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

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[1]*Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029;* [2]*Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52 900, Israel; and* [3]*Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, New York 10032*

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Hurwitz, I., E. C. Cropper, F. S. Vilim, V. Alexeeva, A. J. Susswein, I. Kupfermann, and K. R. Weiss. Serotonergic and peptidergic modulation of the buccal mass protractor muscle (I2) in *Aplysia*. *J Neurophysiol* 84: 2810–2820, 2000. Plasticity of *Aplysia* feeding has largely been measured by noting changes in radula protraction. On the basis of previous work, it has been suggested that peripheral modulation may contribute to behavioral plasticity. However, peripheral plasticity has not been demonstrated in the neuromuscular systems that participate in radula protraction. Therefore in this study we investigated whether contractions of a major radula protraction muscle (I2) are subject to modulation. We demonstrate, first, that an increase in the firing frequency of the cholinergic I2 motoneurons will increase the amplitude of the resulting muscle contraction but will not modulate its relaxation rate. We show, second, that neuronal processes on the I2 muscle are immunoreactive to myomodulin (MM), RFamide, and serotonin (5-HT), but not to small cardioactive peptide (SCP) or buccalin. The I2 motoneurons B31, B32, B61, and B62 are not immunoreactive to RFamide, 5-HT, SCP, or buccalin. However, all four cells are MM immunoreactive and are capable of synthesizing MMa. Third, we show that the bioactivity of the different modulators is somewhat different; while the MMs (i.e., MMa and MMb) and 5-HT increase I2 muscle relaxation rate, and potentiate muscle contraction amplitude, MMa, at high concentrations, depresses muscle contractions. Fourth, our data suggest that cAMP at least partially mediates effects of modulators on contraction amplitude and relaxation rate.

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 (Brezina 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

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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 $MgCl_2$ (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. The sheath overlying the uppermost surface of the ganglion was surgically removed.

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 (Sylgard)–lined plastic culture dishes (Fig. 1*A*). 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 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 $CaCl_2$, 55 $MgCl_2$, and 5 $NaHCO_3$). 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 $CaCl_2$, 132 $MgCl_2$, and 5 $NaHCO_3$). 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. 1*B1*). In contrast, when B62 is stimulated, the part of the I2 muscle that is adjacent to the I1/I3 muscles contracts first (Fig. 1*B2*). 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. 1*B3;* $n = 37$) 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. Hadassah 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

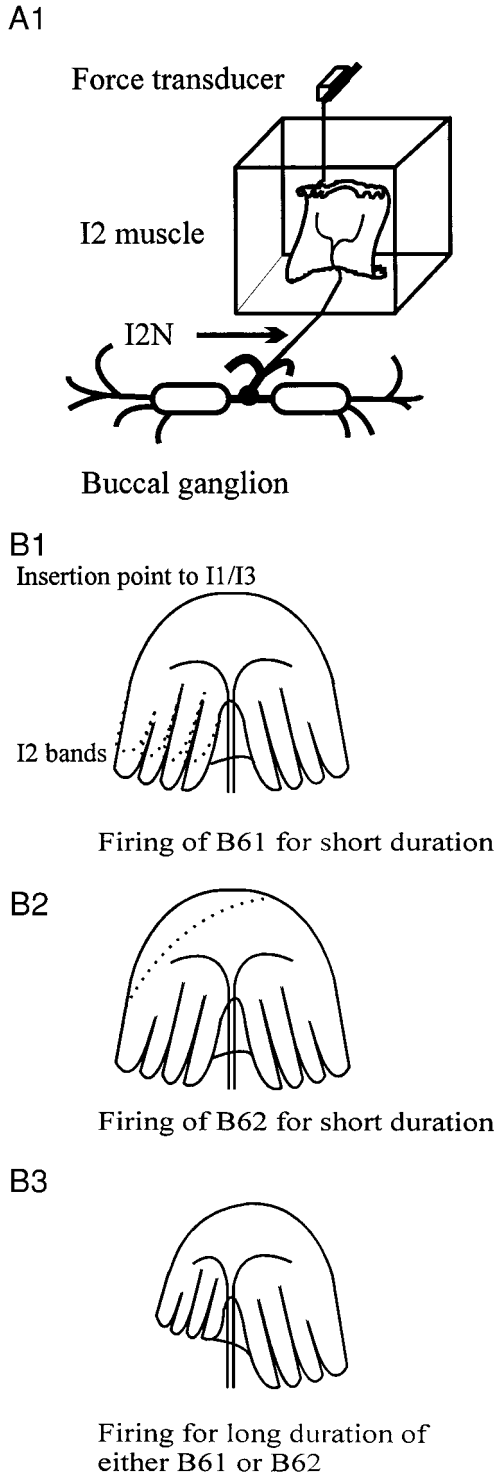

FIG. 1. The I2 neuromuscular preparation. *A*: the buccal ganglion was pharmacologically isolated from the I2 muscle, which was in a separate subchamber. The I2 muscle was pinned along the edge that was originally attached to I1/I3 muscles. The edge of the I2 muscle that was originally attached to the esophagus was hooked to the force transducer. Note that the force transducer was contralateral to the motor neuron that was used to elicit muscle contractions (i.e., motor neurons innervate contralateral muscles). *B*: effect of firing either B61 or B62 on I2 muscle contractions. Contracted states are indicated by the dotted lines in *B1* and *B2*. *B1*: a short burst of activity in B61 results in an I2 muscle contraction along the edge that is normally attached to the esophagus (the side with the obvious bands). *B2*: a short burst of activity in B62 results in an I2 muscle contraction along the edge that inserts into the I1/I3 muscles. *B3*: long duration firing of either B61 or B62 results in an I2 muscle contraction in which both sides of the muscle move toward the center.

