44 Satchell, D. G., and Twarog, B. M., Identification of 5-hydroxytryptamine (serotonin) released from the anterior byssus retractor muscle of *Mytilus californianus* in response to nerve stimulation. Comp. Biochem. Physiol. *59C* (1978) 81–85.
45 Satchell, D. G., and Twarog, B. M., Identification and estimation of dopamine released from the anterior byssus retractor muscle of *Mytilus californianus* in response to nerve stimulation. Comp. Biochem. Physiol. *64C* (1979) 231–235.
46 Twarog, B. M., Responses of a molluscan smooth muscle to acetylcholine and 5-hydroxytryptamine. J. cell. comp. Physiol. *44* (1954) 141–164.
47 Twarog, B. M., The pharmacology of a molluscan smooth muscle. Br. J. Pharmac. *14* (1959) 404–407.

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Peptidergic co-transmission in *Aplysia*: Functional implications for rhythmic behaviors

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Abstract. Despite their ubiquitous presence in the central and peripheral nervous systems, the behavioral functions of peptide co-transmitters remain to be elucidated. The marine mollusc *Aplysia*, whose simple nervous system facilitates the study of the neural basis of behavior, was used to investigate the role of peptidergic co-transmission in feeding behavior. Several novel modulatory neuropeptides were purified, and localized to identified cholinergic motorneurons. Physiological and biochemical studies demonstrated that these peptides are released when the motorneurons fire at frequencies that occur during normal behavior, and that the peptides modify the relationship between muscle contraction amplitude and relaxation rate so as to maintain optimal motor output when the intensity and frequency of feeding behavior change.
Key words. Aplysia; co-transmission; modulation; motorneuron; muscle; peptide.

Our understanding of chemical synaptic transmission has been significantly influenced by the widespread finding that a single neuron may contain and release more than one transmitter substance. In many cases a classical neurotransmitter, such as acetylcholine or GABA, is co-localized with a modulatory neuropeptide[1, 13, 17]. A number of suggestions have been made to explain the functional role of the co-release of classical and peptide transmitters, but despite the impressive progress that has been made in understanding the cellular mechanisms of action of both classical and peptide transmitters, relatively little is known about the behavioral consequences of co-transmission[3, 4]. The challenging task of understanding its behavioral role has not been made any easier by recent reports indicating that many neurons contain more than one peptide co-transmitter (see, for example, Jones et al.[15] and Vincent et al.[32]). Some of the best evidence relating co-transmission to behavior has been obtained in invertebrates[17], but even in these relatively simple systems it has not yet been possible to reach unequivocal conclusions regarding the physiological contribution that even single peptide co-transmitters make to behavior.

We have chosen to study the physiological role of peptidergic co-transmission in a simple neuro-behavioral model system: the feeding behavior of the marine mollusc *Aplysia californica*. Even though the consummatory phases of feeding (biting and swallowing) are highly stereotyped, the size and frequency of these responses are strongly affected by the internal state of the animal, for example, whether the animal is satiated or aroused[28]. Parameters of feeding behavior are also influenced by the nature of the food the animal is ingesting, for example, its size and hardness[16]. In this paper we present a hypothesis, with supporting evidence, that suggests that peptidergic co-transmission plays a major role in maintaining the efficiency of the feeding behavior in the face of such changing behavioral demands.

Let us consider a simple change in feeding behavior that occurs at the beginning of a meal. Figure 1 (A and B) illustrates how the speed and strength of biting change as the animal becomes exposed to food. In the beginning, individual bites are weak and occur at a slow rate. Subsequent bites become stronger and faster until both the strength and speed of biting reach a plateau[28, 34]. At first glance, it may appear that this adjustment of the rate and magnitude of biting is a straightforward task that may be accomplished simply by increasing the biting cycle frequency and firing rates of feeding motorneurons. However, it is by no means obvious how it is achieved when we realize that if animals are to maintain their ability to ingest food, some 15 pairs of muscles must maintain proper phase and intensity relationships in the face of severe biomechanical constraints.

