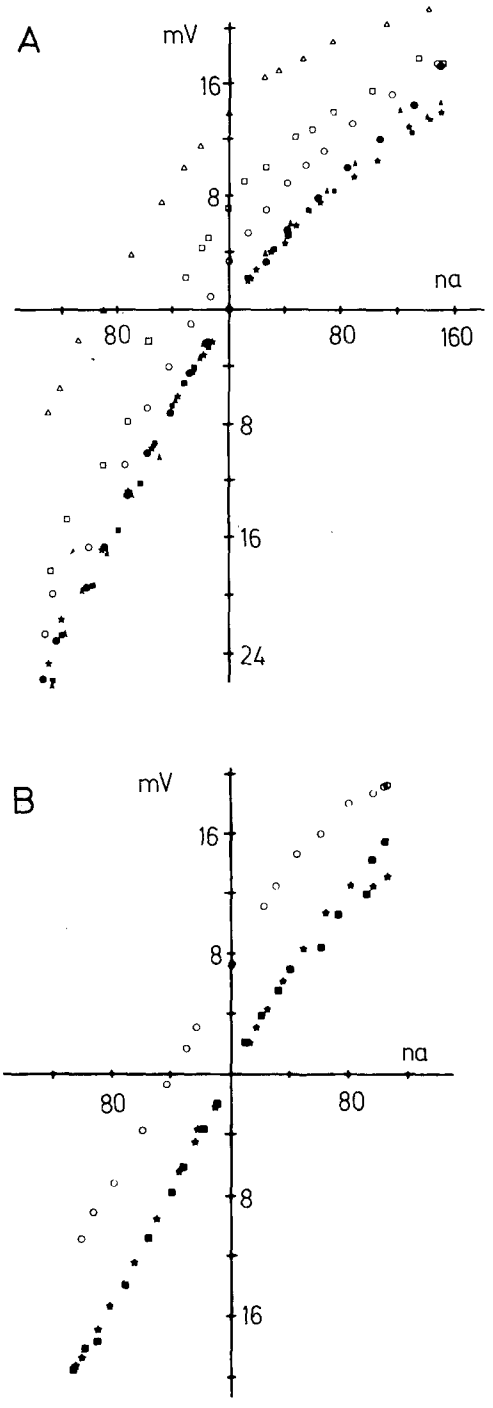

Fig. 4A and B. Effect of acetylcholine and glutamate on current-voltage characteristics of muscle gm6b of *Panulirus*. Points were generated by injection of current pulses before, during, or after application of an agonist. **A** Effects of glutamate. Solid symbols correspond to control and wash curves. Hollow circles: 50 μM glutamate; hollow squares: 80 μM glutamate; Hollow triangles: 150 μM glutamate. **B** Effects of acetylcholine. Solid symbols correspond to control and wash curves. Hollow circles: 8 μM acetylcholine with 10^{-5} g/ml Tensilon

of iontophoretically produced acetylcholine and glutamate potentials was made. Since prolonged depolarization of gm6b muscle fibers produces substantial contracture in normal saline, iontophoretic potentials could only be obtained over membrane potentials of −50 to −120 mV. By extrapolation the iontophoretic potentials to either acetylcholine or glutamate were estimated to reverse above −20 mV, being close to 0 mV in most cases. By inclusion of 20 mM manganese chloride in the saline, contracture was diminished sufficiently to hold membrane potentials up to −20 mV. No significant change in the shape of the membrane potential-iontophoretic potential relationship was observed in the manganese saline. Although iontophoretic potentials approached reversal near −20 mV, actual reversals to either glutamate or acetylcholine could not be obtained, again indicating that the reversal potentials for these conductances are probably around 0 mV. Since the membrane resistance of these fibers decreases substantially at depolarized membrane potentials, these extimated reversal potentials are probably low estimates. Although many errors are involved in this method of estimating a reversal potential, clearly the equilibrium potentials of the conductance changes activated by acetylcholine and glutamate are no less than −20 mV. Thus, the possibility that the depolarization and contracture produced by either acetylcholine or glutamate is the result of some process inconsistent with known mechanisms of excitatory synaptic transmission is unlikely.

