Fast Synaptic Connections From CBIs to Pattern-Generating Neurons in *Aplysia*: Initiation and Modification of Motor Programs

Itay Hurwitz, Irving Kupfermann, and Klaudiusz R. Weiss

1Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York City, New York 10029; 2Interdisciplinary Program in the Brain Sciences, Gonda (Goldschmied) Medical Diagnostic Research Center, Bar Ilan University, Ramat Gan 52900, Israel; and 3Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York City, New York 10032

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Hurwitz, Itay, Irving Kupfermann, and Klaudiusz R. Weiss. Fast synaptic connections from CBIs to pattern-generating neurons in *Aplysia*: initiation and modification of motor programs. *J Neurophysiol* 89: 2120–2136, 2003; 10.1152/jn.00497.2002. Consummatory feeding movements in *Aplysia californica* are organized by a central pattern generator (CPG) in the buccal ganglia. Buccal motor programs similar to those organized by the CPG are also initiated and controlled by the cerebral-buccal interneurons (CBIs), interneurons projecting from the cerebral to the buccal ganglia. To examine the mechanisms by which CBIs affect buccal motor programs, we have explored systematically the synaptic connections from three of the CBIs (CBI-1, CBI-2, CBI-3) to key buccal ganglia CPG neurons (B31/B32, B34, and B63). The CBIs were found to produce monosynaptic excitatory postsynaptic potentials (EPSPs) with both fast and slow components. In this report, we have characterized only the fast component. CBI-2 monosynaptically excites neurons B31/B32, B34, and B63, all of which can initiate motor programs when they are sufficiently stimulated. However, the ability of CBI-2 to initiate a program stems primarily from the excitation of B63. In B31/B32, the size of the EPSPs was relatively small and the threshold for excitation was very high. In addition, preventing firing in either B34 or B63 showed that only a block in B63 firing prevented CBI-2 from initiating programs in response to a brief stimulus. The connections from CBI-2 to the buccal ganglia neurons showed a prominent facilitation. The facilitation contributed to the ability of CBI-2 to initiate a BMP and also led to a change in the form of the BMP. The cholinergic blocker hexamethonium blocked the fast EPSPs induced by CBI-2 in buccal ganglia neurons and also blocked the EPSPs between a number of key CPG neurons within the buccal ganglia. CBI-2 and B63 were able to initiate motor patterns in hexamethonium, although the form of a motor pattern was changed, indicating that non-hexamethonium-sensitive receptors contribute to the ability of these cells to initiate bursts. By contrast to CBI-2, CBI-1 excited B63 but inhibited B34. CBI-3 excited B34 and not B63. The data indicate that CBI-1, -2, and -3 are components of a system that initiates and selects between buccal motor programs. Their behavioral function is likely to depend on which combination of CBIs and CPG elements are activated.

**INTRODUCTION**

The feeding motor system of *Aplysia* is a useful experimental preparation for examining how a neural network generates and controls a variety of behaviors. This system gives rise to consummatory feeding behaviors that are effected by the buccal muscles (for review, see Elliott and Susswein 2002). Contractions of these muscles produce a number of different feeding behaviors, such as biting, swallowing, and rejection (Jing and Weiss 2001, 2002; Kupfermann 1974; Morton and Chiel 1993a,b). All of these behaviors consist of two phases of radula movement, first a radula protraction and then a radula retraction. The protraction-retraction sequence is variably coupled with radula opening and closing movements. The activity of the buccal muscles is organized by a central pattern generator (CPG) contained in the buccal ganglia (Hurwitz and Susswein 1996; Hurwitz et al. 1997; Kirk 1989; Susswein and Byrne 1988; Teyke et al. 1993), which drive the motoneurons that innervate the buccal muscles (Church and Lloyd 1994; Church et al. 1993; Cohen et al. 1978; Hurwitz et al. 1994, 1996, 2000; Morton and Chiel 1993a,b).