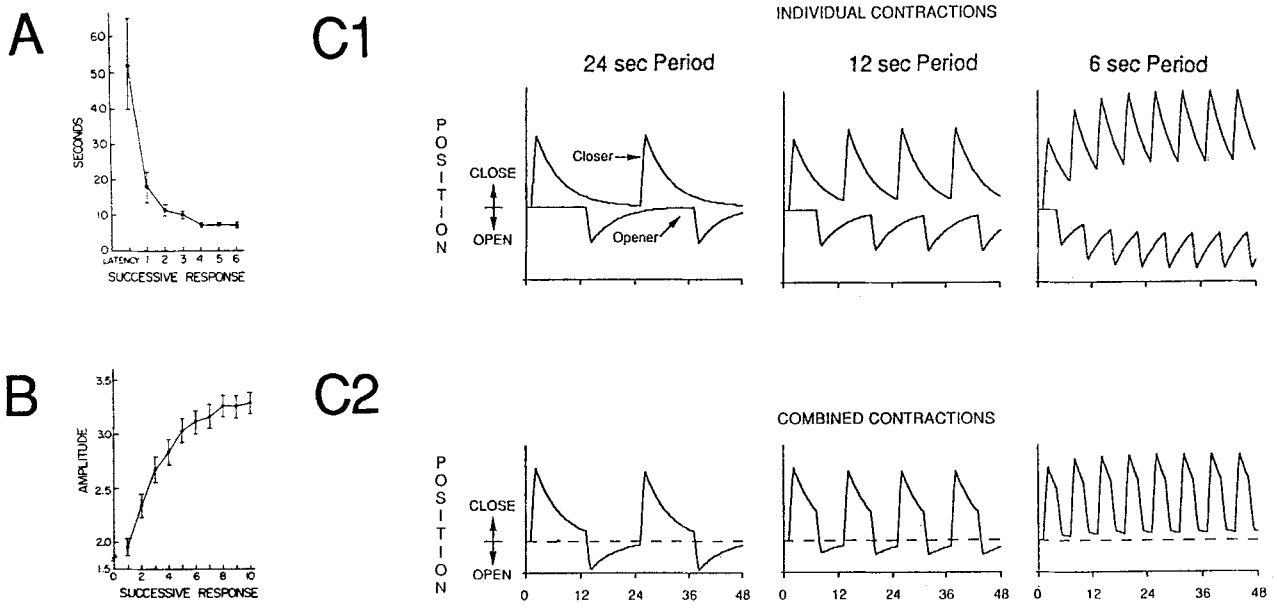

Figure 1. Plasticity of feeding behavior and the problems it poses for the integration of contractions of the muscles that open and close the radula of *Aplysia*. *A*) Decrease of the successive inter-bite intervals of an animal continuously stimulated with food. *B*) Enhancement of the strength of successive biting responses of an animal continuously stimulated with food. The strength of the biting responses was measured on an arbitrary 1-to-4 scale as described by Susswein et al.[28]. *C*) Schematic representation of the effects of increasing frequency of biting on the integrated output of the radula closer and opener muscles. *C1)* shows contractions of the closers and openers separately, *C2)* shows the integrated movement that results from the algebraic sum of these contractions; the dashed line indicates transition between the closed and the open state of the radula. The frequency of the contractions increases in successive columns of *C1* and *C2*. In the 1st column, the cycle period is long enough to allow the muscles to relax fully before the next contraction and a functional motor pattern is produced, i.e., both closing and opening of the radula occur (bottom panel). As the frequency of contractions increases (2nd column), muscles no longer relax fully before the next contraction. Notice the build-up of baseline tension (particularly in the more powerful closer muscle) and the resulting reduction in opening of the radula (bottom panel). As the frequency of contractions increases still further (3rd column), the baseline tension is even greater and the radula no longer opens at all.

For the sake of simplicity, the biomechanical constraints on the feeding neuromuscular system will be illustrated by considering just one of the pairs of antagonistic muscles, those that open and close the radula, a hand-like organ which the animal uses to grasp food. Obviously, if the animal is to grasp its food successfully, the radula has to open and close at the appropriate times. Figure 1 C is the graphic presentation of the output of a simple model that incorporates contractions of a powerful radula closer muscle and a relatively weak radula opener muscle; the top panels of the figure show contractions of the two muscles separately, the bottom panels show the integrated movements, representing opening and closing of the radula, that result from the algebraic sum of these contractions. In the first column the cycle period is long enough to allow each of the muscles to relax fully before the antagonist contracts, thereby assuring the production of a functional motor output (the bottom panel shows that both opening and closing of the radula occur). The second and third panels show that as the biting frequency increases, the muscles no longer relax fully before the next contraction, and the functional output of the system is destroyed. The contractions of the closer muscle predominate and opening of the radula no longer occurs (bottom panel). The situation is even worse when increases in the magnitude of biting are simultaneously incorporated into the model (as shown later in fig. 5); given a constant relaxation rate, the larger the muscle contractions, the longer the time needed for the muscle to relax completely. In this paper, we suggest that peptide co-transmitters in the cholinergic motorneurons of *Aplysia* may function to adjust the relationships between contraction frequency, magnitude, and relaxation rate in order to maintain optimal phase relationships between antagonistic muscle pairs when frequency and strength of biting change.

Neuropeptides in the ARC neuromuscular system

We have studied the accessory radula closer (ARC) muscle (the 'closer muscle' in our model) and its innervation by two cholinergic motorneurons, cells B15 and B16[5], and a modulatory neuron, the serotonergic metacerebral cell[33]. The ARC neuromuscular system has been well characterized[5], and when initial studies indicated that modulatory neuropeptides were present in neuronal terminals and varicosities in the muscle[21], we decided that this preparation would have many advantages for the experimental study of peptide co-transmission.

We first characterized the complement of modulatory neuropeptides present in the innervation of the ARC muscle (fig. 2, A and B). Peptides were purified using reverse-phase high performance liquid chromatography (RP-HPLC). ARC muscles and attached neuronal termi-