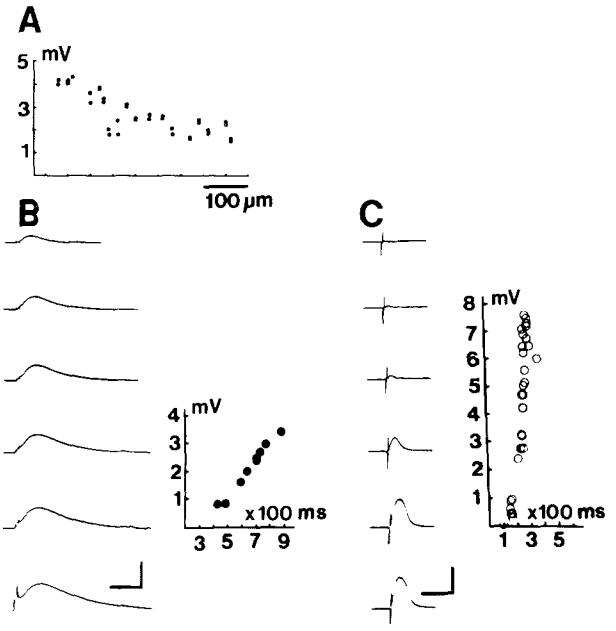

Fig. 5. **A** Iontophoretic map of acetylcholine receptor distribution on muscle gm6b of *Panulirus*. Responses to constant pulses of acetylcholine along the surface of a muscle fiber are plotted. Left end of horizontal axis corresponds to position of the acetylcholine iontophoretic electrode. **B** Effect of increases in size of acetylcholine iontophoretic current pulses on rise time of acetylcholine potential on muscle gm6b. Left: responses to acetylcholine application. Pulse duration was constant at 100 ms and amount of current was varied from top to bottom. Calibration: 2.5 mV; 1 s. Rise time was measured from beginning of iontophoretic pulse (no latency was detectable). Rise time and amplitude of potentials on left generated points in plot on right. **C** Effect of increases in size of glutamate iontophoretic current pulses on rise time of glutamate potentials. Left: intracellular responses to glutamate application. Pulse duration constant at 30 ms and the amount of current was increased from top to bottom. Rise times were measured as in **B** and used to generate the plot on right. Calibration: 5 mV; 1 s

Iontophoretic Studies of Receptor Distributions

Iontophoretic applications of acetylcholine and glutamate to the surface of the dually sensitive gm6b muscle fibers were performed to obtain direct information concerning the distributions of receptors mediating the depolarizing action of these agents.

No matter where an acetylcholine containing iontophoretic electrode was positioned along the surface of a gm6b fiber, ejection of acetylcholine from the micropipette resulted in small, slow rise time potentials (Fig. 5A).

The rise time of iontophoretic potentials generated by substances activating rapid conductance processes is a function of the time it takes for the substance to diffuse from the site of agonist application to the area of membrane at which the greatest number of receptors are activated (del Castillo and Katz, 1955; Dreyer and Peper, 1974). If receptors are localized at a point source some distance from the site of agonist application, once a dose of agonist is reached which completely covers those receptors, as the dose of agonist is further increased, no additional increase in rise time would be expected. However, if receptors are located all over the membrane or at many points on the membrane, as the dose of agonist is increased, receptors at increasingly distant sites will contribute to the iontophoretic potential. As a result the rise time of the response will continue to increase reflecting the increase in diffusion time to the more distant receptors. The rise time of acetylcholine receptors on gm6b was examined as a function of the ejected dose of acetylcholine. As the dose of acetylcholine applied to a single location is increased, so does the rise time of the recorded potentials (Fig. 5B). Additionally, there is no indication of any deviation from the linearity of the risetime-potential relationship. This is consistent with an even distribution of receptors on the muscle fibers or, alternatively, a very frequent occurrence of patches of receptors at intervals of probably less than 30 µm.

Methylene blue staining of nerves to dually sensit-

ive muscles indicates that regions of contact between nerves and muscles occur infrequently. Several hundred micron lengths of muscles are free from nerve contacts. In addition, Atwood et al. (1977) have reported that synaptic areas occur infrequently along the muscle fibers of gm8a in the blue crab, *Callinectes*. As a result, it is very unlikely that the occurrence of acetylcholine receptors at any position along the muscle fiber is indicative of the frequent occurrence of junctional sites. In addition, the absence of significant latencies between the beginning of acetylcholine pulses and the resulting potentials indicates that little diffusion is occurring prior to the activation of the acetylcholine receptors. Thus, the extrajunctional regions of the muscle fibers of dually sensitive muscles contain acetylcholine receptors. Whether acetylcholine receptors may also be involved in synaptic transmission to these muscles is addressed below.

When a search was made on a dually sensitive muscle for sites yielding potentials in response to iontophoresis of L-glutamate, a much different result was obtained. Responsive locations were limited to very circumscribed spots, often difficult to locate. This is similar to the distribution of glutamate-sensitive sites on crustacean muscles thought to receive glutamatergic innervation (Takeuchi and Takeuchi, 1964).

