Two Neuropeptides Colocalized in a Command-Like Neuron Use Distinct Mechanisms to Enhance Its Fast Synaptic Connection

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Koh, H.-Y., F. S. Vilim, J. Jing, and K. R. Weiss. Two neuropeptides colocalized in a command-like neuron use distinct mechanisms to enhance its fast synaptic connection. J Neurophysiol 90: 2074–2079, 2003; 10.1152/jn.00358.2003. In many neurons more than one peptide is colocalized with a classical neurotransmitter. The functional consequence of such an arrangement has been rarely investigated. Here, within the feeding circuit of Aplysia, we investigate at a single synapse the actions of two modulatory neuropeptides that are present in a cholinergic interneuron. In combination with previous work, our study shows that the command-like neuron for feeding, CBI-2, contains two neuropeptides, feeding circuit activating peptide (FCAP) and cerebral peptide 2 (CP2). Previous studies showed that high-frequency prestimulation or repeated stimulation of CBI-2 increases the size of CBI-2 to B61/62 excitatory postsynaptic potentials (EPSPs) and shortens the latency of firing of neuron B61/62 in response to CBI-2 stimulation. We find that both FCAP and CP2 mimic these two effects. The variance method of quantal analysis indicates that FCAP increases the calculated quantal size (\( q \)) and CP2 increases the calculated quantal content (\( m \)) of EPSPs. Since the PSP amplitude represents the product of \( q \) and \( m \), the joint action of the two peptides is expected to be cooperative. This observation suggests a possible functional implication for multiple neuropeptides colocalized with a classical neurotransmitter in one neuron.

INTRODUCTION

In many cases, a single neuropeptide has been shown to be colocalized with a small molecule (classical) transmitter. However, growing evidence indicates that many neurons contain more than one neuropeptide (Merighi 2002). Until recently, the functional consequences of actions of multiple peptide cotransmitters have been studied mostly in the peripheral and were most extensively characterized in the accessory radular closer (ARC) musculature of Aplysia (Cropper et al. 1988; Vilim et al. 2000 1996a,b). In the CNS, however, the investigations of the actions of multiple peptide cotransmitters present in single neurons have been performed only in a few systems. These studies have already yielded important insights into the functional role of such multiple peptide cotransmitters. In particular, in the stomatogastric (STG) systems of the crab and lobster, multiple peptide cotransmitters play an important role in specifying motor programs (Wood et al. 2000) as well as in the generation of fully articulated motor programs (Thirumalai and Marder 2002) and may do so by affecting specific neuronal conductances (Swensen and Marder 2000, 2001).

Although modulation of fast synaptic transmission by neuropeptides is known to occur in the CNS of both vertebrates and invertebrates (Abrams et al. 1984; Dickinson et al. 2001; Montarolo et al. 1988; Parker 2000; Simon et al. 1994), little is known about the effects of multiple colocalized peptides on fast synaptic transmission and therefore about the functional consequences of such actions in the CNS. In this study, we examine the modulatory effects of two colocalized neuropeptides in the Aplysia feeding circuitry. We report that the cholinergic command-like neuron CBI-2 (Hurwitz et al. 2003), in addition to cerebral peptide 2 (CP2; Morgan et al. 2000), contains another peptide feeding circuit activating peptide (FCAP; Sweedler et al. 2002). Furthermore, we show that these two peptides shorten the latency of the initiation of CBI-2 elicited firing of motoneurons B61/62, and that they do so by enhancing synaptic transmission between CBI-2 and B61/62 through apparently different mechanisms.