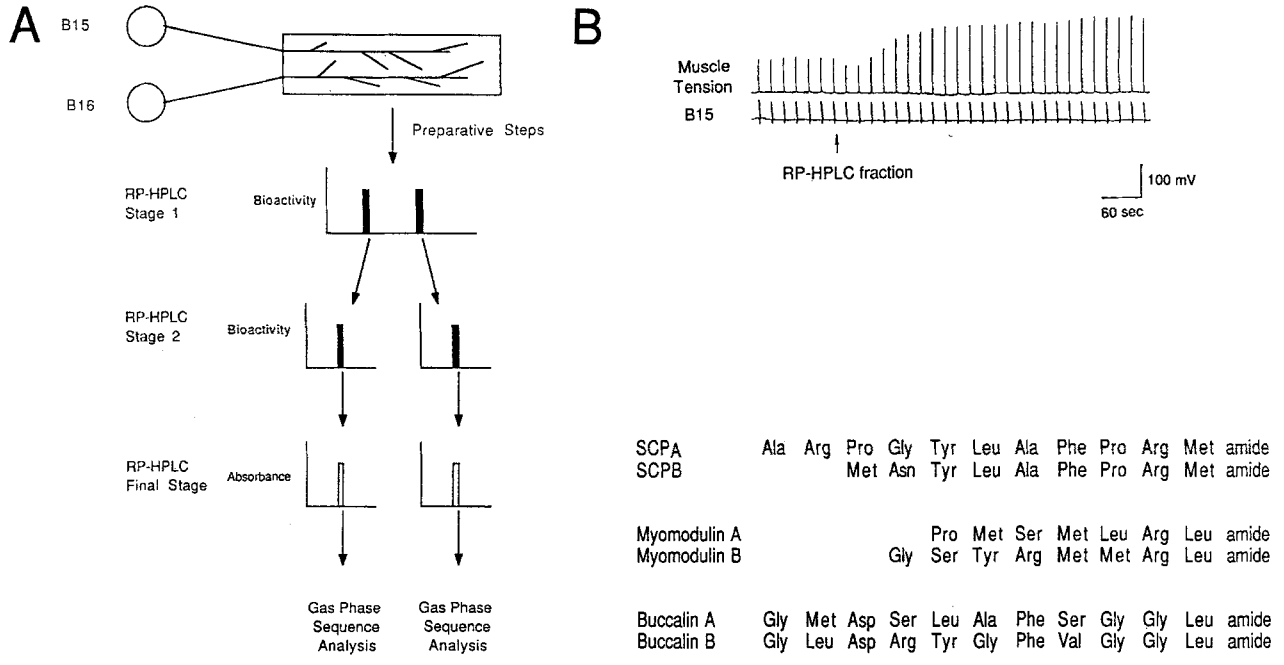

Figure 2. *A*) Schema of the procedure used for purification of peptide co-transmitters from processes of motorneurons B15 and B16 in the AC muscle. ARC muscle extract was pre-purified to remove large proteins and salts, and then subjected to reverse-phase HPLC (RP-HPLC). All of the resulting fractions were bioassayed (see *B*), and all bioactive fractions were rechromatographed until optically pure material was obtained. Optically pure peaks were subjected to gas-phase sequence analysis. *B*, top) Procedure used to test RP-HPLC fractions for bioactivity in the ARC neuromuscular system. Motorneurons were stimulated in bursts (bottom trace) to evoke reproducible muscle contractions (top trace). Lyophilized RP-HPLC fractions were resuspended in artificial seawater and applied directly to the muscle. In the example shown, an RP-HPLC fraction (added at the arrow) increased the size of the contractions. *B*, bottom) Neuropeptides purified from the ARC neuromuscular complex (SCP, small cardioactive peptide).

nals and varicosities were used as the source of material. RP-HPLC fractions were identified for rechromatography by their bioactivity; that is, whether they modulated ARC muscle contractions produced by stimulation of either of the ARC's two motorneurons. With this strategy we were assured that only peptides that are bioactive in the system and are potential co-transmitters would be purified. Material was rechromatographed to optical purity and then subjected to gas-phase sequence analysis. These experiments showed that the ARC neuromuscular complex contains the invertebrate neuropeptide FMRFamide[35] as well as members of three additional multi-peptide families, the small cardioactive peptides (SCPs[10,21]), the myomodulins (MMs[9,12,25]), and the buccalins (BUCs[7,11,23,29]). The SCP family consists of the characterized peptides SCP_A and SCP_B[27]. The MM and BUC families are known each to contain several different peptides, but only two peptides in each family (MMa and b, and BUCa and b) have been fully characterized. Finally, these peptides are not unique to *Aplysia*: the SCPs are present in several different molluscs[19], and it has now been demonstrated that at least one member of both the MM and BUC peptide families is present in *Fusinus*[18].

Preliminary studies suggested that at least some of the purified peptides might indeed be present in the ARC's two motorneurons, B15 and B16. For example, immunocytological staining of buccal ganglia with antibodies raised against the SCPs[20], MMs[26], or BUCs[11] revealed that neurons in the ventral motorneuron cluster (which contains neurons B15 and B16) are peptide-immunoreactive. These results were confirmed in subsequent studies in which physiologically identified B15 and B16 neurons were injected with Lucifer Yellow dye and processed for immunocytochemistry. These experiments indicated that both neurons B15 and B16 are buccalin-immunoreactive[7,11]. However, only neuron B15 is immunopositive for the SCPs[10], and only neuron B16 is immunopositive for MMs[26].

Since antibodies directed against short peptides often cross-react with related peptides, independent verification of these immunocytochemical results was obtained with biochemical techniques. The procedure used in these experiments is outlined in figure 3A. Physiologically identified B15 and B16 neurons were marked with Fast Green dye. The buccal ganglia were then incubated in the presence of radioactive amino acids (e.g., ^{35}S-methionine) long enough for them to be taken up and incorporated into newly synthesized peptides. Dye-labeled neurons were dissected, and radioactively labeled peptides extracted in the presence of nanomolar quantities of cold synthetic peptides, and subjected to several stages of RP-HPLC. Co-elution of radioactive material with a synthetic peptide was taken to indicate that the identified B15 or B16 neuron did indeed contain that particular peptide. Results obtained in these biochemical experiments were consistent with the immunocytochemical results. Thus, neuron B15 was found to contain SCP_A and SCP_B[10] and