The rise times of glutamate potentials were also examined. Figure 5C shows that as the dose and amplitude of glutamate-evoked potentials in a gm6b muscle increase, the rise time of that potential does not substantially vary once a dose is obtained that presumably covers the patch of receptors with agonist. This lack of variation in rise time is particularly striking when compared with the variation in acetylcholine potential rise times and is consistent with the localization of glutamate receptors to specific foci.

Blockade of Presynaptic Transmitter Release

It seemed possible that the effects of either glutamate or acetylcholine might be mediated indirectly by activation of the release of some other excitatory substance from nerve terminals presynaptic to the muscle fibers. To assess this possibility the effect of reducing the calcium content in the physiological saline on both acetylcholine and glutamate potentials was tested (Fig. 6). During a time in which neurally evoked junctional potentials are largely abolished in the absence of calcium, acetylcholine potentials are only slightly diminished (Fig. 6A). In contrast, glutamate iontophoretic potentials are markedly reduced by the removal of calcium (Fig. 6B). This result indicates that acetylcholine-activated depolarization is mediated by a direct effect on the muscle fibers. The reduction in sensitivity to glutamate which has also been observed for crayfish abdominal and claw muscles (Thieffry and Bruner, 1978a, b) could be the result of either a pre- or post-synaptic requirement for calcium. That glutamate is directly acting on the post-synaptic muscle fiber membrane is supported by experiments in which during the abolition of synaptic potentials by addition of manganese or cobalt to normal saline, glutamate potentials are not blocked (Fig. 6C and Lingle, 1979, and in preparation). Thus, both acetylcholine and glutamate receptors are located on the muscle fiber membrane of dually sensitive muscles.

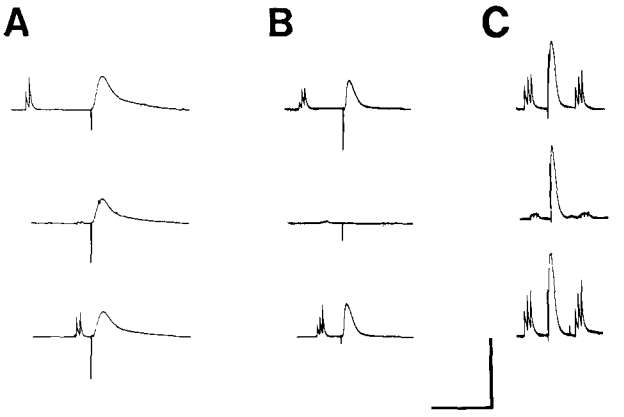

Fig. 6A–C. Effect of blockade of synaptic transmission on acetylcholine and glutamate potentials of muscle gm6b. **A** Top: control EJPs and acetylcholine response; middle: 11 min of 0 calcium, equimolar magnesium saline; bottom: wash. **B** Top: control EJPs and glutamate potential; middle: 8 min of 0 calcium, equimolar magnesium saline; bottom: wash. **C** Top: control EJPs and glutamate potential; middle: 12 min in 20 mM manganese added to normal saline; bottom: wash. Calibration: 6 s; 4 mV in **A** and **B**; 5 mV in **C**

Pharmacological Tests of the Identity of the Transmitter to the Dually Sensitive Muscles

Marder and Paupardin-Tritsch (1980) have provided a pharmacological analysis of cholinergic responses on muscle gm1 in the crab, *Cancer*. They found several known ganglionic nicotinic receptor antagonists and other agents to be effective in antagonizing acetylcholine-evoked responses. If acetylcholine receptors on the dually sensitive muscle are pharmacologically similar to those previously characterized on muscles receiving cholinergic innervation, nicotinic antagonists should be effective in discerning whether acetylcholine is released synaptically onto dually sensitive