Stimulation of the command-like neuron CBI-2 elicits feeding-like buccal motor programs. CBI-2 elicited programs are plastic in that the latency to the onset of protraction (the first phase of the program) becomes shorter when CBI-2 is either stimulated repetitively or prestimulated at high frequencies (Proekt and Weiss 2003; Sanchez and Kirk 2002). Previous work demonstrated that latency shortening is associated with an increase in the amplitude of fast cholinergic excitatory postsynaptic potentials (EPSPs) that CBI-2 evokes in the protraction motoneurons B61/62 (Sanchez and Kirk 2002). However, the fact that CBI-2 contains peptides, and that the stimulation paradigms that shorten the latency are favorable for peptide release in Aplysia, suggested to us that the peptides present in CBI-2 may act to shorten the latency. Here, without showing that the release of peptides is necessary for the CBI-2 elicited latency shortening and the increase of the size of EPSPs, we demonstrate that the two peptides mimic the actions of repetitive or high-frequency stimulation of CBI-2. We proceed to show that the two peptides use different mechanisms to enhance the fast cholinergic EPSPs that CBI-2 elicits in B61/62.

METHODS

Immunocytochemistry

For the purpose of immunocytochemistry, electrophysiologically identified neurons were ionophoretically injected with carboxylfluor...
rescein (Sigma, St. Louis, MO), and the ganglia were fixed and processed for FCAP immunoreactivity as previously described (Sweedler et al. 2002; Vilim et al. 1996b). The previously characterized antibody to FCAP was raised in rats in our laboratory (Sweedler et al. 2002). The secondary antibody was obtained from a commercial source (Rhodamine Red-X Donkey anti-Rat, Jackson ImmunoResearch Laboratories, West Grove, PA). Immunostained ganglia were viewed and photographed with a Nikon Coolpix 990 digital camera mounted on a Nikon Labphot2 microscope (Morrell Instruments, Melville, NY) equipped with epifluorescence and filter sets to visualize rhodamine (Y-2E; EX 540-580/DM 590/BA 600-660) or fluorescein (B-2A; EX 450-490/DM 505/BA 520).

Electrophysiology

Experiments were performed on *Aplysia californica* (125–175 g). Preparations were maintained at 13.8–14.5°C. Extracellular recordings were made using suction electrodes. Axoclamp 2A was used to amplify intracellular signals that were obtained using glass microelectrodes filled with 2 M K acetate and 100 mM KCl. Experiments were performed only if neurons B61/62 had a resting potential more negative than −60 mV, and the data were analyzed only if during the experiment the resting potential was stable (i.e., it did not change by more than 3 mV).

For measurements of the parameters of CBI-2 elicited buccal motor programs, buccal-cerebral ganglia with cerebro-buccal connectives (CBCs) intact were superfused with 40 CaCl₂, 110 MgCl₂, while CBI-2 was stimulated at 2 Hz with 14.5 °

Statistics

All tests involving more than two conditions were done using one-way ANOVA. Post-hoc comparisons used the Bonferroni correction. Statistical analyses were performed using InStat 3.05 software (GraphPad, San Diego, CA).

RESULTS

A recent study which characterized a novel neuropeptide, FCAP, indicated that this peptide (Sweedler et al. 2002) is present in the cerebral M cluster, a set of neurons which, among other cells, also contains several command-like cerebral-buccal interneurons (CBIs) (Hurwitz et al. 1999; Rosen et al. 1991). Furthermore, backfills from the cerebro-buccal connectives demonstrated that some of the M-cluster neurons that stain for FCAP send axons to the CBCs and thus are themselves CBIs (Sweedler et al. 2002). To determine which of the M-cluster CBIs contain FCAP, we injected them with carboxyfluorescein and processed the ganglia for FCAP immunocytochemistry. CBI-1, CBI-2, and CBI-12 stained for FCAP (Fig. 1). Individual CBIs were identified electrophysiologically using the previously described criteria (Jing and Weiss 2001; Rosen et al. 1991). The staining is likely to be specific because previous studies have shown that J staining with this antibody corresponds to that observed with in situ hybridization, and 2) immunostaining is abolished by preadsorption with FCAP (Sweedler et al. 2002).

The CBI that is best characterized in functional terms is CBI-2. This neuron is an effective initiator of motor programs. Motor programs elicited by CBI-2 begin with a protraction phase and the first neurons to be activated, in response to CBI-2 stimulation, are the protraction motoneurons B61 and B62 (Hurwitz et al. 2003). B61 and B62 fire for a period of time before buccal interneurons are activated. Because B61 and B62 are monosynaptically activated by CBI-2, the latency to initiate B61/62 firing is a good indicator of the efficacy of synaptic transmission from CBI-2.