(constant 4-nA hyperpolarizing current) of 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 [S^{35}] methionine, 100 μl 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 [^{35}S] 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 H_2O 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 H_2O and *solvent B* was 0.01 M heptafluorobutyric 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% H_2O and heated to 90°C for 5 min. Homogenates were spun in a clinical centrifuge for 2 min, and the supernatant was stored at −80°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

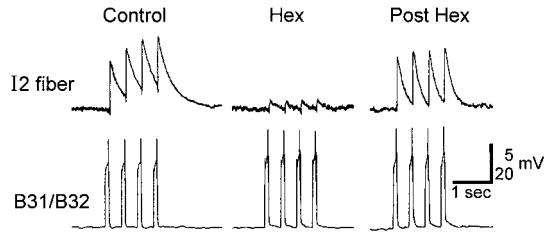

FIG. 2. Effects of hexamethonium (Hex) on excitatory junction potentials (EJPs) in I2 fibers. Firing of B31/B32 evoked ~5 mV EJPs in I2 muscle fibers. These EJPs were reduced in size in the presence of 10^{-3} M hexamethonium. This experiment was conducted in a high divalent cation (HiDi) artificial seawater (ASW).

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^{-6}, 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. 4*A; n* = 7). Furthermore, we observed that when the ACh-elicited contractions decremented, 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. 4*B*) (see also Cohen et al. 1978). Together, these pharmacological experiments support the idea that ACh is a primary neurotransmitter in the I2 neuromuscular system.

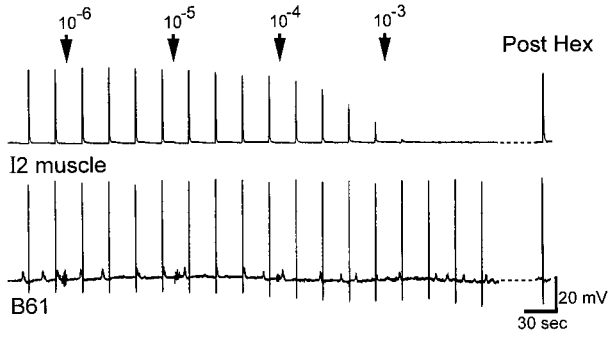

FIG. 3. Effects of hexamethonium on motor neuron elicited muscle contractions. B61 was stimulated in a fixed manner, i.e., the intraburst stimulation frequency, the burst duration, and the interburst interval were all kept constant (*bottom trace*). Hexamethonium was then injected directly into the I2 subchamber, and its concentration progressively increased in a stepwise manner (as indicated by the numbers above the arrows). I2 muscle contractions were depressed at low concentrations of hexamethonium and completely abolished at higher concentrations.

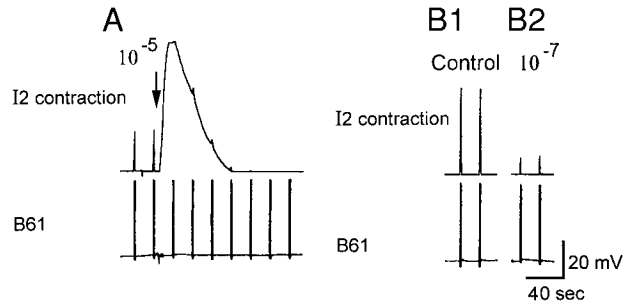

FIG. 4. Effects of acetylcholine on motor neuron elicited muscle contractions. B61 was stimulated in fixed bursts (*bottom trace*), and acetylcholine was injected into the I2 subchamber. *A*: injection of acetylcholine directly evoked a muscle contraction (as marked by the arrow). Note that B61 elicited muscle contractions are not observed following acetylcholine application. *B1*: B61 elicited muscle contractions in the absence of acetylcholine. *B2*: at low concentrations (10^{-7}) acetylcholine decreased the size of motor neuron elicited muscle contractions.

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* =

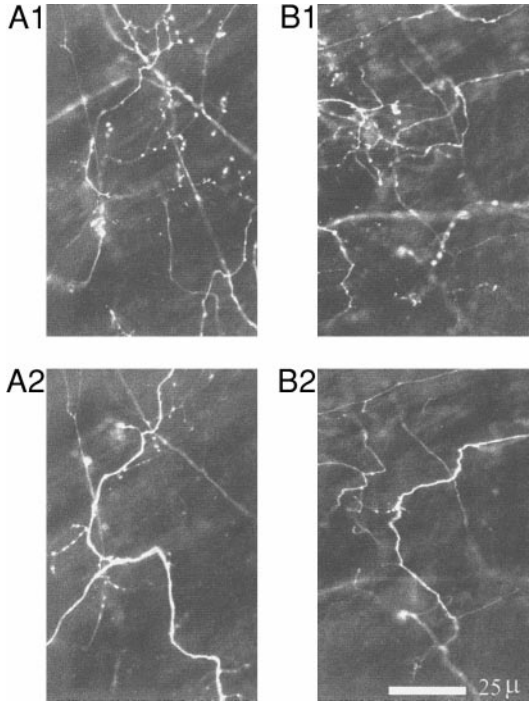

FIG. 5. Serotonin (5-HT), RFamide, and myomodulin (MM)-like stain distinct sets of neuronal processes in the I2 muscle. Processes were double labeled by immunostaining with a MM primary antibody visualized with a Cy-3 secondary antibody, and then either a 5-HT or a RFamide primary antibody visualized with a fluorescein secondary antibody. *A1*: MM-like immunoreactivity, i.e., Cy-3 fluorescence. *A2*: RFamide-like immunoreactivity (i.e., fluorescein fluorescence) in the field shown in *A1*. Processes that are likely to be terminals, i.e., that are comprised of a series of varicosities, are not double labeled. *B1*: MM-like immunoreactivity. *B2*: 5-HT–like immunoreactivity in the field shown in *B1*. Again, structures that are likely to be terminals are not double labeled.