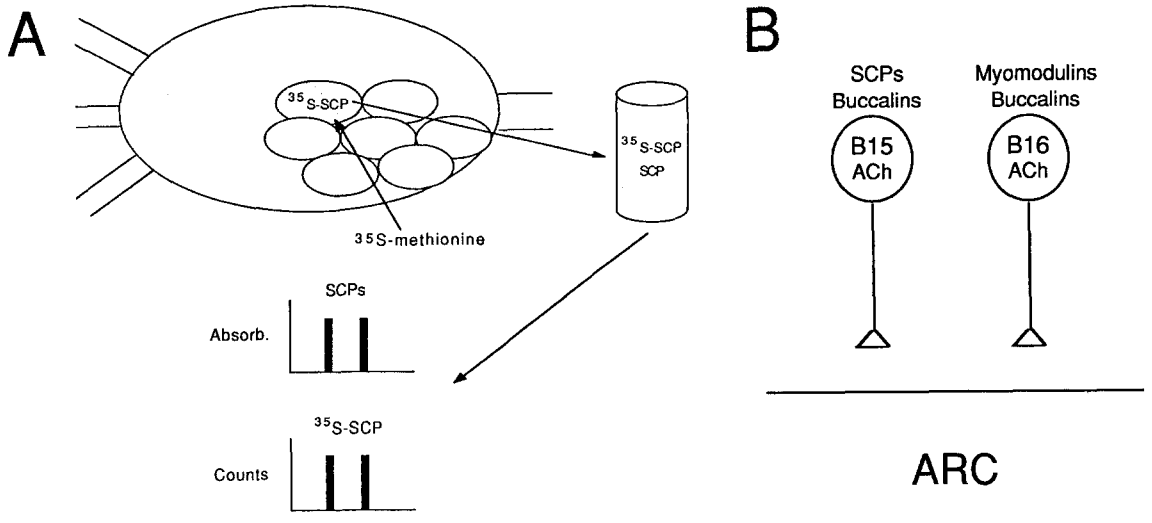

Figure 3. *A*) Schema of the procedure used for in situ radiolabeling of neuropeptides contained in the ARC motorneurons. The motorneurons were physiologically identified and injected with Fast Green dye. The buccal ganglia were incubated in medium containing radiolabeled amino acids, using amino acids present in the peptides of interest. For example, to radiolabel the SCPs the buccal ganglia were incubated in ^{35}S-methionine. Dye-marked neurons were individually dissected and placed in tubes containing nanomolar quantities of the synthetic peptides of interest. This mixture was then subjected to RP-HPLC. The elution time of the synthetic peptides was determined by absorbance measurements, the elution time of radiolabeled peptides by scintillation counting. Positive results were considered to be obtained if a radiolabeled peptide synthesized by one of the ARC motorneurons had chromatographic properties identical to those of a synthetic peptide. *B*) Schema summarizing the localization of peptide cotransmitters in the ARC motorneurons B15 and B16.

at least BUCa and BUCb[11,29], while neuron B16 contains at least MMa and MMb[9,12] and BUCa and BUCb[7,29]. These findings are summarized in figure 3 B. As already noted, these experiments indicated that the ARC peptides are present in families; for instance, there is not just one buccalin, there are at least two structurally related buccalin peptides. This suggested that although there are many peptides in the ARC neuromuscular system, there might be relatively few peptide precursors, for example there might be one large protein that encodes all of the buccalins. Thus, the characterization of the cotransmitter content of the ARC neuromuscular system might be simplified by using molecular-biological techniques to characterize peptide precursors, rather than the biochemical techniques to characterize individual peptides one at a time. Degenerate oligonucleotide probes based on the sequences of one of the MMs and one of the BUCs were therefore used to isolate cDNA clones of large precursor proteins. One of these proteins was found to encode the amino acid sequences of all the MMs that we purified biochemically as well as the amino acid sequences of two novel forms of myomodulin[24]. Another protein encodes the two characterized buccalins as well as 17 additional structurally related peptides[23]. Future experiments will determine whether these additional forms of MMs and BUCs are actually present in neurons B15 and B16.

Actions of neuropeptides in the ARC neuromuscular system

Within a particular peptide family, bioactivity appears to be similar. Thus, both SCP_A and SCP_B enhance the size and relaxation rate of ARC muscle contractions produced by motorneuron stimulation (fig. 4, A and B[10,11,21]). Similar potentiating actions on the size and relaxation rate are exerted by both MMa and MMb[9,12]. In contrast, both BUCa and BUCb decrease the size of motorneuron-elicited muscle contractions but do not affect their relaxation rate (fig. 4 B[7,11,29]). The differences between the inhibitory effects of the BUCs and the potentiating effects of the SCPs and MMs are correlated with differences in the sites of actions of these two types of peptides. The inhibitory BUCs appear to act exclusively presynaptically; buccalin application decreases the amplitude of cholinergic EJPs and muscle contractions evoked by motorneuron stimulation, but has no effect on the amplitude of muscle contractions elicited by direct application of ACh (fig. 4, C and D[7,11]). In contrast, the potentiating SCPs and MMs all have at least some postsynaptic actions, since they do increase the size of the ACh-elicited muscle contractions[9].

Prompted by the finding that the postsynaptic actions of SCPs and MMs are modulatory and probably indirect, we began to investigate signal transduction pathways that might be responsible. Several lines of biochemical and physiological evidence indicate that the SCPs exert their action via a cAMP-dependent protein kinase (PKA). Thus, we found that exposing the ARC muscle to the SCPs caused a dose-dependent increase in cAMP synthesis[21] and increased activation of PKA[14]. Furthermore, addition of the SCPs to muscle homogenates increased phosphorylation of several proteins whose phosphorylation was found to be increased also when PKA was activated directly by addition of cAMP to the homogenate[14]. Using a back-phosphorylation paradigm