Previous studies (Hurwitz et al. 2003; Proekt and Weiss 2003; Sanchez and Kirk 2002) showed that repeated or high-

FIG. 1. CBI-1, CBI-2, and CBI-12 are feeding circuit activating peptide (FCAP) immunoreactive. *A1, B1*: M-cluster neurons of the cerebral ganglion injected with carboxyfluorescein. A2, B2: FCAP immunostaining (rhodamine) in the corresponding visual fields. A: CBI-2 (arrows, both panels) immunostains for FCAP. B: CBI-12 (arrows, both panels) and CBI-1 (arrow heads, both panels) immunostain for FCAP, while CBI-3 (asterisk, both panels) does not immunostain for FCAP (negative control). Scale bar (shown in B2 for all panels), 100 μm.
FIG. 2. The effects of FCAP and cerebral peptide 2 (CP2) on CBI-2 elicited buccal motor programs and excitatory postsynaptic potentials (EPSPs) evoked in B61 by CBI-2. A: effects of FCAP. A1: top traces show a recording of B61/62 activity in response to CBI-2 stimulation. Middle traces show the same recordings but in the presence of $10^{-6}$ M FCAP. FCAP shortened the latency to the onset of B61/62 firing. Bottom traces show the same experiment after washout. Resting membrane potentials: CBI-2/H11005/H11002 $61 \text{ mV}; \text{B61/62/H11005/H11002} 67 \text{ mV}$. A2: grouped data ($n = 5$) for the latency of B61/62 firing. One-way repeated measure ANOVA showed that there was a significant effect of FCAP ($F = 26.49; \text{df} = 2.8; P < 0.001$). In the presence of $10^{-6} \text{ M FCAP, the latency to the onset of B61/62 firing was significantly reduced (} t = 5.94; P < 0.01 \text{). After the wash, the latency recovered. The latency during FCAP was significantly different from after the wash (} t = 6.6; P < 0.001 \text{) but there was no difference between the controls and the postwash values (} t = 0.65; P > 0.05 \text{).}$ A3: grouped data ($n = 4$) for the B61/62 firing frequency during the first 5 s of B61/62 activity (data from the same preparations as in A2). One-way ANOVA showed a significant overall difference ($F = 14.12; \text{df} = 2.8; P < 0.005$). There was a significant effect of FCAP on the firing frequency compared with control ($t = 4.85; P < 0.01$) and postwash conditions ($t = 4.3; P < 0.01$). There was no significant difference between control and postwash conditions ($t = 0.55; P > 0.05$). A4: effects of $10^{-6} \text{ M FCAP on the amplitude of EPSP evoked in B61/62 by CBI-2. Grouped data (} n = 4 \text{) are shown on the left and representative recordings from a single preparation are shown on the right. There was a significant overall difference between conditions (} F = 44.57; \text{df} = 2.6; P < 0.005 \text{). There was a significant effect of FCAP on EPSP amplitude compared with controls (} t = 7.2; P < 0.01 \text{) and postwash conditions (} t = 8.89; P < 0.001 \text{). There was no significant difference between control and postwash conditions (} t = 1.68; P > 0.05 \text{). Resting potential of B61/62 activity in response to CBI-2 stimulation. Middle traces show the same recordings but in the presence of $10^{-6} \text{ M CP2. CP2 shortened the latency to the onset of B61/62 firing. Bottom traces show the same experiment after washout. Resting membrane potentials: CBI-2/H11005/H11002 $58 \text{ mV}; \text{B61/62/H11005/H11002} 63 \text{ mV}$. B2: grouped data ($n = 5$) for the latency of B61/62 firing. One-way repeated measure ANOVA showed that there was a significant difference between conditions ($F = 36.3; \text{df} = 2.8; P < 0.001$). In the presence of $10^{-6} \text{ M CP2, the latency to the onset of B61/62 was significantly shorter than in control (} t = 7.36; P < 0.001 \text{) and postwash (} t = 7.4; P < 0.001 \text{). There was no difference between the controls and the postwash values (} t = 0.04; P > 0.05$.}
frequency (9–15 Hz) stimulation of CBI-2 causes 1) shortening of the latency to the onset of protraction, 2) increase in the firing frequency of B61/62 in subsequent CBI-2 elicited motor programs, and 3) increase in the amplitude of EPSPs evoked in B61/62 by CBI-2. Since it is commonly observed that sustained trains of high-frequency firing facilitate peptide release (Iverfeldt et al. 1989; Vilim et al. 1996a, 2000; Whim and Lloyd 1989), it appeared possible that the two neuropeptides FCAP and CP2 colocalized in CBI-2 may also act to shorten the latency of the firing of B61/62.