5). This immunoreactivity was abolished when the MM antibody was preincubated with synthetic MMa (not shown). This is consistent with MM being the epitope that is recognized by our antibody. In addition, processes in the I2 muscles stained positively with an antiserum that nonspecifically recognizes peptides with the C-terminal RFamide sequence (Fig. 5*A2; n* = 3). The I2 also stained positively with a 5-HT antiserum (Fig. 5*B2; n* = 4). To determine whether immunoreactivity was present in the same or different sets of processes, we performed double-labeling experiments. Fibers that were MM immunoreactive were not immunoreactive to either RFamide or 5-HT (Fig. 5, *A1* vs. *A2*, and Fig. 5, *B1* vs. *B2; n* = 2).

I2 muscles did not show buccalin-like or SCP-like immunoreactivity even when buccalin and SCP immunoreactivity could be seen in ARC muscles (Cropper et al. 1987a; Miller et al. 1992) that were processed in parallel with I2 muscles (*n* = 2; data not shown). We did, however, observe SCP-like immunoreactivity in processes innervating the junction of the I2, I4, and I1/I3 muscles. These processes did not, however, extend into the rest of the I2 muscle. This may account for previous observations of SCP-like immunoreactivity in the I2 muscle (Church et al. 1991).

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, preparations were fixed, 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 [S^{35}] 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). Furthermore, we show in Fig. 6 the absence of immunostaining of neuron B4, in which MM is not detected chromatographically (Church and Lloyd 1991).

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 MMa begins to depress muscle contractions while MMb continues to enhance contraction amplitude. In this

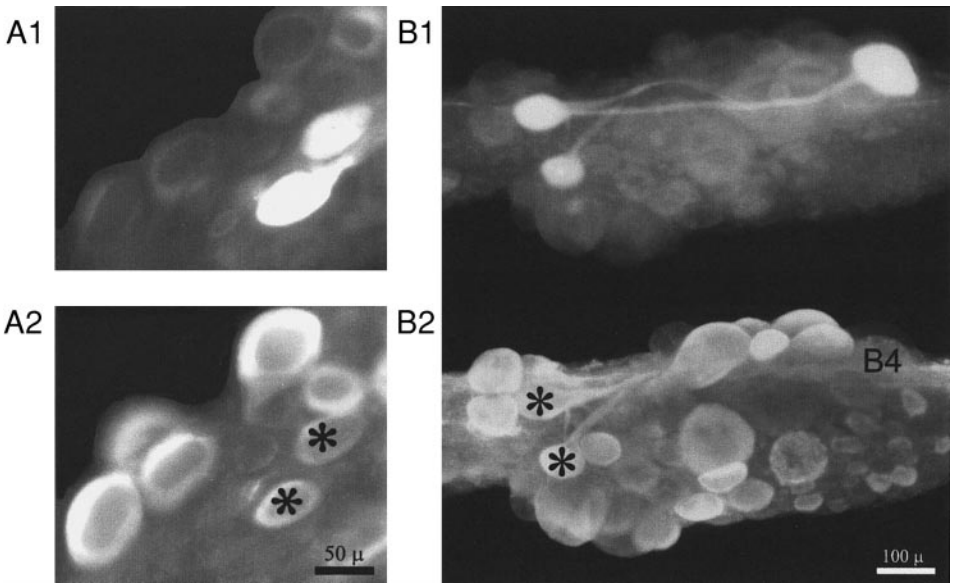

FIG. 6. B31, B32, B61, and B62 all show myomodulin-like (MM) immunoreactivity. *A1*: B31 and B32 injected with biocytin and visualized with avidin/fluorescein. *A2*: MM-like immunoreactivity in the field shown in *A1*. Note that B31 and B32 (marked with asterisks) are MM-immunoreactive. *B1*: B61, B62, and B4 injected with biocytin and visualized with avidin/fluorescein. *B2*: MM-like immunoreactivity in the field shown in *B1*. Note that B61 and B62 (marked with asterisks) are MM-immunoreactive, while no fluorescent signal is present in B4.