The mechanisms of motor program generation have been studied predominantly in isolated buccal ganglia (Hurwitz and Susswein 1996; Hurwitz et al. 1997; Kirk 1989; Susswein and Byrne 1988; Teyke et al. 1993). However, motor programs are strongly influenced by connections from the cerebral ganglion, which senses the presence of the food stimuli that initiate feeding and also modulates feeding behaviors (Rosen et al. 1991). In addition, there is good evidence that complex motor programs can be elicited by a small group of cerebral-buccal interneurons (CBIs). CBI-2, the best studied of these neurons, is capable of driving robust feeding motor programs. Although some of the connections that CBI-2 makes in the buccal ganglion have been described (Hurwitz et al. 1999a; Jing and Weiss 2001, 2002; Sanchez and Kirk 2000), little is known about how the CBIs elicit and control feeding motor programs. To examine the nature of the connections made by different CBIs to various key buccal neurons that function in the initiation of buccal motor programs (BMPs), and the role of the CBIs in shaping the form of BMPs, we have investigated the connections of CBI-2, as well as those of CBI-1 and CBI-3, to key protraction phase neurons that are part of the CPG, the previously identified buccal ganglion neurons B31/B32, B34, and B63. Other studies have focused on the role of additional neurons that function in modifying motor programs so that they become more similar to those seen in specific behaviors, such as ingestion or rejection (Jing and Weiss 2001, 2002; Morgan et al. 2002; Nargeot et al. 1997).
We have found that CBI-2 drives motor programs via ipsilateral and contralateral monosynaptic excitatory postsynaptic potentials (EPSPs) to several protraction-phase interneurons that are capable of initiating feeding motor programs. CBI-2 was found to elicit both fast and slow EPSPs in its followers. The present report characterizes in detail the fast EPSPs elicited by CBI-2. A future report (I. Hurwitz, R. A. DiCaprio, and K. R. Weiss, unpublished data) will characterize in detail the slow EPSPs. The fast EPSPs are apparently cholinergic, and they display a prominent facilitation on repeated stimulation of CBI-2. The facilitation is an integral component of the ability of CBI-2 to recruit key protraction phase interneurons. Other CBIs differentially excite alternate combinations of protraction phase interneurons and may therefore act as modulators of the pattern elicited by the CPG. Thus the ability to initiate and determine the form of motor programs in part arises from the patterns of connectivity of the CBIs to different protraction-phase interneurons.

**METHODS**

The experimental subjects for this study were *Aplysia californica* weighing 150–300 g. They were purchased from Marinus, Long Beach, CA, and from the National Resource for *Aplysia* at the University of Miami. They were maintained at 14–16°C in holding tanks containing aerated, filtered seawater. Before being dissected, animals were immobilized by injection with isotonic MgCl₂ (50% of body weight). In earlier experiments, the buccal and cerebral ganglia were removed with the cerebral-buccal connectives (CBCs) intact with the cerebral ganglia remaining attached via the pleural and pedal connectives to the pleural and pedal ganglia. This allowed us to characterize the synaptic connections from neuron C-PR to neurons CBI-2 and CBI-12, and thereby to identify these neurons. We then characterized the connections of CBI-2 and CBI-12 to the buccal ganglia. The connections from CBI-2 and CBI-12 to buccal ganglia neurons were very different (Sanchez and Kirk 2001). The activity of protraction phase interneurons in CBI-2-elicited motor programs continued to that seen with continuous depolarization even though CBI-2 is coupled to CBI-3, CBI-2 and CBI-12 receive different patterns of synaptic activity from neuron CPR, CBI-2 but not CBI-12 (or any of the other CBIs) elicits a profound facilitation in the protraction-phase neurons, and only CBI-2 elicits an EPSP with an amplitude that exceeds 2 mV.

To generate an action potential in a neuron, 20-ms depolarizing current pulses were injected, and the appearance of one-for-one action potentials was monitored. CBI-2 was fired at 7–12 Hz, for a period that did not exceed 3 min unless otherwise noted.

**Data analysis**

Comparisons between multiple groups were performed using one-way ANOVAs. When only two groups were compared, t-test were used.