To test the potential involvement of FCAP and CP2 in this CBI-2-mediated modulation, we first examined whether these peptides can mimic the effect of high-frequency stimulation of CBI-2 on the latency to the onset of protraction phase and B61/62 firing frequency. Buccal-cerebral ganglia with CBCs intact were superfused with normal ASW throughout the experiment while intracellular recordings were obtained from CBI-2 and B61/62. CBI-2 was stimulated (every 3 min) at a constant frequency until the end of the B61/62 burst (i.e., the end of the protraction phase). We measured two parameters of CBI-2-elicited B61/62 firing, the latency of the onset of B61/62 firing and the B61/62 firing frequency during the first 5 s of activity. The latency to the onset of protraction phase is defined by the period between the first spike of CBI-2 and the appearance of the first action potential potential in B61/62 (double-headed arrows in Fig. 2, A1 and B1). FCAP (10−6 M) significantly reduced the latency (see Fig. 2A2 for grouped data) and also significantly increased the frequency of B61/62 firing (see Fig. 2A3 for grouped data). The same experiments were performed with CP2. In the presence of 10−6 M CP2, the latency to the initiation of firing of B61/62 was significantly decreased (Fig. 2B2), and B61/62 spike frequency was significantly increased (Fig. 2B3).

We next sought to determine whether the effect of FCAP and CP2 on the latency to the onset of B61/62 firing and the B61/62 firing frequency is associated with potentiation of the fast EPSPs that CBI-2 elicits in B61/62. The preparation was superfused with high-Ca2+ ASW (5× Ca2+) to prevent polysynaptic activity and to amplify the size of EPSPs. EPSPs in B61/62 were evoked by stimulating CBI-2 with depolarizing current pulses (20 ms, 1.4 Hz for 3 s, once a minute), each of which elicited a single action potential in CBI-2. Perfusion of 10−6 M FCAP significantly increased the size of the EPSPs (Fig. 2A4). Perfusion of 10−6 M CP2 also significantly increased the size of the EPSPs (Fig. 2B4).

To determine whether the increase in the amplitude of the fast synaptic potentials by either FCAP or CP2 is caused by changes in the resting membrane properties of B61/62, the effect of these peptides on the input resistance of B61/62 was examined by injecting constant current pulses. We found that perfusion of CP2 or FCAP, at the same concentration as that used in the EPSP experiments, had no significant effect on the input resistance of B61/62. In the presence of FCAP the input resistance was 102 ± 1.56% of control values (P = 0.44, n = 3). In the presence of CP2 the input resistance was 105 ± 1.67% of control values (P = 0.19, n = 3).