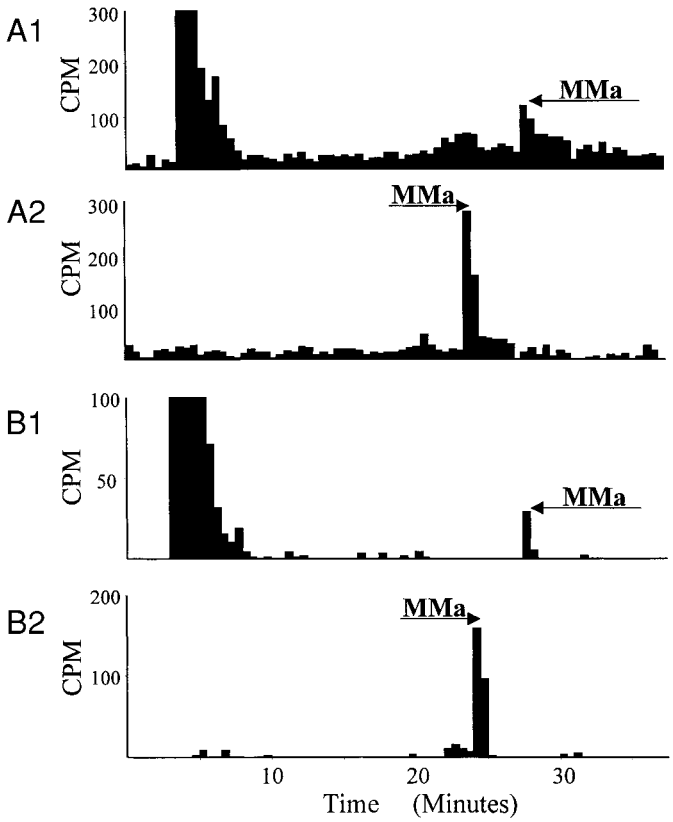

FIG. 7. Comparison of the chromatographic properties of MMa and S^{35} methionine-labeled peptides synthesized by B61/B62 and B31/B32. *A1*: stage 1 of chromatography was performed on 12 B61/B62s in the presence of 0.01 M trifluoroacetic acid (TFA). *A2*: stage 2 of chromatography was performed in the presence of 0.01 M heptafluorobutyric acid (HFBA). *B1*: stage 1 of chromatography was performed on 9 B31/B32s in the presence of 0.01 M TFA. *B2*: stage 2 of chromatography was performed in the presence of 0.01 M HFBA. Note that the motor neurons synthesize radiolabeled peptides that precisely coelute with synthetic MMa (arrows). (Stage 1 of chromatography: 5–5% B in 5 min, followed by 5–50% B in 45 min; 0.5-ml fractions were collected every 0.5 min. In stage 1, for B61/B62 10% of each fraction was counted while for B31/B32 20% was counted. Stage 2 of chromatography: 5–5% B in 5 min, followed by 10–50% B in 40 min; 0.5-ml fractions were collected every 0.5 min and whole fractions were counted.) For the 2nd stage of chromatography of both *A* and *B*, the peak fraction indicated by the arrow and the following fraction were used.

study, therefore we tested the effects of modulators at concentrations ranging from $\sim 10^{-9}$ 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. 8*A1;* 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. 8*A2*). 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. 8*B1*). Figure 8*B2* 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,

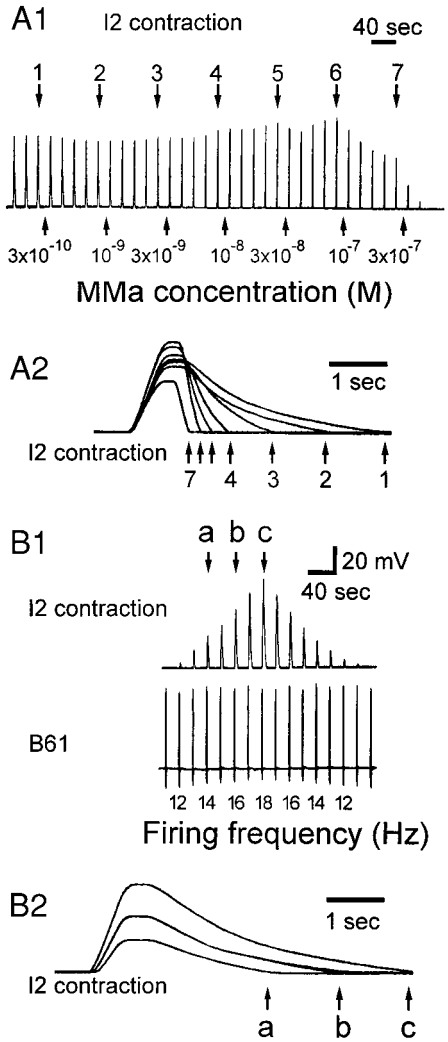

FIG. 8. Effects of MMa on motor neuron elicited I2 contractions. *A1*: B61 was stimulated with fixed bursts, and the concentration of MMa was progressively increased in a stepwise manner (concentrations are indicated below the lower arrows). At lower concentrations, contraction size was moderately increased. At concentrations higher than 3×10^{-8} M, I2 muscle contractions were depressed. *A2*: contractions shown in *A1* at an expanded time scale. The numbers below the arrows correspond to the numbers above the arrows in *A1*. Note that MMa increased the relaxation rate even at the lowest concentration tested (i.e., 3×10^{-10} M). *B1*: B61 was stimulated with a fixed burst duration and interburst interval. However, to vary the amplitude of contractions the intraburst stimulation frequency was progressively increased and then decreased (as indicated by the numbers below the B61 trace). *B2*: contractions shown in *B1* at an expanded time scale. The letters below the contractions correspond to the letters above the arrows in *B1*. The relaxation rate is unchanged, therefore the size of contractions has no effect on the relaxation rate (e.g., c vs. a).

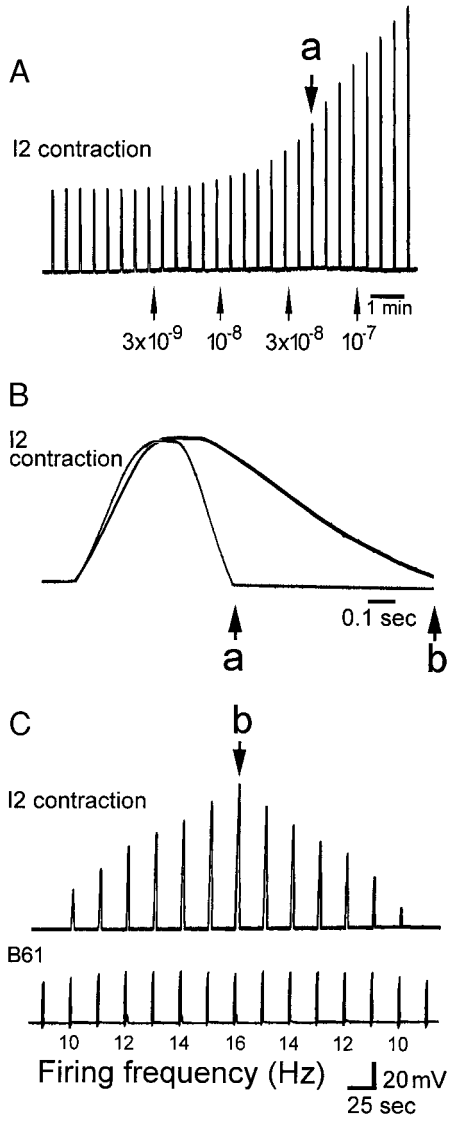

FIG. 9. MMb potentiates I2 muscle contractions and enhances relaxation rate. *A*: repeated bursts of B61 (not shown) were used to produce reproducible contractions of the I2. The MMb concentration was then changed in a stepwise manner (as indicated by the numbers below the I2 trace). Note that the contraction 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).

