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Frequency-dependent release of peptide cotransmitters from identified cholinergic motor neurons in *Aplysia*

(cyclic AMP/modulation/neuromuscular synapse)

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ABSTRACT **We have investigated the release of two peptide cotransmitters from the terminals of a cholinergic motor neuron in *Aplysia*. Identified motor neuron B15 synthesizes the two small cardioactive peptides (SCP) A and B in addition to acetylcholine. A symmetrical pair of B15 neurons innervate symmetrical buccal muscles, termed I5, which are involved in generating biting movements. The amplitude of I5 contractions is enhanced by the SCPs. Intracellular stimulation of one B15 produces depletion of the SCPs from the stimulated muscle as compared to the unstimulated control muscle. Significant depletion requires either high-frequency stimulation or prolonged bursts at lower frequencies. A second cholinergic motor neuron, B16, also innervates I5 but does not synthesize the SCPs. Stimulation of B16 produced no depletion of the SCPs. Exogenous SCPs potently increase cAMP levels in the muscle. If depletion is a reflection of release, it should be possible to demonstrate an effect of B15 stimulation on muscle cAMP levels. Indeed, stimulation of B15 did elevate cAMP levels in I5. Stimulation of B16 had no effect on cAMP levels. Increases in cAMP were observed only when B15 was stimulated in a manner that would produce significantly facilitated acetylcholine release. This facilitation could be produced by increased stimulation frequency, longer burst durations, or shorter interburst intervals. However, B15 is capable of producing cholinergically mediated contractions with stimulation parameters that would not cause release of the SCPs. Thus, B15 appears to function as a purely cholinergic motor neuron when firing slowly, and as a cholinergic/peptidergic neuron when firing rapidly.**

Over the last decade, it has become clear that many neurons contain multiple transmitters, often one or more peptide transmitters with a single conventional transmitter (1, 2). Knowledge of the regulation of release of coexisting transmitters is crucial to our understanding of the physiological roles of each transmitter and the interactions between them (3–5). The metabolism of conventional transmitters and peptide transmitters differs significantly. Conventional transmitters are synthesized enzymatically at synaptic terminals and, once released, usually have high-affinity uptake systems. Thus, there are cellular mechanisms for maintaining homeostatic levels of conventional transmitters. In contrast, peptide transmitters are cleaved from precursors in the neuronal cell body and transported to remote terminals and there is little evidence for efficient reuptake of most peptides. Consequently, during sustained activation, either the rates of peptide release must be very low compared to total content in the terminals or the peptide content must decline.

One class of peptides that have been commonly found to coexist with other transmitters in *Aplysia* neurons are the small cardioactive peptides (SCPs) (6–8). The SCPs are represented by two peptides, SCP_A and SCP_B, which are 11 and 9 amino acids in length, respectively. These peptides have similar sequences, are derived from a common precursor polypeptide (9–11), and have been shown to satisfy many of the criteria required of neurotransmitters (12). They are present in, and synthesized by, individual neurons (6–8) and are contained in membrane-bound dense-core vesicles in central neurons and in varicosities in the central nervous system, muscle, and gut (13, 14). Immunoreactive fibers and varicosities are present at both central and peripheral sites where the SCPs have been shown to potently modulate the efficacy of synaptic transmission (12, 15, 16). Finally, the SCPs are released from identified neurons in culture in a stimulation- and calcium-dependent manner (17).

We have chosen to study cotransmission in a model neuromuscular system in *Aplysia*. This system consists of two bilaterally symmetrical pairs of cholinergic motor neurons (left and right B15 and B16) that innervate a discrete buccal muscle (termed I5; also called the ARC) that is involved in generating biting movements. In addition to acetylcholine, B15 is known to synthesize the SCPs (8, 18). SCP immunoreactivity is localized to dense-core vesicles in the cell body of B15 that are identical to immunoreactive vesicles observed in varicosities in the I5 muscle (8). Application of exogenous SCPs to the I5 muscle enhances the amplitude of motor neuron-driven contractions, primarily by a cAMP-mediated enhancement of excitation–contraction coupling (16, 19, 20). B15 has been shown to synthesize another neuropeptide termed buccalin (21). The other I5 motor neuron, B16, also synthesizes buccalin as well as a second modulatory peptide termed myomodulin (22).