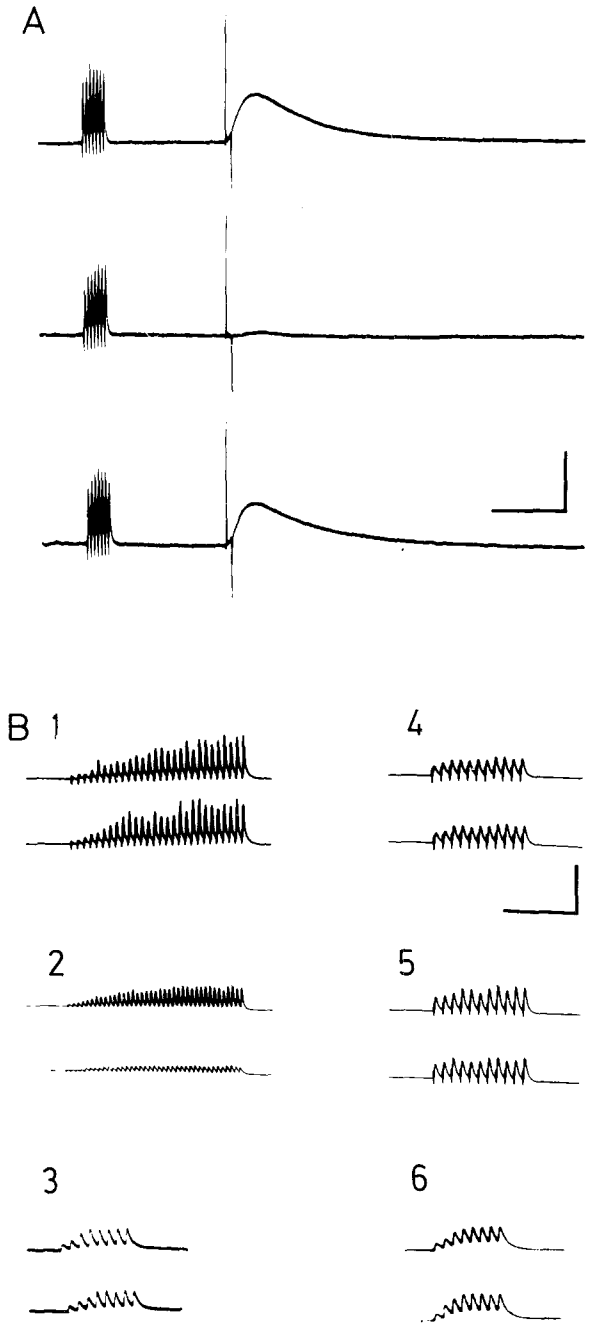

Fig. 7 A and B. Effects of cholinergic antagonists on EJPs and iontophoretic acetylcholine responses on muscle gm6 of *Cancer* and *Panulirus*. **A** Top: control EJPs and intophoretic acetylcholine response; middle: 6 min of 0.5 μM dihydro-β-erythroidine; bottom: wash. Calibration: 4 s; 4 mV. **B1** Top: control; bottom: 0.5 mM trimethapan. **B2** Top: control; bottom: 0.5 mM chlorisondamine. **B3** Top: control; bottom: 100 μM curare. **B4** Top: control; bottom: 100 μM pempidine. **B5** Top: control; bottom: 10 μM dihydro-β-erythroidine. **B6** Top: control; bottom: 1 mM atropine. **B1**, **B2**, **B5**: gm6 of *Cancer*. **B3** and **B6**: gm6b of *Panulirus*. Calibration: 1 s; **B1**: 4 mV; **B2**, **B4**, **B5**: 8 mV; **B3** and **B6**: 10 mV

muscles. The effects of some of these agents on transmission at neuromuscular junctions of dually sensitive muscles were examined. Figure 7A shows an example in which one of these agents, dihydro-β-erythroidine, does not alter transmission to a gm6b muscle, although sensitivity to acetylcholine is practically abolished. Figure 7B shows the effect of several other agents on EJPs to either a gm6b muscle of *Panulirus* or a gm6 muscle of *Cancer*. Table 2 summarizes these experiments in which different agents were tested for ability to block either acetylcholine potentials, glutamate potentials or EJPs on dually sensitive muscles of the various species. In most cases the concentrations employed were at least an order of magnitude above that found to be effective in completely blocking any acetylcholine responses in the muscle fiber. The table also lists the concentrations of antagonist producing half maximal block of cholinergic responses on gm1 of *Cancer* taken from Marder and Paupardin-Tritsch (1980). Since the complete block of all acetylcholine sensitivity in a muscle does not at all alter synaptically activated contractions in a dually sensitive muscle, it is not possible that another pharmacologically different acetylcholine receptor is activated at synaptic sites. There was no indication of any cholinergic innervation in any dually sensitive muscle examined.

It might be argued that the antagonists tested did not have access to the junctional cleft, although cholinergic responses elsewhere on the muscle fiber were blocked. This possibility is unlikely since chlorisondamine, a presumed sympathetic ganglion nicotinic receptor blocker (Volle and Koelle, 1975) was effective in blocking transmission to dually sensitive muscles. Chlorisondamine was similarly effective in blocking iontophoretic glutamate potentials in these muscles (Lingle, Eisen and Marder, submitted).

