Serotonergic and Peptidergic Modulation of the Buccal Mass Protractor Muscle (I2) in Aplysia

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INTRODUCTION

Rhythmic movements driven by neural networks play a major role in the life of both vertebrates and invertebrates. These behaviors may appear to be simple and fixed, e.g., to be driven by a “loop program” that elicits the same phasic movements again and again. Nevertheless, the relationship between the magnitude and the timing of each act can be quite flexible. In part this flexibility results from complex properties of central pattern generating circuits, which have been studied in a number of preparations. Additionally, however, plasticity can be mediated peripherally, i.e., in the neuromuscular systems that execute behavior. Central and peripheral plasticity have been characterized in model systems, e.g., within the context of feeding in Aplysia.

Ingestive feeding behavior in Aplysia consists of stereotypic repetitive biting or swallowing movements (Kupfermann 1974). The rate and specific features of swallowing movements can vary to compensate for changes in the load and width of the ingested food (Hurwitz and Susswein 1992). Both biting and swallowing are also altered as a function of the motivational state of the animal (Weiss et al. 1981). For instance, as animals become food aroused there are progressive increases in the size and speed of biting responses (Kupfermann 1974; Susswein et al. 1978). Food-induced arousal, in part, results from the release of serotonin from the metacerebral cells (MCCs) (Eisenstadt et al. 1973; Gerschenfeld and Paupardin-Tritsch 1974; Gerschenfeld et al. 1978; Rosen et al. 1983; Weinreich et al. 1973; Weiss et al. 1975, 1979, 1986). These neurons make extensive central and peripheral connections and exert modulatory actions. As arousal is developed, there are changes in the movements that constitute a bite. For example, when animals bite they initially weakly protract the radula, a chitinous structure that grasps food. As animals become aroused, the extent of radula protraction increases. These effects are partially mediated by MCC activity (Rosen et al. 1983).

Previous experiments have demonstrated that I2 (intrinsic muscle 2) is the major muscle that mediates radula protraction (Hurwitz et al. 1996). On the basis of the widespread serotonergic innervation of the buccal mass, we hypothesized that serotonin would be present in the I2 neuromuscular system, and that it would modulate parameters of motor neuron elicited muscle contractions that are likely to be altered when food-induced arousal is developed.

Studies of other buccal muscles have indicated that in addition to serotonin released by the MCCs, Aplysia neuromuscular systems can also be modulated by peptides present in the feeding motor neurons themselves (Breznina et al. 1995; Church et al. 1993; Cropper et al. 1987a,b, 1988, 1990b, 1994; Fox and Lloyd 1997; Lloyd et al. 1984; Whim and Lloyd 1990). Thus another major goal of this study was to determine whether there are intrinsic (Cropper et al. 1987a; Katz 1995) modulators present in I2 motor neurons.

The I2 muscle (Howells 1942) was of particular interest because it mediates the protraction phase of feeding (Hurwitz...
et al. 1996) while previously characterized muscles control other movements, i.e., the accessory radula closer (ARC) controls radula closing, the I7–10 controls radula opening, and the I3a control jaw closure (Church et al. 1993; Cohen et al. 1978; Cropper et al. 1987a,b, 1988, 1990a,b, 1994; Evans et al. 1996, 1999; Fox and Lloyd 1997; Lloyd et al. 1984). The fact that the I2 muscle is a radula protractor is important because measurements of bite magnitude during food-induced arousal and satiation have been based on the extent of radula protraction only, since during retraction the radula recedes into the buccal cavity and cannot be visualized. Therefore characterization of the neuromuscular system that controls protraction is central to the attempt to establish a connection between overt behavior and physiological studies performed in vitro. Although there are extensive data regarding modulation of the ARC muscle, recent work indicates that individual buccal muscles exhibit significantly different contractile properties (Evans et al. 1996). Thus one cannot assume that different muscles are modulated in a similar fashion. To determine whether the muscles that generate protraction are also subject to modulation that enhances this phase of behavior, we undertook an investigation of the I2 muscle.

Methods

The experimental subjects for this study were *Aplysia californica* weighing 150–400 g. They were obtained from Marinus (Long Beach, CA) and from the National Resource for *Aplysia* at the University of Miami. They were maintained at 14–16°C in holding tanks containing aerated, filtered seawater. Animals were initially immobilized by injection of isotonic MgCl₂ (50% of body weight) and dissected. In neuromuscular preparations the buccal ganglion and I2 muscle were removed with the I2 nerve intact and attached to the I2 muscle. The buccal ganglion was pinned with the caudal surface up. Muscle. The buccal ganglion was pinned with the caudal surface up.