METHODS

Animals. *Aplysia californica* (100–200 g) were obtained from Marinus (Long Beach, CA), maintained in circulating artificial seawater (ASW) at 15°C, and fed dried seaweed every 3 days.

Stimulation of the Motor Neurons. Animals were immobilized by an injection of isotonic $MgCl_2$. The dissection was similar to that used previously (ref. 18; also see Figure 1 in ref. 19) except that the entire dissection was carried out in high-Mg^{2+} (110 mM, 2× normal) low-Ca^{2+} (2 mM, 0.2× normal) ASW. In brief, the buccal ganglion–buccal mass complex was removed from the animal and all nerves were severed except nerve 3 on the side of I5 muscle to be stimulated. A longitudinal incision was made along the dorsal midline and the mass pinned flat. Muscles overlying I5 were removed and I5 was attached to a transducer (Bionix Isotonic). The buccal ganglion with intact nerve 3 was pinned on a Sylgard platform and desheathed, and the preparation was superfused with normal ASW for 1–2 hr. B15 and B16 were identified by their position, size, and ability to produce contractions in I5 when stimulated (18). The neuron to be studied was impaled with two electrodes (resistance, 5 MΩ) and either hyperpolarized (constant 5 nA) or stimulated to

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Abbreviations: ASW, artificial seawater; SCP, small cardioactive peptide.

fire spikes with brief depolarizing current pulses (10 ms, 100–200 nA) through one electrode while the other monitored membrane potential. A small tube was placed immediately adjacent to the buccal ganglion, which was superfused with high-Mg^{2+} low-Ca^{2+} ASW to suppress central synaptic activity while the bath was superfused rapidly with normal ASW. In many experiments involving cAMP measurements, this normal ASW contained 0.5 mM hexamethonium chloride (Sigma) to block cholinergic contractions (18).

Measurement of SCPs. At the end of a stimulation period, the preparation was rapidly superfused with ice-cold high-Mg^{2+} low-Ca^{2+} ASW and kept in this solution at 4°C for 30 min to permit released SCPs to diffuse from the muscles; I5 muscles were dissected away from the remainder of the buccal mass, weighed, and extracted. The extraction consisted of heating to 100°C for 10 min in 500 μl of 0.02 M heptafluorobutyric acid containing 5 nmol of a carrier peptide, [Tyr^8]substance P. The muscles were then homogenized in polypropylene tubes and centrifuged for 10 min at 10,000 × *g*. The supernatant was filtered and fractionated by HPLC, which consisted of complex gradient elution (three 6-min linear segments from 12% to 25% CH_3CN, from 25% to 28%, and from 28% to 56%) at a flow rate of 2 ml/min on a Brownlee C_8 column. Both H_2O and CH_3CN contained 0.01 M heptafluorobutyric acid. This gradient was designed to separate the SCPs from other neuropeptides found in the muscle and to elute [Tyr^8]substance P and both SCPs at the onset of the steep gradient (third segment) in a single fraction. [Tyr^8]Substance P, which has an amino acid composition similar to those of the SCPs but is completely inactive in the bioassay (see below), was used to prevent losses in the recovery of the SCPs during extraction, HPLC, and bioassay. The fraction containing the SCPs was divided into three aliquots, lyophilized, and taken up in snail saline for bioassay on isolated snail hearts. This bioassay has been described (23) and was used because, over a small range, the increase in the amplitude of spontaneous heartbeats is linearly proportional to the amount of SCPs in the injections (12). Hearts were calibrated before and after test samples with injections of synthetic SCPs (generally 5, 10, and 20 fmol of SCP_B: the two SCPs are equipotent in this assay) to ensure linear responsiveness. All steps from the homogenization through to the bioassay calculations were done blind.

Measurement of cAMP. To determine what substances in I5 muscles caused increases in cAMP, 1 g (wet weight) of muscle (from 25 animals) was extracted as described above, and the extract split in half and run in duplicate through the same HPLC procedure as used for the bioassay. The fractions from one run were dried, taken up in 100 μl of ASW, and incubated with muscle segments for 5 min. The SCP-containing fraction from the duplicate run was dried and run through another HPLC procedure with trifluoroacetic acid as a counterion and a linear gradient from 5% to 53% CH_3CN in 20 min at 2 ml/min. These samples were also dried and tested for activity on I5 muscle segments. Muscle segments were then homogenized in 98% ethanol/2% 2 M HCl at −30°C, stored at least overnight at −30°C, and centrifuged for 10 min at 10,000 × *g*. The supernatant was used for cAMP determinations and the pellet was used for protein determinations. cAMP was measured initially with a radioimmunoassay (Biomedical Technologies, Norwood, MA), although a competitive binding assay (Amersham) was used for most samples. Similar values were obtained with each procedure. The pellet was homogenized in 500 μl of 0.1 M HCl, heated for 10 min at 100°C, and centrifuged as above, and this supernatant was used for protein assays (Pierce). Duplicate cAMP and protein determinations were carried out.

