Differential Modulation of Motor Neurons That Innervate the Same Muscle but Use Different Excitatory Transmitters in *Aplysia*

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**Keating, Christopher and Philip E. Lloyd.** Differential modulation of motor neurons that innervate the same muscle but use different excitatory transmitters in *Aplysia*. *J. Neurophysiol.* 82: 1759–1767, 1999. The medial portion of intrinsic buccal muscle 3 (I3m) is innervated by two excitatory motor neurons, B3 and B9. B3 uses glutamate as its fast transmitter and expresses the neuropeptide FMRFamide, whereas B9 uses acetylcholine as its fast transmitter and expresses the neuropeptide SCP. This preparation was used to study peptidergic modulation of muscles innervated by neurons that use different fast excitatory transmitters. First, we determined the effects of the application of the neuropeptides expressed in these neurons on excitatory junction potentials (EJPs) and contractions. FMRFamide increased the amplitude of EJPs and contractions evoked by B3 while decreasing those evoked by B9. This is the first observation in buccal muscle of a substance that modulates two excitatory neurons innervating the same muscle in opposite directions. SCP increased EJPs contraction amplitude, and the rate of muscle relaxation for both motor neurons. We determined that SCP potently increased cAMP levels in I3m as it does in other buccal muscles. Stimulation of B9 also caused increased cAMP levels in I3m providing independent evidence for SCP release. Finally, stimulation of B9 increased both the contraction amplitude and relaxation rate of B3-evoked I3m contractions in a manner similar to that observed using exogenous SCP. By inhibiting B9’s cholinergic transmission with an antagonist, we were able to observe modulatory effects of B9 in the absence of fast excitatory effects. We found that the magnitude of the modulation was dependent on the firing frequency and did occur at frequencies and patterns of firing recorded previously for B9 during ingestive-like motor programs.

**INTRODUCTION**

The coexistence of multiple transmitters in neurons has proven to be an important means by which neuronal circuits can influence synaptic plasticity. Typically, a neuron contains a single conventional neurotransmitter, such as acetylcholine or glutamate, and one or more families of peptide cotransmitters that can act as modulators of synaptic transmission. Neuromuscular synapses have been used extensively for the study of synaptic physiology. Whereas the role of conventional transmitters in synaptic transmission is well established at neuromuscular synapses, less is known about the precise physiological effects of peptide cotransmitters at these synapses. Modulation of neuromuscular synapses has been extensively studied, especially in invertebrate preparations (Calabrese 1989; Cropper et al. 1987; Jorge-Rivera et al. 1998; Kravitz et al. 1980; Lu et al. 1993; O’Shea and Schaffer 1985; Worden 1998), and we are continuing to investigate these processes using the synapses between motor neurons and their target muscles in *Aplysia*. Specifically, we are studying the motor neurons in the buccal ganglia that generate the cyclic motor output that drives biting and swallowing movements of muscles of the buccal mass (Kupfermann 1974). This system has been used extensively to investigate the neural mechanisms underlying synaptic plasticity (Brezina et al. 1996; Weiss et al. 1992, Whim et al. 1993).