**RESULTS**

**Activity of protraction phase interneurons in CBI-2-elicited motor programs**

A number of CBIs can drive BMPs. However, CBI-2 is the most effective (Rosen et al. 1991). Although a number of studies have examined the properties of CBI-2 and have contributed to characterizing this neuron (Hurwitz et al. 1999a,b; Morgan et al. 2002; Rosen et al. 1991; Sanchez and Kirk 2000; Teyke et al. 1993), the mechanisms that underlie the ability of CBI-2 to initiate BMPs have not been studied systematically. Sustained depolarization of CBI-2 initiates rhythmic activity in the buccal ganglia in which protraction and retraction phases alternate. CBI-2 itself fires during the protraction phase of BMPs and is inhibited during the retraction. In the present study, CBI-2 was stimulated with short pulses that each initiated a single spike. This allowed a fine-grained control of the CBI-2 firing frequency. Using this stimulus, continuous stimulation of CBI-2 was found to initiate rhythmic activity similar to that seen with continuous depolarization even though CBI-2 continued to fire during the retraction. (Fig. 1A). Data to be presented elsewhere (Hurwitz and Weiss, unpublished results) will explain the apparent anomaly that firing in CBI-2 during retraction apparently does not affect BMPs. Because the I₂ nerve contains the axons of motoneurons that innervate and excite a major protraction muscle (the I₂ muscle), the duration of protraction phases can be monitored via extracellular re-
The intracellularly recorded firing of B61/B62 and B31/B32 (Fig. 1, A and B) coincides with the extracellularly recorded activity in the I2 nerve, and previous data have shown that firing of B61/B62 and B31/B32 causes I2 muscle contractions (Hurwitz et al. 1994, 2000). Extracellular recordings can also be used to monitor the duration of the retraction phase via recordings from both the I2 nerve and the RN. The RN contains the axons of neurons (B8) that drive contractions of radula closer muscle (the I4 muscle). Thus the duration of the retraction phase can be monitored by the extracellularly recorded activity in RN that continued after I2 nerve activity ceases (see for details Morton and Chiel 1993a,b; Morgan et al. 2002). Intracellularly recorded activity in neuron B8 largely coincides with the extracellularly recorded activity in RN (Fig. 1A), and I4 muscle contractions.

Intracellular and extracellular patterns of activity were investigated in the I2 and RN nerves and in several key buccal ganglia neurons during motor programs elicited by CBI-2 stimulation (n > 30). Typical recordings are shown in Fig. 1. Tonic firing of CBI-2 induced rhythmic cycles of BMPs with protraction phase interneuron B34 and the B61/B62 motor neurons firing together during the protraction phase (Fig. 1A). B34 and B61/B62 fired out of phase with the firing of B8. Firing of CBI-2 also drives activity in interneuron B63 and in the multi-functional B31/B32 neurons during the protraction phase (Fig. 1B). As in the isolated buccal ganglia, B31/B32 activity is characterized by a sustained plateau depolarization with small, fast depolarizations of a variety of sizes superimposed on the sustained plateau. The largest of these fast depolarizations represents axon spikes that fail to invade the soma (Hurwitz et al. 1994). In addition, B64 is recruited into CBI-2 via intracellular stimulation of B31/B32, B34, B63, and B64. In all BMPs elicited by CBI-2, B64 is active. In isolated buccal ganglia, the retraction phase interneuron B64 remained active throughout the retraction phase (Hurwitz and Susswein 1996), and termination of its activity coincides with
termination phase (monitored by the onset of high-frequency retraction phase. Coincident with the termination of activity in other retraction phase neurons, such as B4 (Hurwitz and Susswein 1996), consistent with observations made in isolated buccal ganglia (Hurwitz and Susswein 1996; Hurwitz et al. 1997), the protraction phase neurons B31/B32, B34, B61/B62, and B63 remained hyperpolarized below their resting membrane potential throughout the retraction phase. Thus similar to observations made in isolated buccal ganglia (Hurwitz and Susswein 1996), the protraction phase neurons were incompletely characterized in the previous studies.

Key buccal interneurons receive monosynaptic excitatory inputs from CBI-2

The preceding data show that CBI-2 can drive BMPs because it drives important elements of the buccal ganglia CPG. However, the CPG is a complex network of electrically and chemically connected neurons, many of which are able to drive BMPs when stimulated (e.g., B31/B32, B34, B63, and B64) (see Hurwitz and Susswein 1996; Hurwitz et al. 1994, 1997; Susswein and Byrne 1988). To characterize in greater detail the mechanisms by which CBI-2 drives BMPs, its synaptic connections to some of the CPG neurons were characterized. Previous data (Rosen et al. 1991; Sanchez and Kirk 2000) reported that B31/B32, B34, and B61/B62 are recruited by CBI-2, but there is no information on possible connections to B63 or to B64, interneurons that are considered central for generating buccal motor programs (Hurwitz and Susswein 1996; Hurwitz et al. 1997, 1999b). In addition, the connections to the other protraction phase neurons were incompletely characterized in the previous studies.