For the stimulation experiments, the bath was rapidly emptied and both I5 muscles were frozen with electronic component-freeze spray (GC-Electronics, Rockford, IL) and processed as described above. All steps after the homogenizations were done blind.

RESULTS

Distribution of the SCPs in Components of the Feeding System. The HPLC–bioassay procedure was used to quantify the SCPs in components of the feeding system (Table 1). Several inferences can be drawn from these data. First, the finding that the concentrations of the SCPs were similar in I5 muscles and in total buccal muscle suggests that the SCP innervation of buccal muscle is relatively uniform. This agrees with the observation that SCPs synthesized in buccal ganglia were transported in similar quantities to all muscles (24). Second, the amount of the SCPs in buccal nerves was only ≈1% of that in buccal muscle. Assuming a transport rate of 50–100 mm/day (25, 26) and an average length for a buccal nerve of 15 mm, SCPs present in buccal ganglia cell bodies could not be transported to buccal muscle in less than 4 hr. Thus, only the SCPs already in the nerves, or 1% of that in buccal muscle, could be replaced in 4 hr. This means that during sustained stimulation, either the SCPs are released from terminals in the muscle at very low levels or their content must decline.

Stimulation of B15 Depletes SCPs from I5 Muscles. To determine whether B15 stimulation could cause a depletion of SCPs from its terminals in I5 muscles, we examined the effects of setting up the dissection, impaling B15, and hyperpolarizing it for 1 hr. Under these conditions, SCP levels in the two bilaterally symmetrical muscles were nearly identical (Fig. 1). Next, the effects of stimulating B15 at an overall frequency of 5 Hz for 1 hr with different paradigms was investigated. Tonic 5 Hz, or 25 Hz for 2 s with 8-s interburst intervals, produced no significant depletion of the SCPs in the stimulated muscles. However, stimulation at 50 Hz for 1 s with 9-s interburst intervals caused a marked depletion from stimulated muscles. B16, the other cholinergic neuron that innervates I5 but that does not synthesize the SCPs, was stimulated with the same paradigm and produced no depletion (Fig. 1). As 50 Hz is very rapid firing for *Aplysia* neurons, the effects of stimulating B15 at lower frequencies with bursts of longer duration was investigated. Stimulation at 25 Hz for 4 s at 6-s interburst intervals (overall frequency of 10 Hz) also depleted the SCPs from the stimulated muscles (Fig. 1).

In the two stimulation paradigms that caused depletion, the difference in amount of SCPs suggests that the peptides were being released at a rate of about 1 fmol per min per μl of muscle volume. This value would be an underestimate of true release rates if an uptake system for the SCPs were present in the I5 muscle. Although diffusion and proteolysis are thought to be the predominant mechanisms for terminating the actions of released peptides, there is also evidence that uptake systems for neuropeptides do exist in some systems (27). We tested the I5 muscle to determine whether it did indeed contain an uptake system for the SCPs. High-specific-activity SCP_A (≈25,000 cpm/pmol) and SCP_B (≈35,000 cpm/pmol) were purified from buccal ganglia la-

Table 1. Measurement of SCPs in components of the feeding system

Tissue	SCP concentration, pmol/g (wet weight)	Total SCP content, pmol	*n*
Buccal ganglia	1910 ± 410	11.7 ± 1.7	3
Buccal nerves*	570 ± 440	1.7 ± 0.5	3
Buccal muscle	166 ± 34	145.3 ± 24.8	3
Muscle I5	191 ± 72	2.5 ± 0.9	12

SCP refers to all SCP-like activity (SCP_A plus SCP_B). Values are mean ± SD.

*Pooled right and left buccal 1, 2, 3, and radula nerves; these nerves provide the innervation of buccal muscle.