The buccal ganglia of *Aplysia* contain many large identifiable motor neurons that innervate discrete muscle fields in the buccal mass and have been shown to express a variety of peptide cotransmitters (Church and Lloyd 1991; Lotshaw and Lloyd 1990). This information allowed us to choose a preparation amenable for studying the role of these transmitters. This neuromuscular preparation consists of the medial region of intrinsic muscle 3 (I3m) that is innervated by six identified motor neurons, all of which express peptide cotransmitters. Three of them express SCP, and three express FMRFamide (Church and Lloyd 1991). In the present study, we focus on B3, which likely uses glutamate as its conventional transmitter and expresses FMRFamide and a second methionine-containing peptide that has yet to be identified, and B9, which is cholinergic and expresses SCP (Church and Lloyd 1991; Fox and Lloyd 1999; Lloyd and Church 1994). These neurons were chosen for two reasons. They use different fast excitatory transmitters, and their fields of innervation overlap extensively. This allowed us to study the effects of peptide cotransmitters released from neuronal terminals in the absence of large postsynaptic effects elicited by the conventional transmitter. Specifically, we could selectively inhibit excitatory junction potentials (EJPs) and contractions produced by B9 using a cholinergic antagonist. We used this preparation to investigate the role of the peptide cotransmitters expressed in B3 and B9. First, we studied the effects of the application of exogenous FMRFamide and SCP on EJPs and contractions evoked by B3 and B9. This is the first example in buccal muscle of the actions of peptide cotransmitters on two motor neurons that innervate the same muscle and use different fast excitatory transmitters. Next, we investigated the effects of stimulating B9 on B3-evoked contractions in the presence of a cholinergic antagonist. B9 was stimulated in a bursting pattern similar to that observed during feeding-like activity (Church and Lloyd 1994). Application of SCP to buccal muscle caused large increases in cAMP levels. Thus measuring the effects of B9 stimulation on cAMP levels in the muscle provides an indirect...
but independent means of demonstrating the release of SCP (Church et al. 1993; Cropper et al. 1990; Whim and Lloyd 1989, 1990). We found that stimulation of B9 both increased cAMP levels in I3m and modulated contractions in a manner very similar to the application of SCP. A major goal of our research is to ascertain the behavioral consequences of peptide release. Thus we determined that peptidergic modulation occurred at physiological firing rates using stimulation paradigms similar to the patterns of activity recorded in B9 during fictive feeding-like activity (Church and Lloyd 1994).

To date, despite extensive investigation, we have been unable to find an effective antagonist for the glutamatergic neuro-muscular receptors (Fox and Lloyd 1999), so we have not been able to carry out the symmetrical experiment of determining the effects of B3 stimulation of B9-evoked contractions.

METHODS

Animals

_Aplysia californica_ (90–300 g) were obtained from Marinus (Long Beach, CA), maintained in circulating artificial seawater (ASW) at 16°C, and fed dried seaweed every 3 days.

Identification of buccal motor neurons

Animals were immobilized with an injection of isotonic MgCl₂ and dissected in a high Mg²⁺ (165 mM; 3× normal), high Ca²⁺ (33 mM; 3× normal) ASW (termed high Ca, Mg ASW). The buccal mass/buccal ganglia complex was removed and bisected along the midline, and all nerves were severed except buccal nerves 2 and 3 (nerve designations from Gardner 1971; muscle nomenclature from Howells 1942; see also Lloyd 1988). The hemiganglion was desheathed and perfused with high Ca, Mg ASW to raise the firing thresholds of neurons in the ganglion. The remainder of the bath containing the I3 muscle was separated by a barrier (except when a perfusion electrode was used; see Measurement of I3m EJPs through which nerves 2 and 3 ran, and was superfused with ASW. Neurons were impaled with two microelectrodes (3–4 MΩ; filled with 3 M K acetate, 30 mM KC1, 0.1% Fast green), one to inject current, and the other to monitor the membrane potential. Motor neurons were identified by their muscle innervation pattern, action potential shape, pharmacological properties, and axonal projections recorded with a suction electrode (Church and Lloyd 1991).

Measurement of I3m contractions

Individual spikes were driven by brief (10–20 ms) depolarizing current pulses. The I3 muscle is contiguous but can be divided into anterior (I3a), medial, and posterior (I3p) segments by the pattern of contractions produced by identified motor neurons. For example, B3 causes contractions of the anterior and medial segments, B9 the medial and posterior segments, and B38 only causes anterior contractions (Church and Lloyd 1991). The anterior extent of I3m is also defined by the location of a white cartilage-like tissue that is confined to the inner side of I3a. Once the region of I3m containing overlapping B3 and B9 innervation was identified, it was mechanically isolated from the other segments by a combination of small incisions and pins. Reproducible submaximal I3m contractions were evoked by stimulating either motor neuron every 100 s at ~15 Hz in 0.5- to 1-s bursts. The long interburst intervals were used to minimize the release of endogenous peptides (Whim and Lloyd 1989). Contractions were monitored with an isotonic transducer (Harvard Apparatus). Experiments were performed at room temperature (~22°C). Peptides to be tested for their actions on evoked contractions were dissolved in ASW and applied via the superfusion. Note that the ganglion was separately superfused with high Ca, Mg ASW and was not exposed to these substances (see Identification of buccal motor neurons).