i.e., 5-HT potentiated the amplitude (Fig. 10*A*) 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 neuromuscular 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. 12*A*) and enhanced relaxation rate (Fig. 12*B; 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. 13*A*). 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

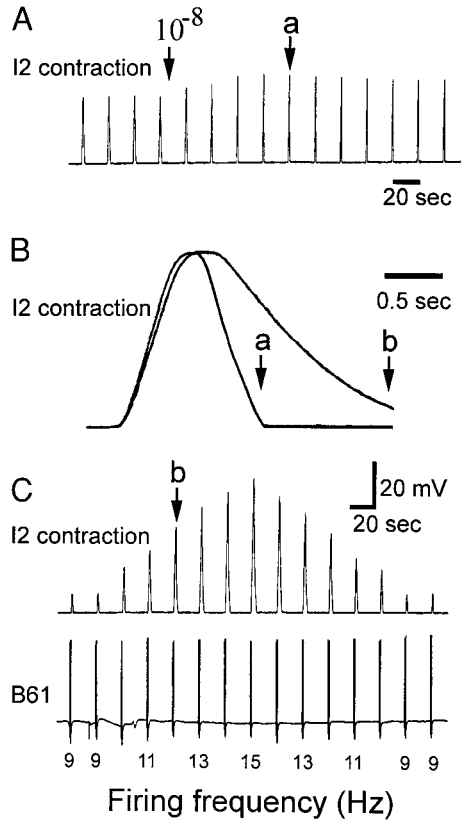

FIG. 10. 5-HT potentiates I2 muscle contractions and enhances their relaxation rate. *A*: B61 was stimulated with bursts of fixed durations while maintaining a constant interburst interval. 10^{-8} M 5HT was applied at the point indicated. *B*: the I2 muscle relaxation rate before (b) and after (a) 5-HT 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).

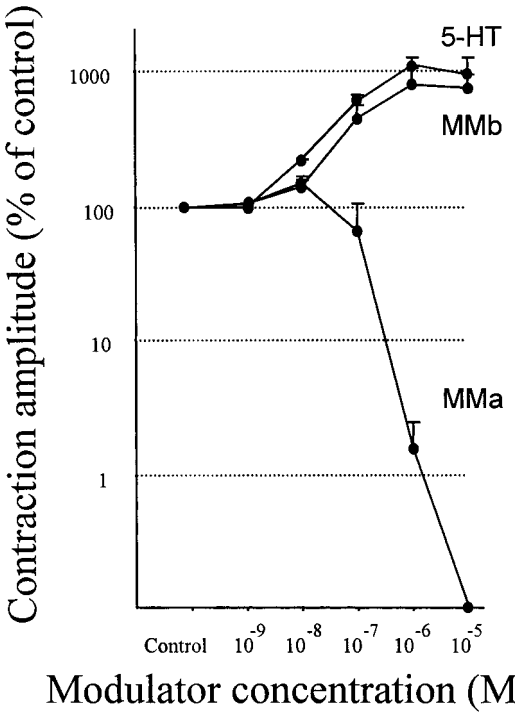

FIG. 11. Effects of modulators on the amplitude of muscle contractions. Notice that 5-HT and MMb increased contraction amplitude at all concentrations tested. In contrast, MMa decreased the contraction size at concentrations above 10^{-8} M ($n = 3$ for each data point).

stimulator of cAMP than MMa parallels the observation that 5-HT is also a stronger potentiator of muscle contractions than MMa. 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. 13*B*). Five different MM concentrations were tested, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. Both MMs produced statistically significant increases

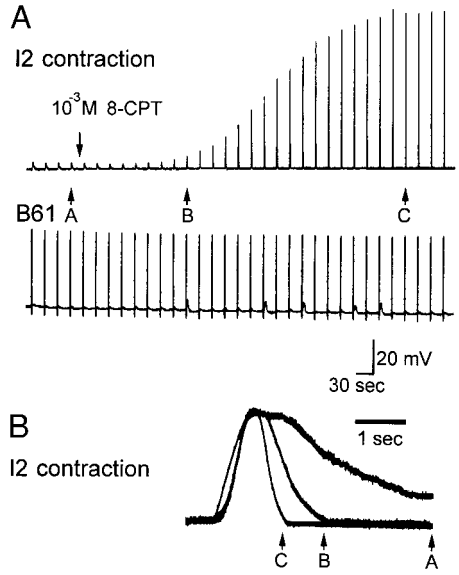

FIG. 12. Effects of 8-CPT cAMP on I2 muscle contraction elicited by B61. *A*: firing of B61 induced contractions that were potentiated by application of [8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic mono phosphate] (8-CPT). *B*: the contractions labeled A–C in *A* at an expanded time base and normalized to the size of the largest contraction shown (i.e., the one labeled C) to compensate for differences in size. Note that 8-CPT enhanced the relaxation rate.