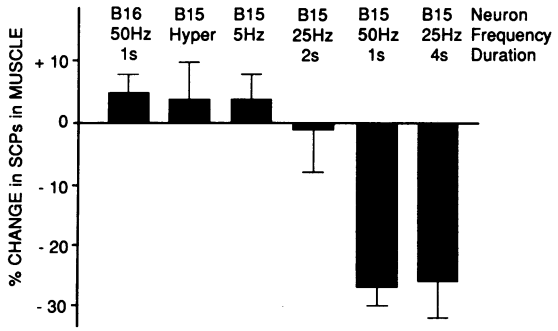

FIG. 1. Effects of stimulating cholinergic motor neuron B15 or B16 on the amount of the SCPs present in I5 muscles. Combined SCP_A and SCP_B bioactivity normalized to wet weight from stimulated muscle was compared to that from the unstimulated contralateral control muscle from the same animal. The interval between the end of a burst and the beginning of the next burst was 9 s for the 1-s bursts, 8 s for the 2-s bursts, and 6 s for the 4-s bursts. Values are mean ± SEM, $n = 4$.

beled with [^{35}S]methionine (10). I5 muscle segments were incubated with each of the labeled peptides (concentration, ≈1 μM) in ASW for 1 hr, then washed for either 20 min or 3 hr under conditions that inhibit release (high-Mg^{2+} low-Ca^{2+} ASW at 4°C), extracted, and run on HPLC. Unhydrolyzed labeled SCP_A and SCP_B were still present in the ASW at the end of the 1-hr incubation, suggesting that the muscle had limited proteolytic activity. By contrast, SCPs were completely hydrolyzed within 5 min in *Aplysia* blood (P.E.L., unpublished observation). There was no detectable labeled SCP in muscle extracts with either the 20-min or the 3-hr wash. These results suggest that the SCPs are not taken up or that they are hydrolyzed soon after uptake and that depletion rates probably represent true release rates.

Stimulation of B15 Enhances cAMP Levels in I5 Muscles. Exogenous SCPs potently stimulate the cAMP levels of isolated I5 muscles (Fig. 2). To minimize variability, these data were generated using control and experimental muscles from the same animal. This is a procedure similar to that used below in the motor neuron-stimulation experiments. SCP_B at concentrations as low as 30 nM produced marked increases in cAMP. The effects of serotonin are shown for comparison. The SCPs are considerably more effective at low concentrations (<1 μM), whereas serotonin has a greater maximal effect on cAMP levels (also see ref. 16). Acetylcholine has no effect on cAMP levels in I5 muscles (20).

To identify the major substances present in I5 that could elevate cAMP levels in the muscle, pooled I5 muscles were fractionated by the same HPLC procedure used in the depletion experiments. The resulting fractions were dried, taken up in ASW, and applied to I5 muscle segments. After 5 min, each muscle segment was homogenized and assayed for cAMP content. Two fractions were found to cause large increases in cAMP. The most potent fraction contained the SCPs, while a somewhat less potent fraction was found with the short retention time of serotonin (Fig. 2). Other fractions had little activity. To determine whether all the activity in the SCP-containing fraction was associated with the SCPs, the same fraction from a duplicate HPLC run was dried and subjected to HPLC with a different counterion and gradient. When these fractions were assayed on muscle segments, two peaks with roughly similar activity and with the same retention times as the two SCPs were found (Fig. 2*C*). Thus, serotonin and the SCPs appear to be the major substances present in I5 muscle extracts that increase cAMP in the muscle.

To determine the feasibility of B15 stimulation causing an increase in muscle cAMP, a very intense stimulation paradigm (50 Hz for 2 s at 3-s intervals for 10 min) was employed.