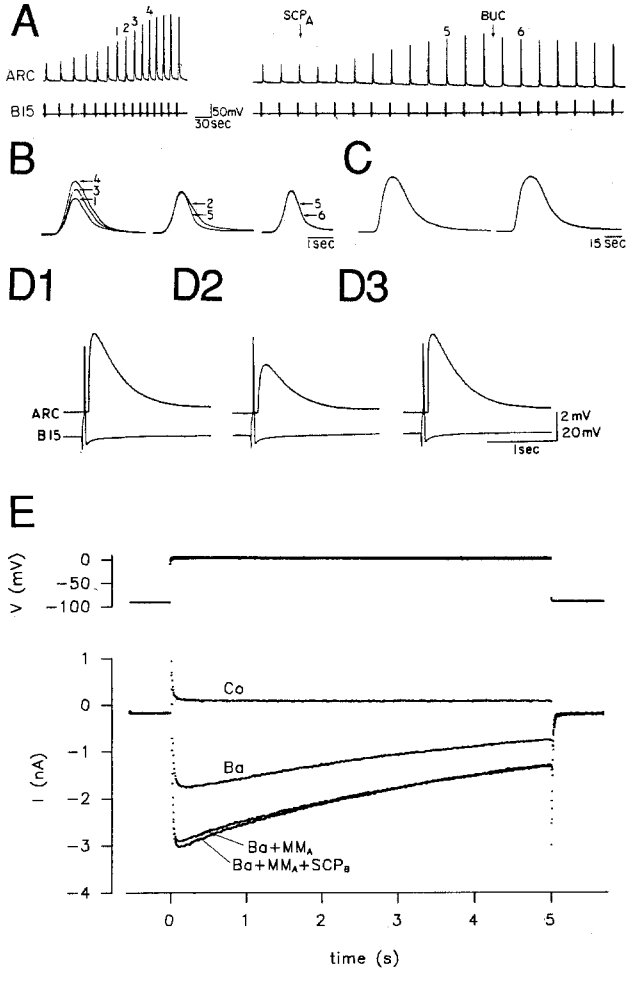

Figure 4. *A*) Effects of synthetic SCP_A and BUCa on the ARC muscle. The top traces are records of ARC contractions, the bottom traces records of stimulation-evoked regular bursts of spikes in motorneuron B15. In *B* contractions 1–6 from *A* are shown at a faster speed. *A*, left) When the size of the ARC contractions is increased by decreasing the inter-burst interval, i.e. increasing post-tetanic potentiation (PTP), the contractions take longer to relax, as is shown by the superimposition of contractions 1, 3, and 4 in *B*, left. In contrast, contractions potentiated by synthetic SCP_A (10^{-9} M) relax faster, as is shown in *B*, middle, where a contraction potentiated by PTP (contraction 2) is superimposed on a contraction potentiated by SCP_A (contraction 5). Addition of synthetic BUCa (10^{-8} M) reduces the size of the contractions without affecting their relaxation rate, as shown in *B*, right, where a contraction potentiated by SCP_A (contraction 5) is superimposed on a contraction subsequently reduced again by BUCa (contraction 6). *C*) Records of contractions produced by direct application of 20 pmoles of ACh to the ARC muscle, before addition of 10^{-6} M BUCa (left) and 7 min after its addition (right). BUCa does not change the contraction size. *D1–D3*) Effect of BUCa on EJPs in the ARC muscle. The top traces are intracellular records from an ARC muscle fiber showing the EJPs elicited by the stimulated spikes in motorneuron B15 that are shown in the bottom traces. *D1*, *D2*, and *D3* are before, during, and after application of 10^{-6} M BUCa, respectively. *E*) Current through L-type Ca channels is increased by the SCPs and MMs in a mutually occlusive manner. Current (bottom) carried by Ba^{2+} through Ca channels, which in other experiments were identified as dihydropyridine-sensitive L-type channels, was elicited in a voltage-clamped dissociated ARC muscle fiber by 5 s voltage steps (top) from −90 to 0 mV in Na^+-free solution containing 460 mM tetraethylammonium and 10 mM 4-aminopyridine to block masking K currents. Bath application of 10^{-6} M MMa increased the Ba current dramatically (typically by 50–100%); subsequent application of 10^{-6} M SCP_B had virtually no effect. In other experiments in which the order of application was reversed, SCP_B similarly increased the Ba current and occluded the effect of MMa. Both the unmodulated und modulated Ba current was completely blocked when the Ba^{2+} in the bath solution was replaced with Co^{2+}.

we extended these in vitro results to the in vivo situation. This paradigm takes advantage of the fact that proteins that have already been phosphorylated in vivo cannot be phorphorylated further in vitro since their phosphorylation sites are already occupied. We found that after intact ARC muscles had been exposed to the SCPs, protein phosphorylation produced by addition of cAMP to homogenates of the muscle was significantly suppressed [14]. Together, these data provide strong biochemical evidence that the SCPs act by stimulating cAMP synthesis, thus leading to PKA activation and protein phosphorylation. The identity of the phosphorylated proteins remains to be determined, but the observation that more than one protein is phosphorylated is consistent with the complexity of the actions of the SCPs. In support of these biochemical experiments, we found that activation of PKA by exogenously applied analogs of cAMP or by forskolin (which stimulates the synthesis of endogenous cAMP) mimicked the actions of the SCPs, that is, both treatments enhanced both the amplitude and the relaxation rate of contractions produced by motorneuron stimulation [14].

Despite the fact that much is known about the mechanism of action of the SCPs little is known about the signal transduction pathway for MM [36]. Evidence has been obtained, however, that indicates that the actions of the SCPs and MMs converge on a molecular level. For example, we have demonstrated that under *in vitro* conditions the MMs are able to phosphorylate several proteins that are also phosphorylated by PKA [14]. Thus, these results strongly suggest that the similarity of the physiological actions of the SCPs and MMs results from the ability of peptides of these two families to phosphorylate common target proteins.

Independent evidence supporting the hypothesis that the actions of the SCPs and the MMs converge on a molecular level was obtained in physiological experiments in which we investigated possible mechanisms responsible for the potentiation of muscle contractions produced by these two peptide families. Using dissociated ARC muscle fibers we found that both the SCPs and the MMs increase a voltage-dependent L-type calcium current (fig. 4E [2]). Since this calcium current is activated in the voltage range that the muscle is brought into when it is stimulated to contract by ACh, it is likely that enhancement of the current by these peptides is involved in their potentiation of the amplitude of the contractions. These experiments are consistent with the idea that the signal transduction pathways activated by the SCPs and the MMs converge on the same molecular targets, one of these being the L-type calcium channel. We reasoned that, if the same calcium channels are indeed modulated by the SCPs and MMs, then application of one of the peptides at a concentration that produces maximal enhancement of the calcium current should occlude further effects of the other peptides. Indeed, we found that in the presence of maximally enhancing doses of SCPs, the

MMs produced no additional enhancement of the calcium current[2]. Similarly, muscles exposed to maximally enhancing concentrations of MMs were no longer affected by the SCPs (fig. 4E[2]). Cyclic AMP and forskolin also increased the calcium current and occluded the effects of both SCPs and MMs[2]. Taken together, these experiments provide physiological support for the hypothesis that the SCPs and the MMs activate a signal transduction pathway that converges on the same muscle proteins to modulate contraction of the muscle.

Behavioral contribution of the ARC peptide co-transmitters

The fact that neuropeptide co-transmitters are present in the ARC neuromuscular system and that they are bioactive when exogenously applied does not prove that release of these peptides occurs in feeding animals. The observation that the SCPs and BUCs are located in dense-core vesicles in motorneuron B15[30], and measurements of peptide depletion from B15 terminals after unphysiologically high frequencies of stimulation[36], indicated that these peptides belong to the releasable pool of transmitters. This, however, does not directly demonstrate that they are released during feeding behavior.