Measurement of I3m EJPs

Neurons were impaled and stimulated as described above. EJPs were recorded via a perfusion electrode (Church et al. 1993; Fox and Lloyd 1997). Briefly, this electrode consisted of a small chamber (250 μl) with a small aperture (~1.5 mm diam) that was positioned to firmly press down on a portion of I3m. The inside of this chamber was superfused rapidly with ASW (~6 volumes/min). The remainder of the muscle outside of the recording chamber was superfused with low Ca²⁺ (0.5 mM; 0.05 × normal), high Mg²⁺ (110 mM; 2× normal) ASW (termed low Ca ASW) to block synaptic transmission and muscle contractions. This procedure confined the contractions to the small area of the muscle covered by the recording chamber and thus markedly reduced movement artifacts in the recordings. Muscle contractions do not begin until after the 10th EJP so at least the 1st 10 EJPs of a burst (at 15 Hz) are recorded in the absence of any movement. EJPs were recorded by extracellular electrodes placed inside and just outside the wall of the perfusion apparatus. Stimulation parameters were similar to those used to evoke contractions. Signals were amplified using a Grass P15D A.C. amplifier. Peptides to be tested for their actions on evoked EJPs were applied in ASW through the perfusion electrode.

Measurement of cAMP

Levels of cAMP in I3 muscle were measured as previously described for the I5 muscle (Whim and Lloyd 1989). Segments of I3m were weighed, incubated at room temperature for 2 h in three changes of ASW, and then for 20 min in ASW containing known concentrations of pep tid e. All incubations were carried out on a rotary shaker in a volume of ASW at least 1,000-fold greater than that of the muscle segment. Muscle segments were extracted in glass homogenization tubes containing 300 μl 1% 1N HCl in ethanol, maintained at ~30°C, homogenized and stored at −20°C. The tubes were centrifuged at 10,000 g, and the supernatants were used for cAMP determinations using a commercial cAMP-radioimmunoassay (Biotechnologies).

cAMP levels in I3 muscle segments were also measured following stimulation of B9. These experiments were performed at 15°C because previous experiments carried out using other buccal neuromuscular preparations indicated that stimulated peptide release increased at lower temperatures (Vilim et al. 1996; Whim and Lloyd 1990). B9 was either hyperpolarized, or stimulated (4-s 10-Hz bursts with 6-s interburst intervals) for 10 min, and muscles were frozen with an electronic component freezer spray (Miller-Stephenson Chemical, Danbury, CT) and separated into anterior, medial, and posterior segments while frozen. Individual segments were then homogenized as described above. cAMP was determined by radioimmunoassay. It was not possible to weigh the frozen muscles so cAMP was normalized to protein determinations using a BCA kit (Pierce Chemicals) carried out on 10,000 g pellets extracted for 10 min in 0.1 N NaOH at 100°C.

RESULTS

Modulation of motor neuron evoked I3m EJPs and contractions by neuropeptides

Superfusion of FMRFamide selectively over I3m differentially modulated EJPs in that B3-evoked EJPs were facilitated and B9-evoked EJPs inhibited (Figs. 1 and 3). FMRFamide also differentially modulated contractions. Figure 2 shows the effects of 10⁻⁸ M FMRFamide on I3m contractions. The amplitude of I3m contractions evoked by B3 were potentiated,
whereas those evoked by B9 were inhibited. These effects increased with concentration for B9 but not for B3 (Fig. 3). FMRFamide also moderately decreased resting tone of I3m. The relaxation rates of I3m contractions evoked by both B3 and B9 were unaffected by FMRFamide. For example, in 10^{-8} M FMRFamide, the relaxation time constants were 99 ± 6% of control for B3 and 101 ± 6% of control for B9 (mean ± SE, n = 4 for each neuron). For B3, similar results were obtained for the effects of FMRFamide on EJPs and contractions in I3a (Church et al. 1993). B9 does not innervate I3a, and the effects of peptides on B9 have not previously been studied. Among buccal muscles that have been studied, this is the first example of a neuropeptide modulating the efficacy of motor neurons innervating the same muscle in opposite directions.