Recording apparatus and bathing solutions

Intracellular recordings were obtained from isolated ganglia or from neuromuscular preparations maintained at room temperature (18–22°C). Neuromuscular preparations, which consisted of the buccal ganglion, I2 nerve, and I2 muscle, were transferred to silicone elastomer (Silgard)-lined plastic culture dishes (Fig. 1A). The I2 muscle was pinned along the edge that was originally attached to the I1/I3 muscle (the edge of the I2 muscle that was originally attached to the esophagus was left free). A plexiglass subchamber was gently placed around the I2 muscle, on top of the I2 nerve, and was sealed with petroleum jelly (Vaseline). A force transducer (Isotonic Transducer “60-3000,” Harvard Apparatus) was used to monitor muscle contractions and was attached to the free end of the I2 muscle. In most experiments, continuous stretching of the muscle was prevented since during the free end of the transducer arm was supported (Evans et al. 1996). In general, both the outside compartment and the muscle subchamber contained artificial seawater (ASW; in mM: 460 NaCl, 10 KCl, 11 CaCl₂, 55 MgCl₂, and 5 NaHCO₃). In some experiments, however, a solution containing high divalent cations (HiDi) was used for blocking polysynaptic activity in the buccal ganglion (in mM: 311 NaCl, 9 KCl, 33 CaCl₂, 132 MgCl₂, and 5 NaHCO₃). This solution blocks polysynaptic activity by raising the threshold for triggering action potentials.

I2 muscle contractions can be elicited when any of its four motor neurons (B61, B62, B31, and B32) are stimulated (Hurwitz et al. 1994). Neurons B31 and B32 cannot be distinguished morphologically. Moreover, because they are strongly electrically coupled, they are difficult to manipulate individually. Consequently, it is difficult to use these motor neurons to elicit a series of controlled muscle contractions with similar parametric features. When B61 is stimulated alone, the “bands” of the I2 muscle contract first, i.e., a response to neuronal stimulation is initially observed along the edge of the I2 that is adjacent to the esophagus (Fig. 1B1). In contrast, when B62 is stimulated, the part of the I2 muscle that is adjacent to the I1/I3 muscles contracts first (Fig. 1B2). Because of the anatomical locus of contractions produced by B62 stimulation, these contractions were more difficult to measure than contractions elicited by B61. When the duration of firing of either B61 or B62 is extended, both parts of the I2 muscle contract (Fig. 1B3; n = 37) perhaps because B61 and B62 are both activated through their electrical coupling. Therefore in most quantitative experiments we triggered I2 contractions by stimulating B61 (at ~18 Hz) in relatively short-duration bursts (~1 s). Results obtained with B61 stimulation were qualitatively confirmed with B62 and B31/B32.

Electrophysiology

For intracellular recording and stimulation, I2 motor neurons were impaled with double-barreled microelectrodes that were made of thin-walled glass and contained 1.9 M potassium acetate and 0.1 M potassium chloride. Electrodes were beveled so that their impedance ranged from 6 to 10 MΩ. In double labeling experiments the potassium acetate in the stimulating electrode was replaced by a 3% solution of biocytin (Sigma) in 1 M potassium acetate. Biocytin electrodes were beveled so that the impedance of the barrel containing the dye was ~10 MΩ and the impedance of the potassium acetate barrel was ~6 MΩ.

Morphology

Immunohistochemistry was performed on whole-mount preparations as previously described (Miller et al. 1991; Vilim et al. 1996a,b; Xin et al. 1999). Preparations were fixed in 4% paraformaldehyde (4°C for 24 h). They were then washed for 2 days in Triton X-100 (diluted 1 to 100). Goat-serum was added at a dilution of 1:200, and 1 h later the first antibody was added at a dilution of 1:250. Primary antisera were as follows: myomodulin (MM) (Miller et al. 1991), buccalin (Miller et al. 1992), RFamide (Cropper et al. 1994), small cardioactive peptide (SCP; kind gift from Dr. R. Scheller, Stanford University), and serotonin (5-HT; kind gift from Dr. Hadasah Tamir, Columbia University). Preparations were incubated for 24 h at 4°C and then washed for 24 h. Secondary antibodies (fluorescently labeled with CY-3) were diluted 1 to 500 and were added for 24 h. Ganglia were then washed for 48 h. In double-labeling experiments two secondary antibodies were used; for MM immunocytochemistry we used an antiserum fluorescently labeled with CY-3, and for RFamide or 5-HT immunocytochemistry we used an antiserum fluorescently labeled with fluorescein. Absorption controls for MM and RFamide were performed by preincubating antibodies with MMa and RFamide A at 10⁻⁵ M. Preincubation controls were not performed for 5-HT.