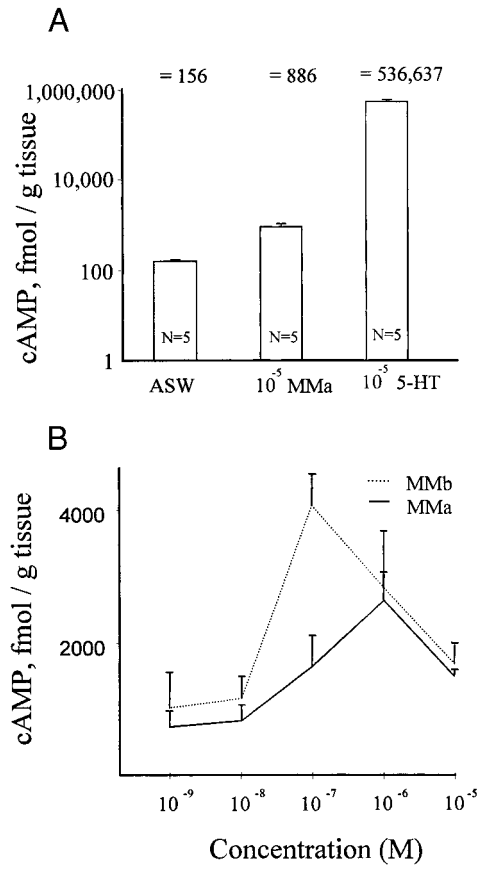

FIG. 13. 5-HT and the MMs increase cAMP levels in the I2 muscle. *A*: I2 muscles were incubated in ASW, 10^{-5} M MMa, or 10^{-5} 5-HT for 10 min. cAMP levels were significantly different in the 3 groups [$F(2,12) = 106.4$, $P < 0.001$]. Post hoc individual comparisons with *t*-tests revealed significant differences between control vs. MMa ($P < 0.05$) and for control vs. 5-HT ($P < 0.001$). *B*: I2 muscles were incubated in MMa or in MMb for 10 min and different MM concentrations were tested; 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. MMb was more effective than MMa in its ability to elevate cAMP levels [$F(1,52) = 2.06$, $P < 0.05$]. The elevation of cAMP levels produced by both MMs was statistically significant [for MMa: $F(4,27) = 4.8$, $P < 0.005$, and for MMb: $F(4,27) = 6.5$, $P < 0.001$].

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 immunocytological 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 mechano-sensory 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 MMa 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 MMa 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 radiolabeled. 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 protraction 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 I7–10 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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REFERENCES

BREZINA V, BANK B, CROPPER EC, ROSEN SC, VILIM FS, KUPFERMANN I, AND WEISS KR. Nine members of the myomodulin family of peptide cotransmitters at the B16-ARC neuromuscular junction of *Aplysia. J Neurophysiol* 74: 54–72, 1995.

BREZINA V, EVANS CG, AND WEISS KR. Enhancement of Ca current in the accessory radula closer muscle of *Aplysia californica* by neuromodulators that potentiate its contractions. *J Neurosci* 14: 4393–4411, 1994a.

BREZINA V, EVANS CG, AND WEISS KR. Activation of K current in the accessory radula closer muscle of *Aplysia californica* by neuromodulators that depress its contractions. *J Neurosci* 14: 4412–4432, 1994b.

BREZINA V, OREKHOVA IV, AND WEISS KR. Optimization of rhythmic behaviors by modulation of the neuromuscular transform. *J Neurophysiol* 83: 260–279, 2000a.

BREZINA V, OREKHOVA IV, AND WEISS KR. The neuromuscular transform: the dynamic, nonlinear link between motor neuron firing patterns and muscle contraction in rhythmic behaviors. *J Neurophysiol* 83: 207–231, 2000b.

BREZINA V AND WEISS KR. The neuromuscular transform constrains the production of functional rhythmic behaviors. *J Neurophysiol* 83: 232–259, 2000.

CHURCH PJ, COHEN KP, SCOTT ML, AND KIRK MD. Peptidergic motoneurons in the buccal ganglia of *Aplysia californica*: immunocytochemical, morphological, and physiological characterizations. *J Comp Physiol [A]* 168: 323–336, 1991.

CHURCH PJ AND LLOYD PE. Expression of diverse neuropeptide cotransmitters by identified motor neurons in *Aplysia. J Neurosci* 11: 618–625, 1991.

CHURCH PJ, WHIM MD, AND LLOYD PE. Modulation of neuromuscular transmission by conventional and peptide transmitters released from excitatory and inhibitory motor neurons in *Aplysia. J Neurosci* 13: 2790–2800, 1993.

COHEN J, WEISS KR, AND KUPFERMANN I. Motor control of buccal muscles of *Aplysia. J Neurophysiol* 41: 157–180, 1978.

CROPPER EC, BREZINA V, VILIM FS, HARISH O, PRICE DA, ROSEN S, KUPFERMANN I, AND WEISS KR. FRF peptides in the ARC neuromuscular system of *Aplysia*: purification and physiological actions. *J Neurophysiol* 72: 2181–2195, 1994.

CROPPER EC, KUPFERMANN I, AND WEISS KR. Differential firing patterns of the peptide containing cholinergic motor neurons B15 and B16 during feeding behavior in *Aplysia. Brain Res* 522: 176–179, 1990a.

CROPPER EC, LLOYD PE, REED W, TENENBAUM R, KUPFERMANN I, AND WEISS KR. Multiple neuropeptides in cholinergic motor neurons of *Aplysia*: evidence for modulatory systems intrinsic to the motor circuit. *Proc Natl Acad Sci USA* 84: 3486–3490, 1987a.

CROPPER EC, PRICE D, TENENBAUM R, KUPFERMANN I, AND WEISS KR. Release of peptide cotransmitters from a cholinergic motor neuron under physiological conditions. *Proc Natl Acad Sci USA* 87: 933–937, 1990b.