**FIG. 1.** Effects of FMRFamide on I3m excitatory junction potentials (EJPs) evoked by B3 or B9. In each pair of traces, the top traces are extracellular recordings from I3m, the bottom traces are intracellular recordings from motor neurons. A: EJPs were evoked by stimulation of B3 at 15 Hz for 0.9 s with 100-s interburst intervals. Superfusion of FMRFamide over I3m facilitated EJPs. B: B3-evoked EJPs with an expanded time scale before (1) and after (2) superfusion with FMRFamide. C: EJPs were evoked by stimulation of B9 at 15 Hz for 0.9 s with 100-s interburst intervals. Superfusion of FMRFamide over the muscle decreased the amplitude of EJPs. D: B9-evoked EJPs before (1) and after (2) superfusion with FMRFamide. In all experiments, the ganglia were separated from the muscle by a barrier to prevent exposure to exogenously applied peptides and selectively superfused with high Ca, Mg artificial seawater (ASW) (see METHODS) to eliminate spontaneous activity. The effects of FMRFamide reversed on wash out. Pooled data are presented in Fig. 3.

**FIG. 2.** Effects of FMRFamide on I3m contractions evoked by B3 or B9. A: top trace is a recording of contractions from I3m; bottom trace is an intracellular recording from B3. Contractions were evoked by stimulation of B3 at 15 Hz in 0.9-s bursts with 100-s interburst intervals. Superfusion of 10^{-8} M FMRFamide over the muscle increased the amplitude of contractions. A decrease in the resting tone of the muscle was also observed. B: superimposed B3-evoked I3m contractions with an expanded time scale before (1) and just after (2) superfusion with 10^{-8} M FMRFamide show that exogenous FMRFamide had no effect on the rate of relaxation of B3-evoked I3m contractions. In this and subsequent figures, contractions were aligned by superimposing motor neuron bursts (not shown). C: top trace is a recording of contractions from I3m; bottom trace is an intracellular recording from B9. Contractions were evoked by stimulation of B9 at 15 Hz for 0.9 s with 100-s interburst intervals. Superfusion of 10^{-8} M FMRFamide over the muscle decreased the amplitude of B9-evoked contractions. D: B9-evoked I3m contractions with an expanded time scale before (1) and just after (2) superfusion with 10^{-8} M FMRFamide show that there was no effect on the relaxation rate of I3m contractions. The effects of FMRFamide reversed on wash out. Pooled data are presented in Fig. 3.
is also the first example of the effects of neuropeptides being examined on two motor neurons that innervate the same muscle but use different fast excitatory transmitters.

Superfusion of SCP selectively over I3m modulated EJPs and contractions evoked by both B3 and B9. At $10^{-8}$ M, SCP did not have a significant effect on EJPs evoked by B3 or B9, whereas $10^{-6}$ M SCP facilitated the amplitude of EJPs evoked by both neurons (Figs. 4 and 6). Figure 5 shows the effects of $10^{-8}$ M SCP on I3m contractions. SCP increased the amplitude and relaxation rates of contractions evoked by both B3 and B9 (Figs. 5 and 6). The effect on contraction amplitude was dose-dependent, whereas the effect on relaxation rate was similar at $10^{-8}$ M and $10^{-6}$ M (Fig. 6). SCP also caused a decline in the resting tone of the I3m. EJPs and contractions evoked by B3 in I3a were also increased by SCP. Although the effects on EJPs were similar, the potentiation of contractions was larger in I3a (Church et al. 1993).

Effects of neuropeptides and motor neuron stimulation on cAMP levels of I3m

As was the case with other buccal muscles, exogenous SCP was very effective at increasing cAMP levels in I3m segments (Fig. 7). Indeed at $10^{-6}$ M, SCP increased cAMP levels ~1,000-fold over control. By contrast, $10^{-6}$ M FMRFamide had no effect on I3m cAMP levels (Fig. 7). We reasoned that if B9 releases SCP from its terminals in I3m, this release should lead to an increase in cAMP levels in the muscle. Levels of cAMP normalized to protein in I3m were compared with cAMP levels in I3a. I3a was used as a control because B9 does not innervate this region of the muscle (Church and Lloyd 1987).
When B9 was hyperpolarized for 10 min, the level of cAMP in I3m was somewhat lower than in I3a (Fig. 8). However, when B9 was stimulated in 10-Hz bursts for 10 min, the level of cAMP in I3m was significantly higher than that in I3a. The increase in cAMP levels produced by B9 stimulation was quite variable (increases ranged from 3- to 10-fold). However, the effects of application of SCP were also quite variable. For example, increases in cAMP caused by $10^{-7}$ M SCP ranged from 20 to over 100-fold. We did not look at the effects of application of SCP on the modulation of B3-evoked EJPs and contractions (Fig. 6).