When specific neurons needed to be identified for immunocytochemistry, the cells were first physiologically identified and were then iontophoretically filled with biocytin (500-ms, 0.5-nA current pulses with alternating polarity for ~10 min), and preparations were kept for 24 h at 14°C. Following the fixation and permeabilization described above, cells were fluorescently labeled by incubating tissue with a streptavidin Bodipy FL conjugate (50 μg/ml; Molecular Probes) in RIA buffer. Ganglia were then processed for immunocytochemistry as described above and were cleared in 50% glycerol in RIA buffer. Preparations were viewed with a Nikon fluorescence microscope and photographed with Tri-X (ASA 400) film.

Biochemical identification of peptides contained in the B61/B62 and B31/B32 neurons

Identified B61/B62 and B31/B32 neurons (Hurwitz et al. 1994; Susswein and Byrne 1988) were marked by intracellular iontophoresis...
Fast green. Buccal ganglia containing labeled neurons were incubated at 14°C for 24 h in 1 ml of a mixture of 50% ASW and 50% Aplysia hemolymph that had been filtered through a 0.22-µm filter. The incubation solution contained 0.5 mCi of [35S] methionine, 100 µl of antibiotics (penicillin and streptomycin each at 50 U/ml), and 2.5 µl of 1 M colchicine dissolved in dimethyl sulfoxide (DMSO). Colchicine was added to inhibit axonal transport, thereby eliminating labeled peptides that might be transported to fibers and terminals near the somata of neurons of interest. At the end of incubation, ganglia were rinsed with 10 ml of the incubation solution without [35S] methionine and were left in this solution for another 30 min. Individual B61/B62 and B31/B32 neurons were then dissected using a freeze substitution method (Ono and McCaman 1980). Cells were transferred to glass microtubes, and peptides were extracted by heating for 10 min in 100 µl of 0.01 M trifluoroacetic acid (TFA) containing nanomolar quantities of synthetic peptides (Cropper et al. 1987b; Lloyd et al. 1987).

Extracted material was subjected to two sequential stages of reverse phase high performance liquid chromatography (RP-HPLC), which were performed using an RP-300 column. Synthetic peptides were identified by absorbance measurements at 215 nm. In the first stage of chromatography, the column was developed at 1 ml/min with 5–5% solvent B in 5 min, followed by 5–50% solvent B in 45 min. Solvent A was 0.01 M TFA in H2O and solvent B was 0.01 M TFA in acetonitrile. Fractions were collected every 30 s. Radioactive peptides were identified by scintillation counting of fraction aliquots (20% of 9 B31/B32s and 10% of 12 B61/B62s). In the second stage of chromatography, the column was developed at 1 ml/min with 5–5% solvent B in 5 min, followed by 10–50% solvent B in 40 min. Solvent A was 0.01 M HFBA in H2O and solvent B was 0.01 M heptfluorobutyric acid (HFBA) in acetonitrile. Fractions were collected every 30 s. Radioactive peptides were detected by counting whole fractions.

**cAMP measurements**

I2 muscles were dissected, weighted, and incubated for 2 h in ASW before treatment with neuromodulators for 10 min. No phosphodiesterase inhibitors were used. After muscles were exposed to modulators, they were homogenized in 65% ethanol and 35% H2O and heated to 90°C for 5 min. Homogenates were spun in a clinical centrifuge for 2 min, and the supernatant was stored at –280°C. cAMP levels were quantified using a commercially available RIA (Amersham).

**Data analysis**

When multiple group comparisons were performed, we used a one-way ANOVA. In post hoc comparisons we used a t-test. The level of statistical significance was set at $P < 0.05$.