In order to ascertain whether these peptides are released under physiological conditions, we determined the patterns and firing frequencies of the two ARC motorneurons in free-moving feeding animals, and then reproduced these patterns and frequencies under experimental conditions in which peptide release and action could be studied quantitatively. We found that in fully aroused animals performing repetitive bite-swallows every 7 s, motorneuron B15 fired for approximately 3.5 s, i.e., half of the duty cycle, at frequencies of up to 12 Hz, while B16 fired at twice these frequencies and began firing approximately 1 s before B15[6, 8]. We then used these parameters to stimulate motorneuron B15 to fire for extended periods in vitro, and demonstrated that such stimulation results in significant depletion of the SCP content of the B15 terminals in the ARC muscle[8]. These experiments provide prima facie evidence that under physiological conditions the SCPs are released from B15 terminals. In view of our immunocytological demonstration of co-localization of buccalins and SCPs in the same dense-core vesicles in the terminals[30], these experiments suggest that buccalins are also released by behaviorally relevant stimulation of motorneuron B15.

Finally, we tested whether the quantities of peptides that are released during behaviorally relevant stimulation of motorneuron B15 are sufficient to exert physiological actions. We were able to demonstrate that such stimulation increases cAMP levels[8], activates PKA in the muscle[14], and, in a back-phosphorylation paradigm, increases protein phosphorylation[14]. The stimulation at the same time produced the appropriate changes in the characteristics of muscle contraction, in other words, increases in contraction size and relaxation rate[8]. Taken together, these findings indicate that under physiological conditions motorneuron B15 indeed releases the SCPs in sufficient quantities to activate the SCPs' signal transduction pathways in the ARC muscle and produce physiological changes in the contractions of the muscle.

Multiple peptide co-transmitters and behavior

The peptides that potentiate contractions, the SCPs and MMs, may be involved in behavioral arousal, which is expressed as increased magnitude and rate of feeding in animals exposed to food stimuli. The function of the co-localized peptides that reduce contraction amplitude, the BUCs, is less obvious. One could hypothesize that, even though co-localized with the potentiating peptides, the BUCs are not released, or are released under different circumstances than the SCPs and MMs. This is unlikely, however, given the immunocytological evidence that the SCPs and BUCs are co-localized not just in the same terminals, but in the same dense-core vesicles[30]. Furthermore, direct measurements of SCP and buccalin release have demonstrated that under various stimulation conditions these peptides are always released in a constant stoichiometric ratio[31]. This requires that in any analysis of the behavioral functions of the peptides the actions of these partially antagonistic peptides be considered jointly. Figure 5 illustrates our thinking about why peptides with seemingly contradictory effects should be co-released. As bite magnitude and frequency increase (fig. 5, second column), as occurs at the beginning of a meal (see fig. 1A), the weaker of the antagonistic pair of muscles, the radula opener, cannot overcome the increasing baseline contraction of the radula closer. The latter does not have enough time to fully relax between bites, and thus the radula does not open. If the increases in contraction amplitude and relaxation rate caused by the potentiating peptides, the SCPs and MMs, are included (fig. 5, third column), then, despite the increased contraction size and frequency, the accompanying increase in relaxation rate of the closer muscle results in a combined movement that almost results in opening of the radula. Finally, when the inhibitory effect of the buccalins on ACh release is also included (fig. 5, last column), the reduction in contraction size allows complete relaxation of the closer muscle between bites. Thus functional opening and closing of the radula can occur despite increased muscle contraction amplitude and frequency.

Our model explains why peptides with partially antagonistic actions should be co-released. The model further implies that the peptides should be released under conditions when the ARC muscle cannot fully relax before contraction of the opener muscle begins. This situation occurs when contractions are large or frequent. To test the model, we manipulated the size and frequency of the contractions using different physiological rates of motorneuron stimulation, and indeed found that when con-