**FIG. 5.** Effect of SCP on I3m contractions evoked by B3 and B9. A: top trace is a recording of contractions from I3m; bottom trace is an intracellular recording from B3. Contractions were evoked by stimulation of B3 at 15 Hz for 0.9 s with 100-s interburst intervals. Superfusion of $10^{-8}$ M SCP over the muscle reversibly increased B3-evoked contractions. B: B3-evoked I3m contractions with an expanded time scale before (1) and just after (2) superfusion with SCP. Note that SCP increased the rate of relaxation. C: top trace is a recording of contractions; bottom trace is an intracellular recording from B9. Contractions were evoked by stimulation of B9 at 15 Hz in 0.6-s bursts with 100-s interburst intervals. Application of $10^{-8}$ M SCP over the muscle reversibly increased B9-evoked contractions. D: B9-evoked I3m contractions with an expanded time scale before (1) and just after (2) superfusion with SCP. Note that SCP increased the rate of relaxation. Pooled data are shown in Fig. 6.

**FIG. 6.** Summary of the modulation of B3- and B9-evoked I3m EJPs and contractions produced by application of SCP at 2 concentrations. Effect of superfusion of SCP on EJPs (top), contraction amplitude (middle), and relaxation time constant of I3m contractions (bottom) evoked by B3 or B9. Note that a decrease in time constant indicates an increase in the rate of relaxation. Values are means ± SE ($n = 4$). Significance was calculated using the paired Student’s t-test (NS, not significant; * $P < 0.05$; ** $P < 0.01$).
of higher stimulation frequencies (e.g., 15 Hz) because extracellular recordings from I3m indicated that B9-evoked EJPs failed before the end of the 10-min stimulation period at frequencies higher than 10 Hz. The failure of somatic spikes to elicit EJPs has been observed previously for neurons innervating I3a and appears to be due to a failure of action potential propagation into the axon (Church et al. 1993; Fox and Lloyd 1998). Failures at higher stimulation frequencies were not observed during the much shorter trains of bursts used for physiological experiments (see Effects of B9 stimulation on contractions evoked by B3). Similar experiments using increases in cAMP levels to indirectly measure SCP release have been carried out in other buccal neuromuscular preparations and have been shown to correlate well with other methods of measuring SCP release (Church et al. 1993; Cropper et al. 1990; Vilim et al. 1996; Whim and Lloyd 1989, 1990).

**Effects of B9 stimulation on contractions evoked by B3**

Finally, we wished to determine whether stimulation of B9 during B3 interburst intervals would have the same effect on subsequent B3-evoked I3m contractions as application of exogenous SCP. We chose to concentrate on contractions rather than EJPs because stimulation of B9 produced increased cAMP levels in I3m that were well below those produced by application of 10^{-7} M SCP at lower concentrations, application of SCP had little effect on EJPs but increased both the amplitude and relaxation rate of contractions (Figs. 6 and 9). These experiments were carried out in the presence of a cholinergic antagonist (3 mM hexamethonium) to suppress B9-evoked I3m contractions (Fig. 9). The degree of suppression varied with stimulation parameters being less effective at higher stimulation frequencies (Whim and Lloyd 1990). Nevertheless, hexamethonium partly or completely blocked contractions evoked by firing B9 at frequencies similar to those recorded in feeding-like motor patterns (Church and Lloyd 1994). In contrast, hexamethonium had no significant effect on the amplitude of B3-evoked I3m contractions. In 3 mM hexamethonium, B3-evoked I3m contraction amplitudes were 95 ± 4% of control