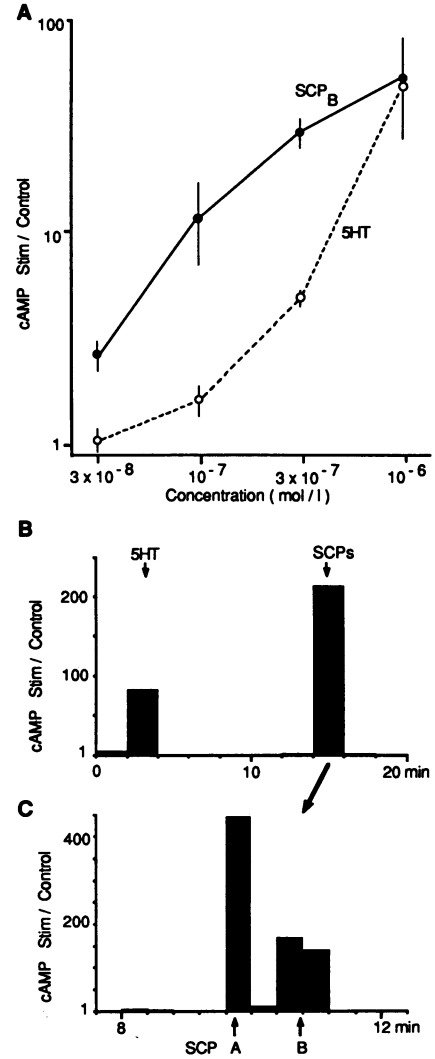

FIG. 2. Effects of substances on cAMP levels in I5 muscles. (*A*) Dose–response curves for synthetic SCP_B (solid line) and serotonin (5-hydroxytryptamine, 5HT; dashed line). I5 muscle segments were incubated for 5 min in ASW containing the indicated concentration of agonist. In all cases, cAMP in stimulated muscles (Stim), normalized to protein, was compared to control muscles from the same animal. Values are mean ± SEM, $n = 3$. (*B*) HPLC analysis of substances present in I5 muscle extracts that were capable of elevating cAMP levels in I5 muscles. The HPLC procedure was the same as that used in the depletion experiments. Fractions that contained serotonin (5HT) and the SCPs are indicated. Myomodulin was eluted in the fraction before the SCPs and buccalin in the fraction before that. (*C*) Fraction containing the SCPs from a duplicate HPLC run to that shown in *B* was subjected to HPLC with a second gradient and a different counterion (see *Methods*) and fractions were tested for their ability to increase cAMP in I5 muscles. Arrows indicate the positions of absorbance peaks of synthetic SCPs from a subsequent run. Note that the SCP_B peak was split by the fraction-collection scheme.

The muscle was superfused with hexamethonium, which blocks muscle cholinergic receptors and prevents contractions (18). This stimulation produced a >300-fold increase in cAMP (Fig. 3). As a control, B16 was stimulated identically. This produced a 1.4-fold rise in cAMP levels, which was similar to that produced by sustained hyperpolarization of B15 (Fig. 3). Less intense stimulation of B15 (25 Hz for 4 s with 6-s intervals for 10 min) also produced very large increases in cAMP (Fig. 3). The magnitude of these increases was the same in the presence or absence of hexamethonium. This result, together with the results from stimulating B16, indicates that cholinergic neuromuscular transmission or the