**RESULTS**

**Acetylcholine as the putative transmitter of the I2 motoneurons**

Many motor neurons in the buccal ganglion utilize acetylcholine as their primary transmitter, and many muscles contract in response to acetylcholine application (Cohen et al. 1978; Evans et al. 1996; Fox and Lloyd 1997; Lloyd and Church 1994). We therefore sought to determine whether hexamethonium, the acetylcholine antagonist that blocks synaptic potentials and contractions of other buccal muscles, could block I2 neuromuscular activity. In the presence of a HiDi solution, firing of B61/B62 induced 3- to 10-mV excitatory junction potentials (EJPs) in I2 muscle fibers, and firing of B31/B32 induced 1- to 5-mV EJPs. These EJPs had a constant latency and a fixed amplitude, i.e., we did not observe facilitation or depression. EJPs were reversibly abolished when...
hexamethonium was added to I2 subchambers (Fig. 2; n = 4 for each motor neuron group). These experiments suggest that the connections of B61/B62 and B31/32 are monosynaptic and cholinergic.

To monitor muscle contractions the cut end of the I2 muscle was attached to a force transducer, and I2 motor neurons were stimulated in fixed bursts. When hexamethonium was added to the I2 subchamber at concentrations of 10^{-2}, 10^{-5}, and 10^{-4} M, muscle contractions induced by stimulation of B61/B62 were reduced in size (Fig. 3). When the hexamethonium concentration was increased to 10^{-3} M, contractions were abolished (n = 5). The blocking effect of hexamethonium was reversible. Similar results were obtained when muscle contractions were elicited by stimulation of B31/B32 (not shown).

We also tested the effect of applying acetylcholine directly to the I2 muscle. At 10^{-5} M acetylcholine induced a powerful long-lasting muscle contraction (Fig. 4A; n = 7). Furthermore, we observed that when the ACh-elicited contractions decreased, presumably because of desensitization of ACh receptors, stimulation of the motor neuron produced a much smaller response than it did before ACh application. The decrement of the motor neuron elicited contractions was unlikely to be a result of muscle fatigue. The size of muscle contraction elicited by motor neuron stimulation also decreased in the presence of low concentrations (i.e., 10^{-9} to 10^{-7} M) of ACh, although at these concentrations ACh itself did not elicit muscle contractions (Fig. 4B) (see also Cohen et al. 1978). Together, these pharmacological experiments support the idea that ACh is a primary neurotransmitter in the I2 neuromuscular system.

**Neuromodulators are present in the I2 muscle**

Many of the muscles of the buccal mass in *Aplysia* receive modulatory input (Cropper et al. 1987b, 1988, 1994; Lloyd et al. 1984). To determine whether the same is true for the I2 muscle, we examined this muscle for the following types of immunoreactivity: MM-like, buccalin-like, SCP-like, RFamide-like, and 5-HT–like. MM immunocytochemistry revealed a dense network of processes (Fig. 5, A1 and B1; n = 5). The blocking effect of hexamethonium was reversible. Similar results were obtained when muscle contractions were elicited by stimulation of B31/B32 (not shown).
B31/B32 and B61/B62 synthesize modulatory neuropeptides

To determine whether any of the I2 muscle motoneurons contain 5-HT or neuropeptides (i.e., MM or RFamide), these neurons were filled with biocytin, and tissue was incubated with avidin labeled with fluorescein. Buccal ganglia were first exposed to those antibodies that stained neuronal processes in the periphery, and then visualized using a secondary antibody labeled with CY-3. Interestingly, all four I2 motoneurons were immunoreactive to MM (Fig. 6) but not to RFamide (not shown; n = 3), or 5-HT (not shown; n = 3). Notice that the signal in B31/B32 and B61/B62 is not due to a breakthrough of fluorescent labeled avidin as neuron B4, which does not contain MM (Church and Lloyd 1991), shows no rhodamine fluorescence. Because various MMs share their C-terminus, the antibodies we used do not distinguish between them.

To determine whether both sets of I2 motor neurons (B31/32 and B61/62) actually synthesize authentic MM, we performed biochemical experiments. Although the gene for MM encodes seven structurally related neuropeptides, only one peptide, MMa, is present in multiple copies (i.e., 10) and is the most abundantly expressed (Miller et al. 1993). I2 motoneurons were incubated in [S35] methionine and then subjected to two sequential stages of RP-HPLC. The counterions used in these experiments were previously employed to purify the MMs (Brezina et al. 1995; Cropper et al. 1987b). Radiolabeled peptides synthesized by B61/B62 and by B31/B32 coeluted with synthetic MMa in both stages of chromatography (Fig. 7). Thus B31/B32 and B61/B62 neurons synthesize MMa. These data are also supported by immunostaining. Previous work has shown that neurons in which MM is not detected chromatographically do not stain for MM (e.g., Cropper et al. 1987b).