CBI-2 monosynaptically excited B63 in addition to also exciting B31/B32 and B34, (Figs. 2, 3, n = 4 for the data in Fig. 2, n = 5 for the data in Fig. 3). There were no direct connections from CBI-2 to B64 in either experiments performed in ASW or HiDi (not shown). Feeding is normally generated by activating bilaterally symmetrical motor neurons, and therefore it was not surprising to find that synaptic potentials elicited by CBI-2 were observed in both ipsilateral and contralateral buccal interneurons, although the synaptic potentials were 30–70% larger ipsilaterally than contralaterally (Fig. 2).

CBI-2 elicited complex synaptic potentials in ipsilateral and contralateral buccal ganglion protraction phase neurons. Using neurons B31/B32 and B34 as examples, Fig. 2 illustrates the basic features of the synaptic potentials elicited by CBI-2. CBI-2 elicited both fast and slow excitatory synaptic potentials in B31/B32, B34, and B63. The fast EPSPs persisted in a solution containing a high concentration of divalent cations (HiDi), in interneurons B63 and B34 (Fig. 3, A and B) and in the motoneurons B31/B32 and B61/B62 (not shown), suggesting that the connections are monosynaptic.

In addition to this fast component, synaptic potentials elicited by CBI-2 also displayed a slow component (Fig. 3A), which also persisted in a HiDi saline (Figs. 3B and 4). The properties of the slow EPSPs will be explored in detail in a future communication (see Hurwitz et al. 1999a).

As was previously reported by Sanchez and Kirk (2000), the fast EPSPs showed pronounced facilitation. In some cases, the first few spikes within a train produced no discernible postsynaptic potentials were fully facilitated, it displayed values of >5 mV. We have found that the fast EPSPs elicited by CBI-2 in B63 displayed within-train facilitation as well as between-train enhancement, even when the ganglia were bathed in HiDi saline (Fig. 4). There was also a between-train enhancement of the slow component of the EPSPs that CBI-2 elicits in B34 and B63 (Fig. 4).
direct excitation that B31/B32 receives from the (Hurwitz et al. 1994; Susswein and Byrne 1988). Thus the calibrations). Furthermore, B31/B32 have a high threshold for 1 mV in B31 (Figs. 2 and 3; note differences in voltage times larger than the ones recorded in B31/B32. For example, B61/B62 were generally twice as large as those in B63, and 10 previous work demonstrated that B61/B62 are not involved in BMP generation (Hurwitz et al. 1994).

The synaptic potentials that CBI-2 elicited in B34 and in B61/B62 were generally twice as large as those in B63, and 10 times larger than the ones recorded in B31/B32. For example, when the amplitude of the facilitated EPSPs reached 10 mV in B34 and 8 mV in B61, it was only 5 mV in B63 and as low as 1 mV in B31 (Figs. 2 and 3; note differences in voltage calibrations). Furthermore, B31/B32 have a high threshold for triggering their typical activity pattern during a BMP, a plateau-like potential in the somata and spiking in the axon (Hurwitz et al. 1994; Susswein and Byrne 1988). Thus the direct excitation that B31/B32 receives from the firing of CBI-2 is not likely to depolarize B31/B32 sufficiently to initiate motor programs. Because B31/B32 receive strong excitatory inputs from B34 and B63 (Hurwitz et al. 1997), we hypothesized that CBI-2 may access the buccal CPG by first activating neurons B34 and B63 and then via a feed forward excitation of neurons B31/B32. To characterize the sequence of activation of B31/B32, B34 and B63 during motor programs elicited by CBI-2, we recorded simultaneously (n = 3) from these followers of CBI-2 while motor programs were elicited by CBI-2 stimulation (Fig. 5). Firing CBI-2 rapidly depolarized neurons B34 and B63, which on reaching threshold began to fire. By contrast, almost no depolarization of B31/B32 was observed until after B34 and B63 were firing. Thus the evidence supports the idea that the strong inputs from B34 and B63 are major contributors to the recruitment of B31/B32 into motor programs.