![Fig. 7](image_url)  
**FIG. 7.** Effects of FMRFamide and 2 concentrations of SCP on cAMP levels in I3m segments. Incubations were for 20 min. cAMP in pmol/mg wet weight were normalized to another I3m segment taken from the same animal. B9 was either hyperpolarized (Hyper) or stimulated at 10 Hz (Stim) in 4-s bursts with a 6-s interburst interval for 10 min. Values are means ± SE (n = 4 for stimulation and n = 3 for hyperpolarization). Experiments were carried out at 15°C. Stimulation of B9 caused a significant increase in cAMP levels in I3m (P < 0.025). (n = 4). B3 was stimulated at 100-s intervals to evoke reproducible submaximal contractions. B9 was then stimulated in a train of bursts during B3 interburst intervals. Stimulation of B9 caused an increase in the amplitude and relaxation rate of subsequent B3-evoked contractions, and the magnitude of these effects were dependent on the frequency of stimulation (Fig. 9). These effects were reversible, with the B3-evoked I3m contractions returning to control values within ~30 min. These responses closely resembled the effects of exogenous SCP on B3-evoked I3m contractions, and it appears likely that these effects of B9 are mediated, at least in part, by the release of SCP from its terminals in I3m.

**DISCUSSION**

Neuromuscular synapses between the buccal motor neurons and their target muscle in *Aplysia* have been used extensively to investigate the physiological role of cotransmitters in modulating synaptic plasticity. Modulation of neuromuscular transmission has now been examined at a variety of buccal muscles in *Aplysia* (Evans et al. 1996; Scott et al. 1997; Weiss et al. 1978, 1992; Whim et al. 1993). A striking conclusion from these studies is that, although there are some basic similarities, there are also fundamental differences in the degree and type of modulation observed, and even in the nature of the modulators found in each of the neuromuscular preparations. Indeed, in the present study, we found some significant differences with other buccal neuromuscular systems. This is the first buccal muscle found to be innervated by motor neurons that use different fast excitatory transmitters. In other preparations, either the excitatory neurons use only acetylcholine (muscles I5 or ARC; Cohen et al. 1978; and I7–10, Evans et al. 1996) or only glutamate (I3a, Fox and Lloyd 1999). In I3m, B3 uses glutamate and B9 is cholinergic. In addition, FMRFamide is the first modulator to have opposite effects on two excitatory motor neurons innervating the same muscle. FMRFamide decreased the amplitude of B9-evoked EJPs and contractions and increased the amplitude of B3-evoked EJPs and contractions. B3 and B9 fire simultaneous bursts during ingestive-like buccal
motor programs (Church and Lloyd 1994). So FMRFamide changes the relative efficacy of two motor neurons that fire together during ingestion-like motor patterns and that have overlapping but different fields of innervation. A modulator that decreases the response to one neuron while increasing the response to another may have little net effect where the fields of innervation overlap but may change the shape of contractions in regions where the innervation does not overlap. Currently little is known about the mechanisms underlying FMRFamide’s effects. It is possible that the changes in EJP amplitude caused by FMRFamide produce the changes in contraction amplitude. FMRFamide increases both EJP and contraction amplitude for B3 and decreases both for B9. We do not know whether these effects are presynaptic or postsynaptic. In another buccal muscle, the inhibitory effects of FMRFamide has been shown to be due, at least in part, to an increase in a K current in muscle fibers (Scott et al. 1997). B3 and B9 functionally innervate the same muscle fibers (Fox and Lloyd 1999), and EJPs and contractions evoked by the two neurons are modulated in opposite directions. Thus it is likely that some aspect of this modulation must occur at sites that are specific to one of the neurons.

An interesting observation is that EJPs and contractions evoked by cholinergic motor neurons (B9, B15, and B16) are inhibited by FMRFamide, whereas those evoked by glutamatergic neurons (B3 and B38) are increased (Church et al. 1993; Cohen et al. 1978; Weiss et al. 1986; L. E. Fox and P. E. Lloyd, personal observation). Of course a much larger sample size is needed to determine how strong this trend is. FMRFamide does inhibit central glutamatergic excitatory postsynaptic potentials between sensory and motor neurons (Mackey et al. 1987), and these synapses are facilitated by SCP. FMRFamide has also been shown to selectively inhibit the effects of a cholinergic motor neuron that innervates the aorta in Aplysia while having no effect on noncholinergic motor neurons innervating the same tissue (Alevizos et al. 1989).