Effects of I2 modulators on contraction size and relaxation rate

Because both 5-HT and MM were detected in the innervation of the I2 muscle, we characterized the effects of these substances on muscle contractions elicited by the I2 motoneurons. (Although RFamide-like immunoreactivity was detected in the I2 system, we could not characterize the bioactivity of this peptide since the structure of the specific RFamide that is present in the I2 innervation is not known.) MM experiments were performed with MMa and MMb since both are encoded by the MMa precursor and previous studies have shown that these two MMs exert the most differing actions (Brezina et al. 1995). These differences are particularly apparent when concentration-dependent effects are compared. For example, in the ARC muscle both MMa and MMb increase the amplitude of muscle contractions at low concentrations, but at higher concentrations MMb begins to depress muscle contractions while MMa continues to enhance contraction amplitude.
study, therefore we tested the effects of modulators at concentrations ranging from $10^{-9}$ to $10^{-5}$ M.

MMa increased contraction amplitude at concentrations lower than $10^{-7}$ M and decreased the amplitude of contractions at higher concentrations (Fig. 8A1; also see Fig. 11 for group data). In addition to increasing the amplitude of muscle contractions, MMa also increased the rate of relaxation of muscle contractions (Fig. 8A2). To determine whether the MM-evoked increase of the relaxation rate was due to the increase of the amplitude of muscle contraction, we increased the amplitude of muscle contractions by increasing the frequency of B61 firing (Fig. 8B1). Figure 8B2 demonstrates that when the amplitude of muscle contractions was increased, relaxation rate was not changed.

At concentrations of up to $10^{-7}$, MMb exerted actions that qualitatively resembled the actions of MMa, i.e., MMb increased the amplitude and the relaxation rate of muscle contractions (Fig. 9, A and B). In this series of experiments, we could again dissociate the modulation of relaxation rate from modulation of the amplitude of muscle contractions since increases of contraction amplitude did not account for increases in relaxation rate (Fig. 9, B and C). Analysis of group data revealed significant quantitative differences between the effects of MMa and MMb on the amplitude of muscle contractions. Unlike MMa, which increases the amplitude of muscle contractions at concentrations up to $10^{-8}$ M and at higher concentrations depresses the contraction amplitude, MMb potentiates muscle contractions throughout the range tested (up to $10^{-5}$ M). Indeed the potentiating effects of MMb grew progressively larger until the concentration of $10^{-6}$ M was reached (group data in Fig. 11).

The actions of 5-HT were similar to those of the two MMs,
5-HT potentiated the amplitude (Fig. 10A) and the relaxation rate of muscle contractions (Fig. 10, B and C). The actions of 5-HT on contraction amplitude were more similar to those of MMb than MMa as 5-HT potentiated I2 muscle contractions at all concentrations tested (group data in Fig. 11). In fact, the two modulators appeared to be approximately equipotent on the amplitude of muscle contractions (Fig. 11). Serotonin and myomodulins stimulate cAMP synthesis in the I2 muscle

cAMP has been implicated as a second messenger in several studies of peptidergic and serotonergic modulation of neuro-

mascular function in Aplysia (Cropper et al. 1990b; Evans et al. 1999; Lloyd et al. 1984; Weiss et al. 1979). To investigate the possible role that cAMP may play in the modulation of the I2 muscle we took two approaches. First, we tested the effect of a cAMP analogue [8-(4-chlorophenylthio)-adenosine 3':5'-cyclic mono phosphate] (8-CPT) on motor neuron elicited muscle contractions. We found that perfusion with this cAMP analogue increased contraction amplitude (Fig. 12A) and enhanced relaxation rate (Fig. 12B; n = 4). Second, we measured cAMP levels after I2 muscles were incubated for 10 min in ASW, 10^{-5} M 5-HT and 10^{-5} M MMA (Fig. 13A). In general, levels of stimulation found in this study are within the range of cAMP stimulation that has been reported for other buccal muscles in Aplysia (e.g., Cropper et al. 1990b; Evans et al. 1999; Lloyd et al. 1984; Weiss et al. 1979). More specifically, one-way ANOVA revealed an overall statistically significant difference (f = 106.4; P < 0.001; df = 2.12) between the three treatments (controls, MMA treated, and 5-HT treated). Individual comparisons using a two-tailed t-test also revealed statistically significant differences. Specifically, when the MMA-treated muscles were compared with controls, the level of cAMP was found to be significantly increased (P < 0.05). Similarly, the level of cAMP in 5-HT–treated muscles was also significantly increased (P < 0.001). The observations that 5-HT is a stronger