To determine whether CBI-2 induces BMPs predominantly via either its excitation of B34 or B63, these neurons were selectively hyperpolarized during CBI-2 firing, thereby preventing them from firing (n = 3 for each neuron). As was seen previously in Fig. 5, when the B34 or B63 neurons were not hyperpolarized, firing in CBI-2 first depolarized neurons B34 and B63, and B31/B32 became depolarized only after they began to fire (Fig. 6A). Hyperpolarizing a single B63 neuron was found to obstruct the expression of a motor program as monitored by activity in B31/B32 (Fig. 6B). Hyperpolarizing B63 also reduced spike activity in the radula nerve (Fig. 6B). These findings extend a previous finding (Hurwitz et al. 1999b) that showed that when BMPs are elicited by tonic stimulation of CBI-2, unilateral hyperpolarization of B63 leads to an interruption of the BMPs until the hyperpolarization is re-

**FIG. 4.** In addition to producing within-train facilitation, stimulating CBI-2 also produced between-train enhancement of the fast EPSPs recorded in B63 and in B34. The recording was in presence of Hidi saline. Note that firing CBI-2 induces EPSPs with both fast and slow components, and the between-train enhancement affects both the fast and the slow components. Note that the lines below the B34 and B63 traces show the resting potential, so that the slow EPSPs can be seen by reference to this potential.

**FIG. 5.** Firing in CBI-2 at a frequency of 12 Hz initiates a BMP that begins within seconds. Spikes in B63 and B34 began ~5 s after activity in CBI-2, whereas spikes in B31 (arrow), which are restricted to the axon, and do not invade the soma, began much later, after ~10 s. Note the onset of activity in the I2 nerve that represent firing in motoneurons B61/B62. Firing later in the I2 nerve represents both B31/B32 and B61/B62 activity.

**FIG. 6.** CBI-2 initiates BMPs via B63. **A:** firing in CBI-2 drives protraction-phase neurons B31, B63, and B34. These neurons are inhibited at the start of retraction. Note that B8 activity as recorded from the RN persists during both protraction and retraction phases. B: B63 was hyperpolarized (arrows), preventing its firing in response to activity in CBI-2. This prevented CBI-2 from driving a BMP. Specifically, although B34 fired in response to CBI-2 stimulation, B31 did not fire, and the retraction phase, as monitored via RN activity and the onset of a rapid inhibition (presumably from firing B64), did not appear. Note that B31 and B34 did display an inhibition with a slower onset than usually seen when B64 fires, and presumably this is driven by neurons other than B64. C: when B34 was hyperpolarized and prevented from firing (arrows), activity in CBI-2 successfully drives a BMP, indicating that B34 is not necessary for driving a BMP via CBI-2 (compare C with A).
of the retraction phase was shortened when the B34 neurons allowed to
curred at the same time relative to the onset of stimulation of potentials was shorter when the two B34s were hyperpolarized.
the duration during which the B31/B32 axon generated action
motion programs (Fig. 7). Speci
predominantly due to
I2 nerve that innervates the I2 muscle, which has a major role
releasing B34 neurons from hyperpolarization restored the characteristic CBI
ring of protraction phase motor neuron
B63 did not prevent the typical protraction phase activity in
this nerve. In CBI-2-induced BMPs, activity in the I2 nerve is
ing CBI-2 still is able to drive a BMP. Specifically, B31 was active, and its activity was terminated via a fast onset of inhibition. However, hyperpolarizing both B34 neurons decreased the time B31/B32 was maximally depolarized and delayed the onset its activity. Note that during B34 hyperpolarization, the duration of the retraction phase was also reduced (both firing of B8 and the postprotraction hyperpolarization of B31 were shortened). C: releasing B34 neurons from hyperpolarization restored the characteristic CBI-2-elicted BMP. The duration of B8 firing and the duration of the post protraction hyperpolarization of B31 were restored (compare C with B). Note that the B34 neurons were somewhat depolarized in this panel.
leased. These experiments also monitored spike activity in the I2 nerve that innervates the I2 muscle, which has a major role in effecting protraction (Hurwitz et al. 1996). Hyperpolarizat-
B63 did not prevent the typical protraction phase activity in
nerve. In CBI-2-induced BMPs, activity in the I2 nerve is
predominantly due to firing of protraction phase motor neuron B61/B62, which independently receives a direct, strong monosynaptic input from CBI-2 (Sanchez and Kirk 2000). This excitation is likely to drive B61/B62 firing even in the absence of inputs from the buccal ganglion CPG.
Unlike the unilateral hyperpolarization of B63, unilateral hyperpolarization of B34 (n = 5) did not obstruct the expression of CBI-2-elicted motor programs (Fig. 6C). Bilateral hyperpolarization of B34 (n = 3), however, affected buccal motor programs (Fig. 7). Specifically, the slope of depolarization of B31/B32 in response to CBI-2 stimulation was shallower when the two B34s were hyperpolarized (Fig. 7). Also, the duration during which the B31/B32 axon generated action potentials was shorter when the two B34s were hyperpolarized. Interestingly, the transition from protraction to retraction occurred at the same time relative to the onset of stimulation of CBI-2 stimulation, independent of whether the two B34s were allowed to fire or were hyperpolarized. However, the duration of the retraction phase was shortened when the B34 neurons were hyperpolarized, and in the absence of B34 activity, the