FMRFamide has been shown previously to inhibit EJPs and contractions in the ARC (I5) buccal muscle (Weiss et al. 1986). However, in this muscle, the neuronal source of FMRFamide has not been identified, and the physiological ligands for this inhibition may actually be three peptides, termed the RFamide peptides, that have sequence similarity to FMRFamide and that were found to be synthesized in one of the motor neurons innervating the muscle (Cropper et al. 1994). We cannot exclude the possibility that these peptides also act in I3m, but it is clear that at least three of the motor neurons that innervate I3m synthesize authentic FMRFamide (Church and Lloyd 1991). However, in that study, peptides were identified by labeling with radioactive methionine, and this residue is not present in the RFamide peptides, so it remains possible that any of the motor neurons innervating I3m could synthesize the three peptides or indeed any other peptides that do not contain methionine residues.

Application of SCP potentiated the amplitude of EJPs and contractions evoked by both B3 and B9 in I3m. SCP may have presynaptic actions but clearly has postsynaptic actions in muscle fibers. It increases cAMP levels, and the magnitude of this increase indicates that it must occur predominantly in muscle fibers and not in neural elements that make up a very small proportion of the muscle volume. In addition, SCP increases the relaxation rate of contractions. Relaxation occurs after the EJPs have decayed and as such is a property of the muscle fibers themselves. Indeed, in another buccal muscle, the rate of relaxation has been correlated with the degree of cAMP-
dependent phosphorylation of a contractile protein (Probst et al. 1994).

Stimulation of B9 mimics the actions of SCP on I3m. In these experiments, B9 evoked EJPs, and contractions were selectively inhibited using a cholinergic antagonist. Both application of SCP and stimulation of B9 caused increased cAMP levels in the muscle. The magnitude of the increase in cAMP produced by B9 was similar to those observed when other SCP-containing motor neurons were stimulated at frequencies observed during ingestion-like motor programs (Church et al. 1993; Cropper et al. 1990; Whim and Lloyd 1989). Both application of SCP and stimulation of B9 produced very similar increases in the amplitude and rate of relaxation of muscle contractions. Finally, the effects produced by stimulation of SCP-containing motor neurons were independent of the fast excitatory transmitter used by the neurons. For example, the effects on cAMP levels and muscle contractions were similar for neurons that used ACh (B9) or glutamate (B38) (Church et al. 1993). In the present study, we were able to to look for the first time at the immediate effects of peptide cotransmitter release from an excitatory motor neuron in the absence of large contractions evoked by the neuron’s fast excitatory transmitter. The potentiation of B3-evoked I3m muscle contractions was observed when firing B9 at rates and in patterns similar to those observed during ingestive-like buccal motor programs, suggesting that modulation of I3 contractions occurs during feeding behaviors. It is also likely that other motor neurons that innervate I3m contribute to the modulation of EJPs and contractions. Indeed, of the six motor neurons known to innervate I3m, three synthesize FMRFamide (B3, 4, 5) and three synthesize SCP (B6, 9, and 10) (Church and Lloyd 1991). These neurons all fire bursts during ingestive-like buccal motor programs (Church and Lloyd 1994). Thus it is likely that peptides released from their terminals modulate their own synaptic efficacy on muscle fibers as well as the efficacy of other motor neurons that innervate I3m. It is difficult to estimate how the observed modulatory effects would affect contractions during feeding when all of the motor neurons innervating I3m fire bursts approximately at the same time (Church and Lloyd 1994). It is possible that simultaneous activation of the motor neurons would cause near maximal contraction amplitude and the modulatory effects on amplitude would not be important. However, it is difficult to imagine that the increased rate of relaxation would not have behavioral implications. Indeed, modeling studies of another buccal muscle (ARC or I5) indicate that increased relaxation rates are particularly important behaviorally (Weiss et al. 1992). However, this preparation differs from I3m in that each motor neuron releases a complement of peptide cotransmitters that both increase and decrease the amplitude of contractions, and increase the rate of relaxation so that the net modulatory effect may be the increase in relaxation rate.

B3 also innervates the I3a region, and the effects of SCP on contraction amplitude are considerably larger in I3a than I3m (Church et al. 1993). This raises the possibility that in the intact I3, released SCP would not only increase the amplitude of a B3-evoked contraction, but also change its shape by potentiating contractions more effectively in I3a than in I3m. SCP also potentiated contractions evoked by motor neurons innervating another buccal muscle (I5 or ARC), but SCP did not facilitate EJPs in this muscle (Brezina et al. 1994; Lloyd et al. 1984). Apparently, the mechanisms of action of modulatory neuropeptides in buccal muscles are varied.

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REFERENCES