![Figure 9](http://jn.physiology.org/2816/)

**FIG. 9.** MMb potentiates I2 muscle contractions and enhances relaxation rate. A: repeated bursts of B61 (not shown) were used to produce reproducible contractions of the I2. The MMb concentration was then changed in a stepwise manner (as indicated by the numbers below the I2 trace). Note that the contraction amplitude increased. B: a contraction from A (i.e., the one labeled a) and a contraction from C (i.e., the one labeled b) at an expanded time scale. Note that MMb enhanced muscle relaxation rate even though the superimposed contractions are of comparable amplitude. C: the control contraction needed for the comparison shown in B was generated by stimulating B61 with bursts of fixed duration and with a constant interburst interval. The intraburst stimulation frequency was, however, progressively increased and then decreased (as indicated by the numbers below the B61 trace).

![Figure 10](http://jn.physiology.org/2816/)

**FIG. 10.** 5-HT potentiates I2 muscle contractions and enhances their relaxation rate. A: B61 was stimulated with bursts of fixed durations while maintaining a constant interburst interval. 10^{-8} M 5HT was applied at the point indicated. B: the I2 muscle relaxation rate before (b) and after (a) 5-HT application. Notice that at this concentration 5-HT had a profound effect on relaxation rate but exerted a modest effect on contraction amplitude. C: the control contraction needed for the comparison shown in B was generated by stimulating B61 with bursts of fixed duration and with a constant interburst interval. The intraburst stimulation frequency was, however, progressively increased and then decreased (as marked by the number below B61 trace).
stimulator of cAMP than MMa parallels the observation that 5-HT is also a stronger potentiator of muscle contractions than MMb. Since MMb is also a stronger potentiator of contraction amplitude than MMa, we sought to determine whether MMb is also a stronger stimulator of cAMP (Fig. 13B). Five different MM concentrations were tested, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M. Both MMs produced statistically significant increases in cAMP (MMa $df = 4.27$; $f = 4.8$; $P < 0.005$, and for MMb $df = 4.27$; $f = 6.5$; $P < 0.001$). Maximal stimulation occurred at $10^{-7}$ M for MMb and $10^{-8}$ M for MMa. There was statistically significant overall difference between the effects of the two MMs ($t$-test $df = 1.52$; $f = 2.06$; $P < 0.05$).

**DISCUSSION**

Behavioral observations of biting have been limited to radula protraction (when the radula retracts, it recedes from view into the buccal cavity). In contrast, physiological studies that were used to support the hypothesis that modulatory processes contribute to the enhancement of muscle contractions were performed on muscles that control other movements, i.e., the ARC and I7-I10, which mediate radula opening and closing, and the I3a, which mediates jaw closure (Church et al. 1993; Cohen et al. 1978; Cropper et al. 1987a,b, 1988, 1990a,b, 1994; Fox and Lloyd 1997; Lloyd et al. 1984). In this study we investigated modulatory control of the I2 muscle, a major buccal muscle that protracts the radula (Hurwitz et al. 1996). Importantly, recent work on different buccal muscles has in-
dicated that muscles display distinct contractile and biophysical properties (Evans et al. 1996). Thus these observations make it imperative that the organization of modulation of protraction muscles be determined as a first step toward establishing a connection between the overt behavior of the animal and the modulatory processes that may underlie the behavior.