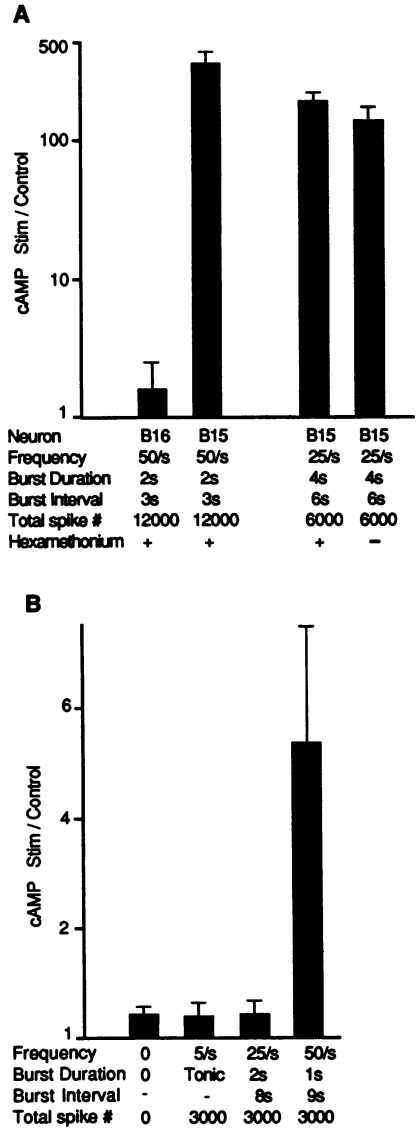

FIG. 3. Effects of stimulating cholinergic motor neurons B15 and B16 on cAMP levels in I5 muscles. cAMP normalized to protein of stimulated muscle (Stim) was compared to that of the unstimulated contralateral control muscle from the same animal. Burst interval refers to the period from the end of a burst to the beginning of the next burst. (*A*) Comparisons of the effects of intense stimulation of B15 and B16 and less intense stimulation of B15 in the presence or absence of the cholinergic antagonist hexamethonium (0.5 mM). Overall stimulation duration was 10 min. Note logarithmic scale. Values are mean ± SEM, $n = 4$ except for the no-hexamethonium experiment, where $n = 3$. (*B*) Effects of hyperpolarizing B15 or of stimulating B15 at an overall rate of 5 s^{-1} with different stimulation paradigms. Values are mean ± SEM, $n = 4$, except for the B15 hyperpolarization experiment (0 spikes), where $n = 2$.

muscle contractions themselves were minimally involved in the changes in cAMP levels produced by B15 stimulation.

The effects of B15 stimulation on cAMP levels in I5 were so marked that we investigated the possibility that there were additive or synergistic actions between the SCPs and the other modulatory peptide present in B15, buccalin. As expected from the fractionation experiment, buccalin on its own had little effect on cAMP levels (1 μM buccalin, 1.2 ± 0.3-fold stimulation, mean ± SEM, $n = 4$). It did not enhance the effectiveness of the SCPs (1 μM SCP_B, 135 ± 30-fold stimulation; 1 μM SCP_B and 1 μM buccalin, 94 ± 20-fold stimulation, $n = 4$ for each). These results suggest that there is little interaction between the SCPs and buccalin in their effects on cAMP levels.

Elevation of cAMP Levels Shows the Same Frequency Dependence as Depletion of the SCPs. Next, the effects of stimulating B15 with an overall firing frequency of 5 Hz with the same spike patterns used in the SCP-deletion experiments were tested. The only difference between the experiments was the duration, which was 1 hr in the depletion experiments and 10 min in the cAMP experiments. Tonic stimulation of B15 at 5 Hz or stimulation at 25 Hz for 2 s with 8-s intervals, yielded small (≈1.4-fold) increases in cAMP that were not different from those produced by sustained hyperpolarization (Fig. 3). However, stimulation at 50 Hz for 1 s with 9-s intervals produced a marked increase in cAMP. Thus, the frequency dependence of the cAMP enhancement accurately paralleled the frequency dependence of the depletion of SCPs from terminals in the muscle. That is, stimulation at 25 Hz for 2 s with 8-s intervals produced neither peptide depletion nor marked increases in cAMP, whereas either 50 Hz for 1 s with 9-s intervals or 25 Hz for 4 s with 6-s intervals produced both effects. We propose that depletion reflects release from B15 terminals of the SCPs, which then act to enhance muscle cAMP levels.

Dependence of cAMP Levels on B15 Stimulation Parameters. We wished to determine which stimulation parameters were important in regulating the release of the SCPs as measured indirectly from increased cAMP levels. First, B15 stimulation frequency was systematically lowered while the other parameters were kept constant (4-s bursts with 6-s intervals for 10 min). Elevation of cAMP levels was marked at 25 Hz (Fig. 3*A*) and still observed at 20 Hz but had disappeared by 17.5 Hz (Fig. 4). When the interburst interval was halved (4-s bursts with 3-s intervals for 10 min) stimulation of B15 at 15 Hz produced marked increases in cAMP whereas stimulation at 12.5 Hz had little effect (Fig. 4). Thus, the critical frequency for elevating cAMP levels was lowered by either increasing burst duration or decreasing interburst interval.

DISCUSSION

The results from the depletion experiments strongly suggest that the SCPs are released during stimulation of B15. Further, this release is steeply dependent on stimulation frequency,

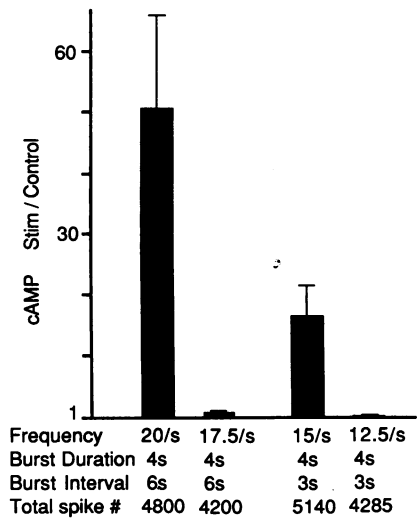

FIG. 4. Effects of stimulating motor neuron B15 on cAMP levels in I5 muscles. cAMP normalized to protein of stimulated muscle (Stim) was compared to that of the unstimulated contralateral control muscle from the same animal. Burst interval refers to the period from the end of a burst to the beginning of the next burst. Values are mean ± SEM, $n = 4$.