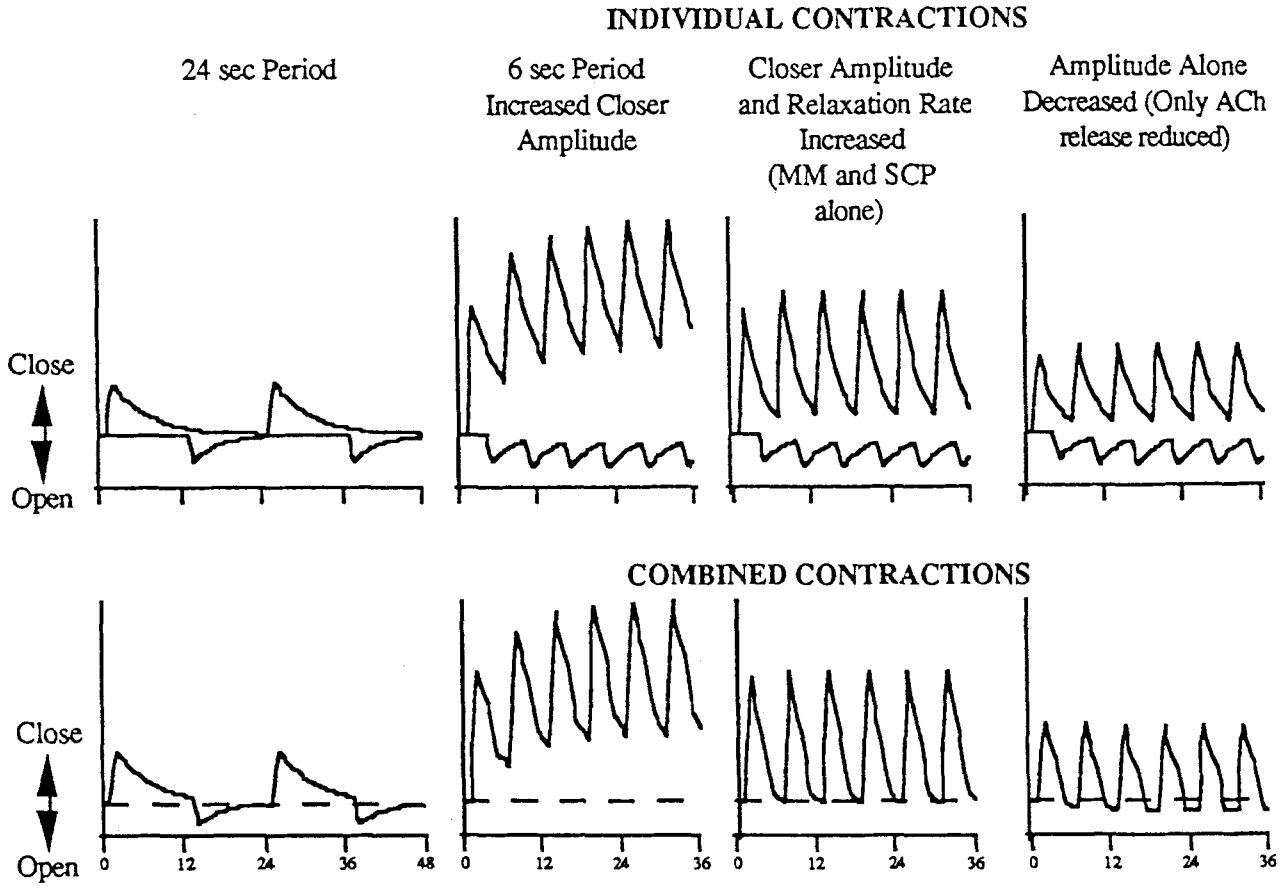

Figure 5. Peptidergic modulation integrates the functional output of radula openers and closers. This figure is presented in the same way as fig. 1 C. In the 1st column, the cycle period is long enough to allow the muscles to relax fully before the next contraction. In the 2nd column the frequency and amplitude of the contractions have been increased; the muscles no longer relax fully between contractions (top panel) and radula opening no longer occurs (bottom panel) since the weaker muscle, the radula opener, cannot overcome the contractions of the radula closer. In the 3rd column, the contraction frequency and amplitude are the same as in the 2nd column, but the relaxation rate of the contractions has also been increased; this situation is thus similar to the changes produced by SCP or MM application. The contractions can now relax to a larger degree, but (bottom panel) still not sufficiently to restore functional opening of the radula. In the last column the action of buccalin has been incorporated. Buccalin reduces the amplitude of the MM- or SCP-potentiated contractions (although the reduced contractions are still larger than those in control conditions, in column 1) without affecting their relaxation rate. These three peptides thus work in concert to ensure a functional motor output (opening and closing of the radula) as strength and frequency of biting increase during arousal.

traction size was made larger by increasing either the within-burst frequency of motorneuron firing, or the burst duration, peptide release per spike increased[31]. Similarly, when contraction frequency was increased by shortening the inter-burst interval, peptide release per spike also increased[31]. Independent support for our model came from experiments in which we took advantage of the observation that at elevated temperatures (but still within the temperature range animals encounter in their habitat) muscle contractions are reduced in size. As predicted, this decrease in contraction size, which leads to a reduction in the time needed for relaxation, was accompanied by a reduction in the release of peptides[31]. Thus all of our release data are consistent with the idea that peptidergic co-transmission in the ARC neuromuscular complex serves to allow regulation of the duration of contractions under conditions of changing contraction amplitude and frequency.

Conclusions

In order to study the role of peptidergic co-transmitters in motorneurons we have developed a simple neuro-behavioral preparation consisting of one muscle and its innervation. We have purified and determined the structure of peptidergic co-transmitters that are present in the two cholinergic motorneurons that innervate this muscle. Each of the motorneurons contains two families of peptides. The peptides of one family enhance the amplitude and relaxation rate of the muscle contractions, by postsynaptically modulating membrane ion currents or excitation-contraction coupling mechanisms in the muscle. The peptides of the other family depress the amplitude of the contractions by presynaptically inhibiting the release of ACh, the primary transmitter of the motorneurons. The combined actions of the potentiating and depressing peptides allow the system to vary both the amplitude and the

relaxation rate of the contractions. This type of modulation may be necessary to maintain proper phasing of feeding behavior when the intensity and frequency of the behavior change.

1 Bartfai, T., Iverfeldt, K., Brodin, E., and Ögren, S. O., Functional consequences of coexistence of classical and peptide neurotransmitters. Prog. Brain Res. *68* (1986) 321–330.

2 Březina, V., Evans, C. G., Kupfermann, I., and Weiss, K. R., Modulation of ion currents in the ARC muscle of *Aplysia*: Convergent actions of serotonin, SCPs and myomodulins. Soc. Neurosci. Abstr. *17* (1991) 1305.

3 Calabrese, R. L., Modulation of muscle and neuromuscular junctions in invertebrates. Semin. Neurosci. *1* (1989) 25–34.

4 Campbell, G., Cotransmission. A. Rev. Pharm. Toxicol. *27* (1987) 51–70.

5 Cohen, J. L., Weiss, K. R., and Kupfermann, I., Motor control of buccal muscles in *Aplysia*. J. Neurophysiol. *41* (1978) 157–180.

6 Cropper, E. C., Kupfermann, I., and Weiss, K. R., Differential firing patterns of the peptide-containing cholinergic motor neurons B15 and B16 during feeding behavior in *Aplysia*. Brain Res. *522* (1990) 176–179.

7 Cropper, E. C., Miller, M. W., Vilim, F. S., Tenenbaum, R., Kupfermann, I., and Weiss, K. R., Buccalin is present in the cholinergic motor neuron B16 of *Aplysia* and it depresses accessory radula closer muscle contractions evoked by stimulation of B16. Brain Res. *512* (1990) 175–179.

8 Cropper, E. C., Price, D., Tenenbaum, R., Kupfermann, I., and Weiss, K. R., Release of peptide cotransmitters from a cholinergic motorneuron under physiological conditions. Proc. natl. Acad. Sci. USA *87* (1990) 933–937.

9 Cropper, E. C., Vilim, F. S., Alevizos, A., Tenenbaum, R., Kolks, M. A. G., Rosen, S., Kupfermann, I., and Weiss, K. R., Structure, bioactivity, and cellular localization of myomodulin B: A novel *Aplysia* peptide. Peptides *12* (1991) 683–690.

10 Cropper, E. C., Lloyd, P. E., Reed, W., Tenenbaum, R., Kupfermann, I., and Weiss, K. R., Multiple neuropeptides in cholinergic motor neurons of *Aplysia*: Evidence for modulation intrinsic to the motor circuit. Proc. natl. Acad. Sci. USA *84* (1987) 3486–3490.

11 Cropper, E. C., Miller, M. W., Tenenbaum, R., Kolks, M. A. G., Kupfermann, I., and Weiss, K. R., 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* (1988) 6177–6181.