CROPPER EC, TENENBAUM R, GAWINOWICZ-KOLKS MA, KUPFERMANN I, AND WEISS KR. Myomodulin: a novel bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia. Proc Natl Acad Sci USA* 84: 5483–5486, 1987b.

CROPPER EC, TENENBAUM R, GAWINOWICZ-KOLKS MA, KUPFERMANN I, AND WEISS KR. Structure and action of buccalin: a modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of *Aplysia californica. Proc Natl Acad Sci USA* 85: 6177–6181, 1988.

DEODHAR D, KUPFERMANN I, ROSEN SC, AND WEISS KR. Design constrains in neural circuits underlying behavior. In: *Flexibility and Constraint in Behav-*

ioral Systems, edited by Greenspan RJ and Kyriacou CP. New York: Wiley, 1994, p. 41–52.

EISENSTADT M, GOLDMAN JE, KANDEL ER, KOIKE H, KOESTER J, AND SCHWARTZ JH. Intrasomatic injection of radioactive precursors for studying transmitter synthesis in identified neurons of *Aplysia californica. Proc Natl Acad Sci USA* 70: 3371–3375, 1973.

EVANS CG, ROSEN S, KUPFERMANN I, WEISS KR, AND CROPPER EC. Characterization of a radula opener neuromuscular system in *Aplysia. J Neurophysiol* 76: 1267–1281, 1996.

EVANS CG, VILIM FS, HARISH O, KUPFERMANN I, WEISS KR, AND CROPPER EC. Modulation of radula opener muscles in *Aplysia. J Neurophysiol* 82: 1339–1351, 1999.

FIORE L AND MEUNIER JM. Synaptic connections and functional organization in *Aplysia* buccal ganglia. *J Neurobiol* 10: 13–29, 1979.

FOX LE AND LLOYD PE. Serotonin and the small cardioactive peptides differentially modulate two motor neurons that innervate the same muscle fibers in *Aplysia. J Neurosci* 17: 6064–6074, 1997.

FOX LE AND LLOYD PE. Role of cAMP in the short-term modulation of a neuromuscular system in *Aplysia. J Neurophysiol* 83: 1567–1579, 2000.

GERSCHENFELD HM, HAMON M, AND PAUPARDIN-TRITSCH D. Release of endogenous serotonin from two identified serotonin-containing neurons and the physiological role of serotonin re-uptake. *J Physiol (Lond)* 274: 265–278, 1978.

GERSCHENFELD HM AND PAUPARDIN-TRITSCH D. On the transmitter function of 5-hydroxytryptamine at excitatory and inhibitory monosynaptic junctions. *J Physiol (Lond)* 243: 457–481, 1974.

HORN CC, BENJAMIN PR, WEISS KR, AND KUPFERMANN I. Decrement of the response of a serotonergic modulatory neuron (the metacerebral cell) in *Aplysia*, during repeated presentation of appetitive (food) stimuli. *Neurosci Lett* 267: 161–164, 1999.

HOWELLS HH. The structure and function of the alimentary canal of *Aplysia punctata. Q J Microsc Sci* 83: 357–399, 1942.

HURWITZ I, GOLDSTEIN RS, AND SUSSWEIN AJ. Compartmentalization of pattern-initiation and motor functions in the B31 and B32 neurons of the buccal ganglia of *Aplysia californica. J Neurophysiol* 71: 1514–1527, 1994.

HURWITZ I, NEUSTADTER D, MORTON DW, CHIEL HJ, AND SUSSWEIN AJ. Activity of the I2 muscle of *Aplysia californica* during normal feeding movements. *J Neurophysiol* 75: 1309–1326, 1996.

HURWITZ I AND SUSSWEIN AJ. Adaptation of feeding sequences in *Aplysia oculifera* to changes in the load and width of food. *J Exp Biol* 166: 215–235, 1992.

KATZ PS. Intrinsic and extrinsic neuromodulation of motor circuits. *Curr Opin Neurobiol* 5: 799–808, 1995.

KATZ PS AND FROST WN. Intrinsic neuromodulation: altering neuronal circuits from within. *Trends Neurosci* 19: 54–61, 1996.

KUPFERMANN I. Feeding in *Aplysia*: a simple system for the study of motivation. *Behav Biol* 10: 1–26, 1974.

KUPFERMANN I AND WEISS KR. Activity of an identified serotonergic neuron in free moving *Aplysia* correlates with behavioral arousal. *Brain Res* 241: 334–337, 1982.

LLOYD PE AND CHURCH PJ. Cholinergic neuromuscular synapses in *Aplysia* have low endogenous acetylcholinesterase activity and a high-affinity uptake system for acetylcholine. *J Neurosci* 17: 6722–6733, 1994.

LLOYD PE, FRANKFURT M, STEVENS P, KUPFERMANN I, AND WEISS KR. Biochemical and immunocytological localization of the neuropeptides FMRFamide, SCP_A, SCP_B to the neurons involved in the regulation of feeding in *Aplysia. J Neurosci* 7: 1123–1132, 1987.

LLOYD PE, KUPFERMANN I, AND WEISS KR. Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in *Aplysia. Proc Natl Acad Sci USA* 81: 2934–2937, 1984.

MILLER MW, ALEVIZOS A, CROPPER EC, KUPFERMANN I, AND WEISS KR. Distribution of buccalin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica. J Comp Neurol* 320: 182–195, 1992.

MILLER MW, ALEVIZOS A, CROPPER EC, VILIM F, KARAGOGEOS D, KUPFERMANN I, AND WEISS KR. Localization of myomodulin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica. J Comp Neurol* 314: 627–644, 1991.