burst duration, and interburst interval. Stimulation of B16, another cholinergic motor neuron, which does not contain the SCPs, did not cause depletion. The effects of motor neuron stimulation on muscle cAMP levels provide independent support that the SCPs are being released. Again, stimulation of B15, but not B16, produced large increases in muscle cAMP, and these increases occurred only when B15 was stimulated with a paradigm that produced depletion. Taken as a whole, these results provide convincing evidence that the SCPs are being released from the terminals of B15 in muscle. The only result that does not fit readily with this proposal is that stimulation at 25 Hz for 4 s with 6-s interburst intervals produced depletion of the SCPs similar to that of stimulation at 50 Hz for 1 s with 9-s interburst intervals but produced a much larger increase in cAMP in the muscle. We have no explanation for these results; however, there is no reason to expect that cAMP levels should be directly proportional to the release of the SCPs.

The increase in cAMP observed with the more intense stimulation paradigms corresponds to the increases expected from incubating the muscle in 1–10 μM exogenous SCPs. This value contrasts with the release rate for SCPs of about 1 fmol per min per μl of muscle volume estimated from the depletion experiments. Peptides released at this rate for 10 min would only reach a concentration of 1 μM if they were confined to a volume equal to 1% of the muscle. This volume would be reasonable if the SCP receptors were localized very close to release sites. Currently, nothing is known about the localization of the SCP receptors relative to B15 terminals.

The results from the depletion experiments suggest that there are stimulation parameters that produce very little release of the SCPs. The cAMP experiments suggest that this threshold is very sharp. For example, stimulation at 17.5 Hz for 4 s with 6-s intervals yielded cAMP levels that were not different from control, whereas increasing the frequency to 20 Hz produced an elevation of about 50-fold. This threshold can be lowered by increasing burst duration or decreasing interburst interval. For example, this threshold frequency for 4-s bursts dropped to below 15 Hz when the interburst interval was decreased from 6 s to 3 s. It should be emphasized that rates of release of the SCPs necessary to produce measurable elevation of cAMP levels are likely to be higher than those necessary to produce threshold modulatory effects. In comparison, cholinergic junction potentials are produced by single B15 spikes and muscle contractions are observed with stimulation frequencies as low as 5 Hz. Thus, B15 terminals may release only acetylcholine during low-frequency firing, but both acetylcholine and the SCPs during high-frequency firing.

Although we know of no other case where the release of peptides from single neurons has been systematically analyzed, a number of studies have addressed peptide release during nerve or field stimulation. In general, these studies gave results similar to those presented here. Higher frequency stimulation was required for the release of peptides than for conventional transmitters (3). However, it is clear that the stimulation frequencies required to produce peptide release is specified primarily by the properties of the neurons and not the transmitters themselves. For example, release of the SCPs from B15 requires considerably higher firing frequencies than the 2- to 3-Hz stimulation that effectively release the SCPs from B1 and B2 neurons in culture (17).

Release of the SCPs from B15 terminals occurs specifically when the motor neurons are firing at high frequency and with short interburst intervals. In a behavioral sense, this would be most likely to occur during food-induced arousal, when the amplitude and frequency of buccal muscle contractions associated with biting movements are maximal. This is also the time at which the serotonergic metacerebral neurons (MCCs) are most active (28). Intracellular stimulation of the MCC has been shown to increase cAMP levels and enhance motor neuron-driven contractions in the I5 muscle (20). Apparently, serotonin release from the terminals of the MCC acts in concert with the SCPs to enhance cAMP levels in the muscle and modulate contractions. Thus, motor neuron B15 is well suited to release the SCPs to produce larger contractions and more rapid rates of relaxation during food-induced arousal. Interestingly, in a study in which both MCCs were lesioned by intracellular injection of proteolytic enzymes, the animals demonstrated a marked deficit in the increased frequency of biting but little deficit in the increased magnitude of biting during arousal (29, 30). One explanation of these observations is that during food-induced arousal, a significant component of the modulation of contractions of buccal muscles may be due to the release of the SCPs.

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