In view of the widespread serotonergic innervation of the buccal musculature, we expected that neural processes on the I2 muscle would contain 5-HT. We also expected that 5-HT would increase the size of motor neuron elicited I2 contractions. This is indeed what we found. Additionally, we found that as in other buccal muscles (Fox and Lloyd 1997; Lloyd et al. 1984; Rosen et al. 1983; Weiss et al. 1975, 1979) 5-HT increased relaxation rate and therefore shortened contraction duration. Modeling and experimental studies in the ARC neuromuscular system have suggested that increases in relaxation rate are important to accommodate the increase in the speed of biting that occurs in parallel with the increase in bite magnitude (Deodhar et al. 1994; Weiss et al. 1992, 1993). In particular, experimental studies have shown that when contraction amplitude is increased in unmodulated ARC muscles, relaxation rate does not change (Cropper et al. 1990b). Consequently, contraction duration increases. We found that this is also true for the I2 muscle. Modeling studies have suggested that these types of increases in contraction duration may become a problem when behavior is executed rapidly (Brezina and Weiss 2000; Brezina et al. 2000a,b; Deodhar et al. 1994; Weiss et al. 1992). Thus behavior becomes inefficient because individual muscles fail to completely relax between contractions and antagonistic muscles are coactive, and therefore the two sets of muscles work against each other. Experimental studies (Yu et al. 1999) of the visco-elastic properties of the I2 muscle indicate that this muscle may exert braking, i.e., opposing actions on retraction movements, especially when the retraction movements are rapid. The braking actions of I2 on the retraction phase will depend on the speed with which the I2 muscle relaxes. In this study we demonstrated that the relaxation rate of I2 can be modulated by 5-HT and by the MMs. Since the two forms of modulation also enhance the size of contractions, it is likely that the two modulatory actions will act jointly to assure that in face of increased amplitude of I2 contractions this muscle will not provide additional braking of the retraction phase that follows I2 contractions.

Considerable evidence indicates that all serotonergic input to the buccal muscles has as its source the extrinsic modulatory neuron (the MCC). Thus the 5-HT in I2 is also presumably from this extrinsic source. We sought to determine whether there are also modulatory transmitters intrinsic to the I2 neuromuscular system. To localize modulatory peptides we initially used immunocytochemical techniques. We found that antibodies raised against RFamide and MM stained neural processes and varicosities on I2 muscles. Immunopositive neurons were not detected in the muscle, indicating that somata of immunopositive neurons were located elsewhere. The I2 motor neurons were not, however, RFamide immunopositive. A similar situation exists in the ARC neuromuscular system where RFamide peptides are present in terminals on the ARC muscle but are not present in the ARC motor neurons. The RFamide like peptides in the ARC neuromuscular system may originate from the S cluster of buccal mechanosensory neurons, which send their process to buccal muscle (Fiore and Meunier 1979; Lloyd et al. 1987). Since the S cells project extensively to buccal muscles, they may also innervate the I2 muscle.

We found that both sets of I2 motor neurons, i.e., B31/32 and B61/62, are MM immunoreactive. Although the antiserum used in this study does not distinguish between the nine members of the MM family, our biochemical data are consistent with the idea that MMs is present in B31/32 and B61/B62. At least six of the other MMs are likely to be present in the I2 motor neurons since multiple copies of MMs are present on the MM precursor that additionally encodes single copies of MMb, MMd, MMf, MMg, MMh, and MMi (Miller et al. 1993). We did not find evidence that the other two MMs (C and E) are present in the I2 motor neurons. A peak of radioactivity with a relatively long retention time that could correspond to MMc was not observed in B31/B32 or B61/B62. We would not have expected to see MMe in biochemical experiments since MMe does not contain methionine and therefore would not be radio-labeled. Thus immunocytochemical and biochemical experiments indicate that I2 motor neurons do in fact contain intrinsic modulators, i.e., members of the MM peptide family.

Interestingly low doses of the most abundant MM, MMa, exert modulatory effects that are very similar to those of 5-HT; they increase the size and relaxation rate of motor neuron elicited I2 contractions. This might seem redundant. Data, however, suggest that the release of extrinsic and intrinsic modulators may predominately occur at different times during a feeding sequence. For example, recordings from intact animals have indicated that the MCCs are active during the appetitive phase of feeding (Horn et al. 1999; Kupfermann and Weiss 1982), but that their firing slows down precipitously when food is ingested (Kupfermann and Weiss, unpublished observation). Radula protrusion does not occur during appetitive behaviors; consequently, for the most part the I2 motor neurons are not likely to be active until consummatory feeding begins. Thus the extrinsic modulator 5-HT is likely to be present in the highest concentrations during appetitive feeding, whereas the intrinsic I2 modulators, the MMs, are likely to be present during consummatory feeding.