MILLER MW, BEUSHAUSEN S, STAMM S, VITEK A, KUPFERMANN I, BROSIUS J, AND WEISS KR. The myomodulin-related neuropeptides: characterization of a gene encoding a family of peptide cotransmitters in *Aplysia. J Neurosci* 13: 3357–3367, 1993.

ONO JK AND MCCAMAN RE. Identification of additional histaminergic neurons in *Aplysia*: improvement of single cell isolation techniques for in tandem physiological and chemical studies. *Neuroscience* 5: 835–840, 1980.

PROBST WC, CROPPER EC, HEIERHORST J, HOOPER SL, JAFFE H, VILIM F, BEUSHAUSEN S, KUPFERMANN I, AND WEISS KR. cAMP-dependent phosphorylation of *Aplysia* twitchin may mediate modulation of muscle contractions by neuropeptide cotransmitters. *Proc Natl Acad Sci USA* 91: 8487–8491, 1994.

ROSEN SC, KUPFERMANN I, GOLDSTEIN RS, AND WEISS KR. Lesion of a serotonergic modulatory neuron in *Aplysia* produces a specific defect in feeding behavior. *Brain Res* 260: 151–156, 1983.

SUSSWEIN AJ AND BYRNE JH. Identification and characterization of neurons initiating patterned neural activity in the buccal ganglia of *Aplysia. J Neurosci* 8: 2049–2061, 1988.

SUSSWEIN AJ, WEISS KR, AND KUPFERMANN I. The effects of food arousal on the latency of biting in *Aplysia. J Comp Physiol* 123: 31–41, 1978.

VILIM FS, CROPPER EC, PRICE DA, KUPFERMANN I, AND WEISS KR. Peptide cotransmitter release from motorneuron B16 in *Aplysia californica*: costorage, corelease, and functional implications. *J Neurosci* 20: 2036–2042, 2000.

VILIM FS, CROPPER EC, PRICE DA, LESSER W, KUPFERMANN I, AND WEISS KR. Release of peptide cotransmitters in *Aplysia*: regulation and functional implications. *J Neurosci* 16: 8105–8114, 1996a.

VILIM FS, PRICE DA, LESSER W, KUPFERMANN I, AND WEISS KR. Costorage and corelease of modulatory peptide cotransmitters with partially antagonistic actions on the accessory radula closer muscle of *Aplysia californica. J Neurosci* 16: 8092–8104, 1996b.

WEINREICH D, MCCARMAN MW, MCCARMAN RE, AND VAUGHN JE. Chemical enzymatic and ultrastructural characterization of 5-hydroxytryptamine-containing neurons from ganglia of *Aplysia californica* and *Tritonia diomedia. J Neurochem* 20: 969–976, 1973.

WEISS KR, BREZINA V, CROPPER EC, HEIERHORST J, HOOPER SL, PROBST WC, ROSEN SC, VILIM FS, AND KUPFERMANN I. Physiology and biochemistry of peptidergic cotransmission in *Aplysia. J Physiol (Lond)* 87: 141–151, 1993.

WEISS KR, BREZINA V, CROPPER EC, HOOPER SL, MILLER MW, PROBST WC, VILIM FS, AND KUPFERMANN I. Peptidergic cotransmission in *Aplysia*: functional implications for rhythmic behaviors. *Experientia* 48: 456–463, 1992.

WEISS KR, CHIEL HJ, KOCH U, AND KUPFERMANN I. Activity of an identified histaminergic neuron, and its possible role in arousal of feeding behavior in semi-intact *Aplysia. J Neurosci* 6: 2403–2415, 1986.

WEISS KR, COHEN JL, AND KUPFERMANN I. Potentiation of muscle contraction: a possible modulatory function of an identified serotonergic cell in *Aplysia. Brain Res* 99: 381–386, 1975.

WEISS KR, COHEN JL, AND KUPFERMANN I. Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia. J Neurophysiol* 41: 181–203, 1978.

WEISS KR, KOCH UTJ, MANDELBAUM DE, AND KUPFERMANN I. Neural and molecular mechanisms of food induced arousal in *Aplysia californica. Adv Physiol Sci* 23: 305–344, 1981.

WEISS KR, MANDELBAUM DE, SCHONBERG M, AND KUPFERMANN I. Modulation of buccal muscle contractility by the serotonergic metacerebral cells in *Aplysia*: evidence for a role of cyclic adenosine monophosphate. *J Neurophysiol* 42: 791–803, 1979.

WHIM MD AND LLOYD PE. Frequency-dependent release of peptide cotransmitters from identified cholinergic motor neurons in *Aplysia. Proc Natl Acad Sci USA* 86: 9034–9038, 1989.

WHIM MD AND LLOYD PE. Neuropeptide cotransmitters released from an identified cholinergic motor neuron modulate neuromuscular efficacy in *Aplysia. J Neurosci* 10: 3313–3322, 1990.

WHIM MD AND LLOYD PE. Modulation of peptide release from single identified *Aplysia* neurons in culture. *J Neurosci* 12: 3545–3553, 1992.

WHIM MD AND LLOYD PE. Differential regulation of the release of the same peptide transmitters from individual identified motor neurons in culture. *J Neurosci* 14: 4244–4251, 1994.

XIN Y, HURWITZ I, PERRINS R, EVANS CG, ALEXEEVA V, WEISS KR, AND KUPFERMANN I. Actions of a pair of identified cerebral-buccal interneurons (CBI-8/9) in *Aplysia* that contain the peptide myomodulin. *J Neurophysiol* 81: 507–520, 1999.

YU SN, CRAGO PE, AND CHIEL HJ. Biomechanical properties and a kinetic simulation model of the smooth muscle I2 in the buccal mass of *Aplysia. Biol Cybern* 81: 505–513, 1999.