This result suggests that the failure of the other cholinergic blockers to block transmission of glutamate potentials is not due to limited access to junctional sites.

These pharmacological studies eliminate the possibility that acetylcholine is the transmitter released by nerves terminating on the dually sensitive muscles. In contrast, the notable absence of substances effective in antagonizing synaptic potentials or glutamate potentials at presumed glutamatergic synapses in Crustacea does not permit a pharmacological test of the glutamatergic nature of transmission to dually sensitive muscles. However, the reduction of both EJPs and glutamate potentials on dually sensitive muscles by chlorisondamine is consistent with the idea that synaptic transmission to these muscles is mediated by the same receptor activated by glutamate.

Table 2. Effect of pharmacological agents on acetylcholine potentials, glutamate potentials, or EJPs in dually sensitive muscles

Substance	ED[a]$_{50}$ ACh response gm 1 *Cancer*	Maximum concentration tested on dual-sensitive muscles	% block of ACh response on dual-sensitive muscles at max. conc.	% block of glutamate response on dual-sensitive muscles at max. conc.	% block gm6b *P. inter.*	% block gm6 EJPs *H. ameri.*	% block gm6 EJPS *C. bor.*
Dihydro-β-erythroidine	30 nM	10 μM	100	0	0	0	0
Pempidine	200 nM	500 μM		0	0	0	0
Mecamylamine	200 nM	50 μM		0	0		
Trimethapan	2 μM	500 μM		0	0	0	0
Chlorisondamine	5 μM	500 μM	100	50 (100 μM)[b]	60 (100 μM)[b]		60 (100 μM)[b]
Curare	10 μM	100 μM	100	0	0		
Decamethonium	15 μM	100 μM		0	0		0
Atropine	25 μM	1 mM	100	0	0		
Picrotoxin	40 μM	1 mM		0			
Hexamethonium	50 μM	500 μM			0		
Strychnine		50 μM		0	0		
Glutamate-γ methylester		2 mM		0	0		
L-aspartate		2 mM		0	0		
D-glutamate		1 mM		0	0		

[a] Data taken from Marder and Paupardin-Tritsch (1980)
[b] Taken from Lingle, Eisen and Marder (submitted)

Discussion

This study has shown that several muscles within the stomatogastric system of several decapod species display receptors to both acetylcholine and glutamate. On the basis of the distribution and pharmacological characteristics of the receptors, the single excitatory motor innervation to these dually sensitive muscles is most likely to be glutamatergic. The acetylcholine receptors are diffusely distributed on the muscle fibers. That the acetylcholine receptors can in fact be considered extrajunctional is supported by the apparent absence of junctional regions over large areas of such muscle fibers. In contrast, no sites along a dually sensitive muscle fiber are insensitive to acetylcholine. However, in some decapod species the occurrence of these acetylcholine receptors is reduced or absent on the homologous muscles.

These findings resolve apparent discrepancies among previous investigations (Marder, 1976; Atwood et al., 1977; King, 1976) and indicate that the most probable transmitter of the LG, MG, and DG neurons of the stomatogastric ganglion is glutamate. Two issues raised by these results are addressed below. First, the significance of the use of both cholinergic and glutamatergic excitatory motor innervation within this system is considered in the context of the current information concerning stomatogastric ganglion motor transmitters. Second, the significance of the extrajunctional receptors on some muscles is discussed.

Cholinergic and Glutamatergic Excitatory Motor Transmission in the Decapod Foregut

Until recently, crustacean neuromuscular junctions were thought to use glutamate exclusively as excitatory transmitter, while acetylcholine was considered the transmitter used by crustacean sensory nerves (Barker et al., 1972). Clearly both cholinergic and glutamatergic types of excitatory motor transmission are present in the decapod foregut. For *Panulirus*, the putative cholinergic neurons are the 2 PDs, the VD, and 4 GM, and the LPG (Marder, 1976) and the 2 CDs. All of these neurons innervate extrinsic muscles. The putative glutamatergic neurons are the LP, the 8 PYs, the IC, and AM, and the LG, MG, and DG. The LG, MG, and DG innervate muscles that display extrajunctional acetylcholine receptors. Figure 8 schematically illustrates the distribution of muscles of the three types of sensitivity. No muscle receives both cholinergic and glutamatergic excitatory innervation.