The results described above show that both FCAP and CP2, two peptides that co-exist in CBI-2, enhance the size of the fast synaptic potentials that CBI-2 elicits in B61/62. Even though these peptides seem to have nearly the same effects on the CBI-2-elicited program and the synaptic potentials that CBI-2 elicits in B61/62, the preceding experiments could not indicate whether the potentiating actions of FCAP and CP2 involve the same or different cellular mechanisms. We pursued this question using the variance method of quantal analysis (Hubbard et al. 1969). The preparation was superfused with 4× Ca2+/2× Mg2+ ASW to block polysynaptic activity and generation of spontaneous buccal motor programs. EPSPs were evoked in B61/62 by stimulating CBI-2 continuously at 2 Hz. Mean quantal size (q, response to a single unit quantum) and mean quantal content (m, number of quanta released by a single action potential spike) were calculated from the analysis of the distribution of a population of EPSPs (more than 350) evoked under a specific condition (before or during the superfusion of peptide, or after washout) (Fig. 3, A1, 3, and 4 for FCAP, B1, 3, and 4 for CP2). In the presence of 10−6 M FCAP there was a significant increase in the variance of the EPSPs (Fig. 3A2) as compared with the two control conditions (before and after FCAP). Consistent with this increase in variance we found that the calculated quantal size was significantly increased (Fig. 3A3). The calculated quantal content was not affected (Fig. 3A4). In contrast, 10−6 M CP2 did not significantly affect the variance (Fig. 3B2); consistent with this there was no significant effect on the calculated quantal size. However, CP2 significantly increased the calculated quantal content (Fig. 3B4) with little effect on the calculated quantal size (Fig. 3B3).

**DISCUSSION**

Although the overall sequence of *Aplysia*’s feeding movements is stereotyped, individual responses can differ significantly depending on animal’s history and motivational state (Kupfermann 1974; Susswein et al. 1984). One noticeable alteration of feeding responses is the shortening of the latency to initiate protraction. The shortening of the latency to initiate CBI-2 elicited motor programs also occurs when CBI-2 is prestimulated at higher frequencies or is stimulated repeatedly (Proekt and Weiss 2003; Sanchez and Kirk 2002). Repeated activation of feeding responses in intact animals leads to a progressive shortening of the latency of feeding responses. Since CBI-2 is activated by food stimuli, the shortening of the latency, which occurs when CBI-2 is repeatedly activated, may represent a neural correlate of behavioral plasticity.

Previous work in the *Aplysia* feeding circuit has implicated
peptidergic actions in the generation of different motor programs (Jing and Weiss 2001; Morgan et al. 2002) as well as in the plasticity of these programs (Morgan et al. 2000). However, the potential contribution of peptides to the shortening of the latency of CBI-2 elicited motor programs has not been investigated previously. Here we report that CBI-2 contains FCAP in addition to CP2, a neuropeptide that has been previously localized to CBI-2. We find that in the isolated nervous system both peptides act to shorten the latency of motor programs that are elicited by stimulation of CBI-2. Previous studies, in which the latency to initiate motor programs was shortened through high-frequency or repeated stimulation of CBI-2 or by stimulation of serotonergic modulatory neurons (Proekt and Weiss 2003; Sanchez and Kirk 2002), reported that this phenomenon was associated with an increase in the size of EPSPs that CBI-2 elicits in B61/62. Furthermore, when the cholinergic EPSPs were pharmacologically reduced, the latency to initiate motor programs increased (Hurwitz et al. 2003). Thus the size of these EPSPs plays an important role in the initiation of motor programs. In our study, both peptides which shortened the latency to initiate the programs also increased the size of EPSPs.

Our studies were designed to investigate the possibility that FCAP and CP2 can mimic the effects of repetitive or high-frequency stimulation of CBI-2. We investigated whether the actions of these peptides are sufficient but not whether they are necessary to mimic the effect of CBI-2 stimulation. At this time, we cannot exclude the possibility that other more classical mechanisms (e.g., similar to those involved in post-tetanic potentiation and long-term potentiation) may also mediate the effects of repetitive or high-frequency stimulation of CBI-2.

Homosynaptic enhancement of fast synaptic transmission by co-release of a peptide has been shown to occur at the neuromuscular junction of Aplysia (Fox and Lloyd 2001). Here, we studied the actions of two peptides that are contained in the same presynaptic neuron that is located and exerts actions within the CNS. Our finding that both FCAP and CP2 converge on the same target, the CBI-2 to B61/62 synapse, is reminiscent of reports of several peptides converging on the same cellular targets in the crab STG system (Blitz et al. 1999; Swensen and Marder 2000; Thirumalai and Marder 2002). In the case of the STG these peptides exerted the same physiological actions by activating the same ionic current. Indeed, actions of different peptides occluded each other. To determine whether the actions of FCAP and CP2 depend on the same or different physiological mechanisms to potentiate the CBI-2 to B61/62 synapse, we studied the effects of these two peptides on the calculated quantal content and the calculated quantal size. We found that the two peptides potentiated the synaptic connection between CBI-2 and B61/62 through apparently different mechanisms. CP2 increased the calculated quantal content while FCAP increased the calculated quantal size. Thus the mode of multiple peptide actions described here appears to be distinct from those analyzed in the crab STG.