An understanding of the relative role of the extrinsic (5-HT) and intrinsic (peptide) modulation of buccal muscles must take into consideration that the effects of these modulators are apparent only when the muscle begins to contract. Consequently, although 5-HT is released in the I2 neuromuscular system during appetitive behaviors, its effects will not be manifested until consummatory feeding is triggered. Since the released 5-HT will dissipate as consummatory feeding progresses (Horn et al. 1999; Kupfermann and Weiss 1982), 5-HT might primarily modulate I2 muscle contractions that occur at the beginning of a feeding sequence. In contrast, studies of peptide release in other Aplysia neuromuscular systems have suggested that peptide release progressively increases when motor neurons are repeatedly activated (Vilim et al. 1996a,b; Whim and Lloyd 1992, 1994). In fact, peptide release may not occur at all during a single burst of activity (Vilim et al. 1996a,b, 2000). Thus although effects of 5-HT and MM on the I2 muscle will both be manifested during consummatory feeding, serotonergic effects may predominate early in an ingestive sequence, whereas effects of MM may predominate as feeding progresses.

This work has established that the I2 muscle contains two types of modulators: those that are intrinsic (i.e., originate in
the motoneurons) and those that are extrinsic (i.e., originate in cells that are not motoneurons) (Cropper et al. 1987a; Katz 1995; Katz and Frost 1996). Previously, intrinsic and extrinsic modulators were shown to elevate the levels of cAMP in other muscles: the ARC, the I-10 complex, and the I3 muscles (Church et al. 1993; Cropper et al. 1990b; Evans et al. 1999; Fox and Lloyd 2000; Lloyd et al. 1984; Probst et al. 1994; Weiss et al. 1978, 1979; Whim and Lloyd 1989, 1990). In these muscles, cAMP was shown to potentiate muscle contractions and to increase muscle relaxation rate. In this paper, we established that incubation of the I2 muscle with 8-CPT-cAMP also increases the amplitude and the relaxation rate of muscle contractions. In addition, the extrinsic modulator (5-HT) and the intrinsic modulators (MMa or MMb) elevate cAMP levels in the I2 muscle. Thus cAMP is a candidate second messenger for the actions of both the intrinsic and extrinsic modulators.

However, our data concerning the mechanisms by which I2 muscle contractions/relaxations are modulated suggest that cAMP may not be the sole player. Several lines of evidence suggest that other unknown second messengers might be involved. First, different modulators produced similar effects in the I2 muscle, but do not produce similar levels of cAMP synthesis. For example, 5-HT and MMb both potentiate I2 muscle activity, but yet, 5-HT increase cAMP synthesis more than MMb did. Second, increases in the potentiation of I2 muscle do not always go along with increases in cAMP synthesis. Namely, increasing MMb concentration increases the muscle potentiation but decreases cAMP synthesis levels. Third, in the ARC muscle, MMa was shown to activate both cAMP synthesis that up-modulates an L-type calcium current (Brezina et al. 1994a) and, in addition, an unknown second messenger responsible for potassium current activation (Brezina et al. 1994b). High concentrations of MMa decrease the contraction size of I2 muscle, which can be related to activation of K current that tends to decrease the depolarization of the muscle and therefore to diminish the activation of the voltage-dependent L-type calcium current (Brezina et al. 1994a). The fact that MMa produce similar bioactive effects both in the I2 and ARC muscles suggests that similar currents driven by similar second messengers may be activated. Other studies in the I7-I10 and the I3 muscle describe some degrees of dissociation between modulation of muscle contractions and cAMP elevations (Evans et al. 1999; Fox and Lloyd 2000). Independent of the detailed explanations of the dissociations of cAMP synthesis and potentiation of muscle contractions of the I2 muscle, it is important to note that when one combines the finding that intrinsic and extrinsic modulators stimulate cAMP with the finding that cAMP application mimics the actions of modulators, the most parsimonious interpretation of our data suggests that, at least in part, cAMP mediates the actions of modulators on muscle contractions.

In summary, in this study we sought to determine the identity and define the actions of modulators that may be present in the neuromuscular system that plays a major role in generating the protraction phase of feeding. We showed that the MM peptides are present in the motoneurons that innervate this muscle and that the muscle also receives serotonergic modulation. The peptides as well as 5-HT increase the amplitude and relaxation rate of the I2 contractions. Because the I2 muscle is a major protractor and observations of the buccal mass during feeding are largely restricted to this phase of behavior, these findings begin to bridge the gap between the behavior and the in vitro physiology of the animal. Thus these findings will be incorporated into computer simulations (e.g., Brezina and Weiss 2000; Brezina et al. 2000a,b) that are being developed to understand how activity of different buccal muscles becomes integrated to generate appropriate output of the buccal mass during feeding behavior.

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