The use of the two excitatory motor transmitters raises the question: are there any indications within this system as to why a cell uses a particular transmitter? Marder (1976) partially addressed this question

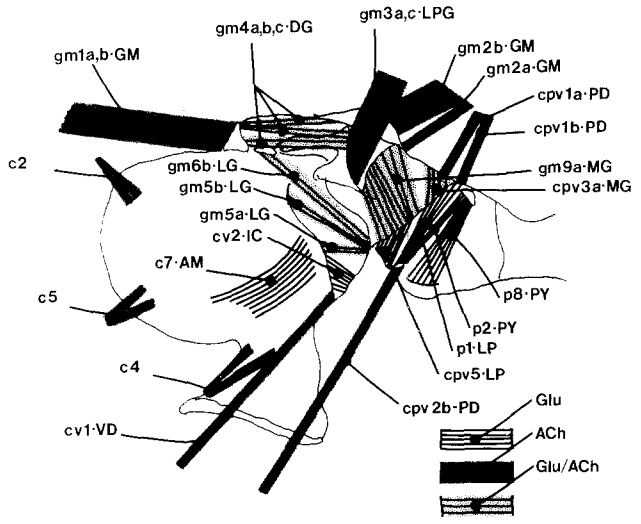

Fig. 8. Representation of lateral view of musculature of *Panulirus interruptus* foregut illustrating distribution of muscles of each of three classes of sensitivity. Nomenclature corresponds to muscle name followed by name of innervating neuron(s)

following the initial demonstration of the cholinergic nature of some of the foregut motor neurons. From a consideration of the lack of variation in facilitation and contraction properties of extrinsic and intrinsic muscles, there is no difference in synaptic or mechanical function that might account for the use of the two transmitters at the neuromuscular junctions.

The clear morphological segregation of muscles receiving cholinergic and glutamatergic innervation (Fig. 8) suggests that the basis for the particular transmitter used at a muscle may be explained from developmental or evolutionary processes. The primitive foregut may have been composed of an intrinsic musculature supported by non-muscular ligaments as is the case for the crustacean hindgut and heart. Neurons that may have primarily subserved a pattern-generating function within a foregut of entirely intrinsic muscles might have subsequently acquired motor functions coincident with the appearance of an extrinsic musculature. The prediction would be that cells employ transmitters according to a functional hierarchy resulting from the temporal appearance of transmitter systems during the evolution of the organism. Exceptions might arise when neurons are involved in dual functions or the original functions become modified.

Examination of known chemical and electrical connectivity among neurons within the stomatogastric ganglion (Selverston et al., 1976) fails to reveal any clear functional hierarchy between cholinergic and glutamatergic neurons and, in fact, within the ganglion both transmitter types make synapses on both types of neurons. Further information concerning connectivity among stomatogastric neurons may provide insight to this problem.

Extrajunctional Receptors on Foregut Muscles

The occurrence of extrajunctional receptors is not a unique phenomenon. They have been identified on many invertebrate neuronal somata (Asher and Kehoe, 1975; Sargent et al., 1977; Marder and Paupardin-Tritsch, 1978), insect (Cull-Candy, 1976), molluscan (Taraskevitch et al., 1977), and vertebrate (Fambrough, 1979) muscle, and vertebrate axons (Sabelli and May, 1975; Brown and Marsh, 1978). As yet the significance of such receptors is not known. In general, these receptors are thought to possibly correspond to chemical inputs localized at other sites on the cell. Thus, it is surprising that some stomatogastric muscles may display receptors to a substance not evidently released onto those cells, at least from the known motor neuronal inputs. Several possible interpretations of the presence of these receptors follow.

First, these receptors may be indicative of the input of acetylcholine from non-motor neuron sources onto certain regions of the muscle fibers. No peripheral nerve plexus can be seen. However, acetylcholine released from sensory neurons might activate these receptors. Dendritic regions of sensory cells in the posterior stomach nerve contain choline acetyltransferase, take up choline by a high affinity transport system, and synthesize and store acetylcholine (Auerbach, 1978). The intrinsic and extrinsic gastric mill musculature which includes the dually sensitive muscles receives an arborization of sensory fibers from this nerve (Dando and Maynard, 1974). Sufficient acetylcholine could be released or leaked out to produce effects on the dually sensitive muscles. Two arguments can be raised against this idea. First, the sensory fibers do not ramify extensively over these muscles, but terminate near ossicles. Second, other muscles of the foregut with similar sensory fiber ramifications are not sensitive to acetylcholine. It seems likely that these muscle fibers are displaying receptors to a substance not released onto those fibers.