Because in our analysis we had to assume a Poisson distribution and could not reject the possibility of an underlying binomial distribution, caution has to be exercised in giving a physiological interpretation to the calculated $m$ and $q$. However, independent of whether the calculated quantal content represents a presynaptic change, the quantal size a postsynaptic change, or the alterations of calculated $m$ and $q$ are interpreted.

**FIG. 3.** Effect of FCAP and CP2 on the amplitude, the calculated quantal size ($q$), and the calculated quantal content ($m$) of EPSPs evoked in B61/62 by CBI-2. A: effects of FCAP. Af: effects of FCAP on the size of the EPSPs. There was an overall difference between conditions ($F = 38.1; df = 2.8; P < 0.001; n = 5$). In the presence of FCAP EPSPs were significantly larger than in control ($t = 8.01; P < 0.001$) or postwash conditions ($t = 7.01; P < 0.001$), but there was no difference between control and postwash conditions ($t = 1.0; P > 0.05$). A2: effects of FCAP on the variance of EPSP amplitude. In the 3 conditions there was a significant overall difference ($F = 16.99; df = 2.8; P < 0.005$). In the presence of FCAP the variance was significantly larger than in control ($t = 5.4; P < 0.01$) and postwash conditions ($t = 4.57; P < 0.01$), but there was no difference between control and postwash conditions ($t = 0.85; P > 0.05$). A3: effects of FCAP on the calculated quantal size. In the 3 conditions there was a significant overall difference ($F = 20.03; df = 2.8; P < 0.001$). In the presence of $10^{-6}$ M FCAP, the quantal size was significantly larger than in control ($t = 6.1; P < 0.001$) and postwash conditions ($t = 4.5; P < 0.01$), but there was no difference between control and postwash ($t = 1.61; P > 0.05$). A4: the quantal content was not affected by FCAP as there was no overall difference between the 3 conditions ($F = 2.18; df = 2.8; P > 0.05$). B: effects of CP2. B1: effects of CP2 on the size of the EPSPs. There was an overall difference between conditions ($F = 10.14; df = 2.8; P < 0.01; n = 5$). In the presence of CP2 EPSPs were significantly larger than in control ($t = 3.93; P < 0.05$) or postwash ($t = 3.86; P < 0.05$) conditions, but there was no difference between control and postwashout conditions ($t = 0.07; P > 0.05$). B2: there was no significant overall difference between the three conditions ($F = 0.39; df = 2.8; P > 0.05$). B3: there was no significant overall difference in calculated quantal size between the 3 conditions ($F = 0.68; df = 2.8; P > 0.05$). B4: there was a significant overall difference in calculated quantal content between the 3 conditions ($F = 13.46; df = 2.8; P < 0.005$). In the presence of $10^{-6}$ M CP2, the calculated quantal content was significantly larger in control ($t = 4.53; P < 0.01$) and postwash ($t = 4.45; P < 0.01$) conditions, but there was no difference between control and postwash ($t = 0.08; P > 0.05$). Asterisks indicate statistically significant differences.
in a different manner, our analysis separated two components each of which contributes to the final size of the EPSP. Because the differential effects of the two peptides were observed at the same synapse, it is likely that these effects indeed reflect the fact that each peptide utilizes a distinct mechanism to potentiate the EPSP. Since the final size of the EPSP represents the product of the calculated $m$ and $q$, the joint actions of FCAP and CP2 could produce a cooperative effect. The cooperative effect of the two peptides may represent an efficient mechanism for potentiating synaptic transmission in some cases where neurons contain more than one modulatory neuropeptide.

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**DISCLOSURES**

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**REFERENCES**