12 Cropper, E. C., Tenenbaum, R., Kolks, M. A. G., Kupfermann, I., and Weiss, K. R., Myomodulin: A bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. Proc. natl. Acad. Sci. USA *84* (1987) 5483–5486.

13 Hökfelt, T., Johansson, O., and Goldstein, M., Chemical anatomy of the brain. Science *225* (1984) 1326–1334.

14 Hooper, S. L., Probst, W. C., Kupfermann, I., and Weiss, K. R., SCP and myomodulin utilize separate signal transduction pathways and converge on common substrates in the ARC muscle of *Aplysia*. Soc. Neurosci. Abstr. *17* (1991) 1305.

15 Jones, E. G., De Phelipe, J., Hendry, S. H. C., and Maggio, J. E., A study of tachykinin-immunoreactive neurons in monkey's cerebral cortex. J. Neurosci. *8* (1988) 1206–1224.

16 Kupfermann, I., Feeding behavior in *Aplysia:* A simple system for the study of motivation. Behav. Biol. *10* (1974) 1–26.

17 Kupfermann, I., Functional studies of cotransmission. Physiol. Rev. *71* (1991) 683–731.

18 Kobayashi, M., and Muneoka, Y., Structure and action of molluscan neuropeptides. Zool. Sci. *7* (1990) 801–814.

19 Lloyd, P. E., The small cardioactive peptides: A class of modulatory neuropeptides in *Aplysia*. Trends Neurosci. *9* (1986) 428–431.

20 Lloyd, P. E., Mahon, A. C., Kupfermann, I., Cohen, J. L., Scheller, R. H., and Weiss, K. R., Biochemical and immunocytological localization of molluscan small cardioactive peptides in the nervous system of *Aplysia californica*. J. Neurosci. *5* (1985) 1851–1861.

21 Lloyd, P. E., Kupfermann, I., and Weiss, K. R., Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in *Aplysia*. Proc. natl. Acad. Sci. USA *81* (1984) 2934–2937.

22 Lloyd, P. E., Kupfermann, I., and Weiss, K. R., Sequence of small cardioactive peptide A: A second member of a class of neuropeptides in *Aplysia*. Peptides *8* (1987) 179–184.

23 Miller, M. W., Cropper, E. E., Eisinger, K., Vilim, F., Tenenbaum, R., Beushausen, S., Brosius, J., Kupfermann, I., and Weiss, K. R., The buccalin neuropeptide family in *Aplysia*: Purification of buccalin C and sequence of additional peptides predicted by a cDNA clone. Soc. Neurosci. Abstr. *15* (1989) 665.

24 Miller, M. W., Stamm, S., Cropper, E. C., Vilim, F. S., Beushausen, S., Brosius, J., Kupfermann, I., and Weiss, K. R., Characterization of cDNA clone encoding multiple myomodulin-related neuropeptides in *Aplysia*. Soc. Neurosci. Abstr. *17* (1991) 1305.

25 Miller, M. W., Vilim, F. S., Cropper, E. C., Alevizos, A., Tenenbaum, R., Karagogeos, D., Kupfermann, I., and Weiss, K. R., Structure and distribution of myomodulin related peptides in *Aplysia*. Soc. Neurosci. Abstr. *16* (1990) 307.

26 Miller, M. W., Alevizos, A., Cropper, E. C., Vilim, F. S., Karagogeos, D., Kupfermann, I., and Weiss, K. R., Localization of myomodulin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica*. J. comp. Neurol. *314* (1991) 627–641.

27 Morris, H. R., Panico, M., Karplus, A., Lloyd, P. E., and Riniker, B., Identification by FAB-MS of the structure of a new candidate peptide from *Aplysia*. Nature *300* (1982) 643–645.

28 Susswein, A. J., Weiss, K. R., and Kupfermann, I., The effects of food arousal on the latency of biting in *Aplysia*. J. comp. Physiol. A *123* (1978) 31–41.

29 Vilim, F. S., Cropper, E. C., Rosen, S. C., Tenenbaum, R., Kupfermann, I., and Weiss, K. R., Structure, cellular localization and modulatory action of buccalin B–A novel bioactive peptide from *Aplysia californica*. J. Neurosci. (submitted).

30 Vilim, F. S., Kupfermann, I., and Weiss, K. R., Ultrastructural localization of SCP and buccalin-like immunoreactivity in the accessory radula closer muscle of *Aplysia*. Soc. Neurosci. Abstr. *16* (1990) 306.

31 Vilim, F. S., Price, D. A., Lesser, W., Kupfermann, I., and Weiss, K. R., Direct measurement of peptide co-transmitter release following intracellular stimulation of a single identified motorneuron in *Aplysia*. Soc. Neurosci. Abstr. *17* (1991) 1305.

32 Vincent, S. R., Johansson, O., Hökfelt, T., Skirboll, L., Elde, R. P., Terenius, L., Kimmell, J., and Goldstein, M., NADPH-diaphorase: A selective histochemical marker for striatal neurons containing both somatostatin- and avian pancreatic polypeptide-like immunoreactivities. J. comp. Neurol. *217* (1983) 252–263.

33 Weiss, K. R., Cohen, J. L., and Kupfermann, I., Modulatory control of buccal musculature by a serotonergic neuron (Metacerebral Cell) in *Aplysia*. J. Neurophysiol. *41* (1978) 181–203.

34 Weiss, K. R., and Kupfermann, I., Serotonergic neuronal activity and arousal of feeding in *Aplysia californica*. Soc. Neurosci. Symp. *3* (1977) 66–89.

35 Weiss, K. R., Lloyd, P. E., Cropper, E. C., Frankfurt, M., and Kupfermann, I., FMRF-amide is present in the ARC muscle of *Aplysia* and depresses its contractions. Soc. Neurosci. Abstr. *12* (1986) 947.

36 Whim, M. D., and Lloyd, P. E., Frequency-dependent release of peptide cotransmitters from identified cholinergic motor neurons in *Aplysia*. Proc. natl. Acad. Sci. USA *86* (1989) 9034–9038.

0014-4754/92/050456-08$1.50 + 0.20/0