Second, these receptors may be indicative of the use of a different transmitter by the innervating neuron at its central synapses. This possibility is also unlikely. In a study of the synapses of identified neurons within the stomatogastric ganglion of *Panulirus*, King (1976) showed that the DG neuron contains synaptic vesicles similar to those of the putative glutamatergic neurons, the LP and PY, and distinct from those found in the cholinergic PD terminals. In addition the LG, MG, and DG make IPSPs on various cells of the ganglion. These IPSPs are similar in time course to IPSPs of the putative glutamatergic LP and PY and can be distinguished from slow IPSPs of the cholinergic PD and VD neurons by the picrotoxin-

sensitivity of the former (Maynard, 1972). Within the stomatogastric ganglion, glutamate-activated K^+ and Cl^- inhibitory conductance increases are blocked by 1 to 10 μM picrotoxin (Marder and Paupardin-Tritsch, 1978). This argues that the IPSPs activated by the LG, MG, and DG within the ganglion involve a glutamatergic transmission as is observed for the peripheral neuromuscular connections of these neurons.

Third, the neurons innervating the dually sensitive muscles might be electrically coupled to other neurons which do use acetylcholine as transmitter. Through such coupling may pass sufficient acetylcholine or some unknown substance to induce the expression of cholinergic receptors on post-synaptic cells, even though acetylcholine does not contribute significantly to the transmitter released by those cells. Further study of electrical connections within the stomatogastric ganglion is required to address this possibility. Clearly, the LG, MG, and DG only innervate muscles that display both glutamate and acetylcholine receptors, while other neurons of the ganglion innervate muscles with either only glutamate or only acetylcholine receptors. This indicates that there may be some specificity in the types of receptors a cell innervated by a given neuron may express. However, such expression may be either the consequence of innervation by a particular neuron or serve to place constraints on the identity of the innervating neuron.

Fourth, the extrajunctional receptors may be molecules involved in some cellular function unrelated to cholinergic function.

Finally, the receptors may be without physiological significance simply representing biochemical and genetic noise inherent to biological systems. Alternatively, these receptors may be a vestigial expression of the innervation of these muscles by cholinergic neurons at earlier developmental stages.

Most of this work was done at the University of Oregon in partial fulfillment of the PhD requirements of the Department of Biology, and was supported by NIH grants GM 00336, MH14281, NS-10614 (to D.L. Barker), USPHS Biomedical Science Grant 5 S07 RR-7080-11 to the University of Oregon and a Sigma Xi research grant-in-aid. Some experiments were completed during a Muscular Dystrophy Association Postdoctoral Fellowship at Brandeis University with the support of NSF grant BNS 78-15399 and the McKnight Foundation (to E. Marder). I would like to thank David Barker and Eve Marder for their interest and encouragement. I would also like to thank Eve Marder and Judith Eisen for participation in some of the experiments shown in Fig. 7.

References

Ascher, P., Kehoe, J.S.: Amine and amino acid receptors in gastropod neurons. In: Handbook of psychopharmacology, Vol. 4. Iversen, L.L., Iversen, S., Synder, S.H. (eds.), pp. 263–310. New York: Plenum Press 1975

Atwood, H.L., Govind, C.K., Jahromi, S.S.: Excitatory synapses of blue crab gastric mill muscles. Cell Tissue Res. **177**, 145–158 (1977)

Auerbach, A.L.: Acetylcholine metabolism in a crustacean sensory nerve. Ph.D. thesis, University of Oregon, Eugene, Oregon (1978)

Barker, D.L., Herbert, E.A., Hildebrand, J.G., Kravitz, E.A.: Acetylcholine and lobster sensory neurons. J. Physiol. **225**, 205–229 (1972)

Brown, D.A., Marsh, S.: Axonal GABA-receptors in mammalian peripheral nerve trunks. Brain Res. **156**, 187–191 (1978)

Colton, C.K., Freeman, A.R.: Dual response of lobster muscle fibers to L-glutamate. Comp. Biochem. Physiol. **51**C, 275–284 (1975)

Cull-Candy, S.G.: Two types of extrajunctional L-glutamate receptors in locust muscle fibers. J. Physiol. **255**, 449–464 (1976)

Dando, M.R., Maynard, D.M.: The sensory innervation of the foregut of *Panulirus argus* (Decapoda Crustacea). Mar. Behav. Physiol. **2**, 283–305 (1974)

Del Castillo, J., Katz, B.: On the localization of acetylcholine receptors. J. Physiol. **128**, 157–181 (1955)

Dreyer, F., Peper, K.: The acetylcholine sensitivity in the vicinity of the neuromuscular junction of the frog. Pflügers Arch. **348**, 273–286 (1974)

Fambrough, P.M.: Control of acetylcholine receptors in skeletal muscle. Physiol. Rev. **59**, 165–227 (1979)

Govind, C.K., Atwood, H.L., Maynard, D.M.: Innervation and neuromuscular physiology of intrinsic foregut muscle of the blue crab and spiny lobster. J. Comp. Physiol. **96**, 185–204 (1975)

King, D.G.: Organization of crustacean neuropil. I. Patterns of synaptic connections in lobster stomatograstric ganglion. J. Neurocytol. **5**, 207–237 (1976)

Lea, T.J., Usherwood, P.N.R.: The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle fibers. Comp. Gen. Pharmacol. **4**, 333–350 (1973)

Lingle, C.J.: Responses of spiny lobster stomatogastric musculature to glutamate and acetylcholine. Neurosci. Abstr. **2**(1), 349 (1976)

Lingle, C.J.: The effects of acetylcholine, glutamate, and biogenic amines on muscles and neuromuscular transmission in the stomatogastric system of the spiny lobster, *Panulirus interruptus*. Ph.D. Thesis, University of Oregon, Eugene (1979)

Marder, E.: Acetylcholine as an excitatory neuromuscular transmitter in the stomatogastric system of the lobster. Nature **251**, 730–731 (1974)

Marder, E.: Cholinergic motor neurons in the stomatogastric system of the lobster. J. Physiol. **257**, 63–86 (1976)

Marder, E., Paupardin-Tritsch, D.: The pharmacological properties of some crustacean neuronal acetylcholine, gamma-aminobutyric acid, and L-glutamate responses. J. Physiol. **280**, 213–236 (1978)

Marder, E., Paupardin-Tritsch, D.: The pharmalogical profile of the acetylcholine response of a crustacean muscle. J. Exp. Biol. (in press) (1980)

Maynard, D.M.: Simpler networks. Ann. N.Y. Acad. Sci. **193**, 59–72 (1972)

Maynard, D.M., Dando, M.R.: The structure of the stomatogastric neuromuscular system in *Callinectes sapidus, Homarus americanus*, and *Panulirus argus* (Decapoda Crustacea). Philos. Trans. R. Soc. (London) Biol. **268**, 161–220 (1974)

Otsuka, M., Kravitz, E.A., Potter, D.D.: Physiological and chemical architecture of a lobster ganglion with particular reference

to gamma-aminobutyrate and glutamate. J. Neurophysiol. **30**, 725–752 (1967)

Pantin, C.F.A.: Notes on microscopical techniques for zoologists. Cambridge: Cambridge University Presse 1961

Sabelli, H.C., May, J.: Chemical excitability of axons: excitatory and inhibitory effects of putative neurotransmitters and modulators on frog sciatic nerves. Experientia **31**, 1049–1051 (1975)

Sargent, P.B., Yau, K.-W., Nicholls, J.G.: Extrasynaptic receptors on cell bodies of neurons in central nervous system of the leech. J. Neurophysiol. **40**, 446–452 (1977)

Selverston, A.I., Russell, D.F., Miller, J.P., King, D.G.: The stomatogastric nervous system: structure and function of a small neural network. Prog. Neurobiol. **7**, 215–290 (1976)

Shinozaki, H., Shibuya, I.: A new excitant quisqualic acid: effects on crayfish neuromuscular junction. Neuropharmacology **13**, 665–672 (1974)

Takeuchi, A., Takeuchi, N.: The effect on crayfish muscle of iontophoretically applied glutamate. J. Physiol. **170**, 296–317 (1964)

Taraskevitch, P.S., Gibbs, D., Schmued, L., Orkand, R.K.: Excitatory effects of cholinergic, adrenergic, and glutaminergic agonists on a buccal muscle of *Aplysia*. J. Neurobiol. **8**, 325–336 (1977)

Thieffrey, M., Bruner, J.: Sensibilité des fibres musculaires d'écrevisse au glutamate et au médiateur naturel dans des solutions pauvres en calcium. C. R. Acad. Sci. (Paris) **286**, 1813–1816 (1978a)

Thieffrey, M., Bruner, J.: Direct evidence for a presynaptic action of glutamate at a crayfish neuromuscular junction. Brain Res. **156**, 402–406 (1978b)

Volle, L., Koelle, G.B.: Ganglionic stimulating and blocking agents. In: The Pharmacological basis of therapeutics. Goodman, L.S., Gilman, A. (eds.), pp. 565–574 New York: McMillan 1975