Facilitation enhances the ability of CBI-2 to recruit protraction neurons
The preceding data, as well as a previous report (Sanchez and Kirk 2000), indicated that the fast EPSPs from CBI-2 to protraction-phase interneurons undergo a prominent facilitation and the facilitated EPSPs summate (see Figs. 2–4). These findings suggested that aspects of the control of BMPs by CBI-2 could depend on the frequency of CBI-2 firing, and the concomitant build-up and the decay of facilitation that may depend on different firing frequencies. We examined this possibility, by exploring systematically the effect of different frequencies of CBI-2 firing on the protraction-phase interneurons.
Facilitation and summation of the EPSPs elicited by CBI-2 affected the ability of CBI-2 to initiate firing in the protraction-phase neurons. When the CBI-2 firing was set to duration of 1.5 s at frequency of 10 Hz, the amplitude of the facilitated EPSPs was below threshold for firing both B34 and B61 (Fig. 8A). Increasing the firing frequency to 15 Hz caused a corresponding large increase in the amplitude of individual EPSPs from 2 to 4 mV (tested on the 5th EPSP) but did not cause firing in either protraction phase interneuron (Fig. 8B). An increase in CBI-2 firing frequency to 20 Hz caused an increase of the EPSP to 6 mV and firing in B61 (Fig. 8C), and a further increase in CBI-2 firing frequency recruited both B61 and B34 (n > 15, Fig. 8D). Thus the ability of CBI-2 to drive protraction-phase neurons is dependent on the CBI-2 firing frequency, and the resultant facilitation of the fast EPSPs.

FIG. 8. The ability of CBI-2 to initiate activity in protraction-phase neurons depends on facilitation and summation of the EPSPs that CBI-2 elicits. Firing of CBI-2 for 1.5 s elicited facilitating EPSPs in B34 and B61. A and B: at firing frequencies <20 Hz, CBI-2 did not drive action potentials in B61 or B34. C: at a firing frequency of 20 Hz, CBI-2 generated only 1 action potential in B61. D: by contrast, when CBI-2 fired at 25 Hz, several action potentials in both B61 and B34 were elicited. Note that both B61 and B34 remained depolarized for several seconds after the termination of CBI-2 stimulation.
neurons (n/H11005) and the latency of the subsequent initiation of the EPSPs that CBI-2 elicits in B63 and B34 and the latency for close to a minute, thereby affecting the ability of subsequent EPSPs to become progressively more effective in initiating activity in protraction phase interneurons B34 and B63. The 1st stimulus train was delivered for 2 s at 15 Hz, and the 2nd train was set to 7 Hz and lasted until the CBI-2 follower neurons began to fire. A: when the interval between the 1st and 2nd train was set to 3 s, the onset of firing in the follower cells occurred at 2.5 s for B34, and at 3 s for B63. B: at a 10-s interval, the B34 latency was 4.2 s and the B63 latency was 4.8 s. C: at a 40-s interval between the 2 trains, the latency for firing in B34 was 4.9 s and the B63 latency was 5.7 s. D: at a 60-s interval, both B34 and B63 latencies were ~6.5 s. Longer intervals lead to a similar latency (not shown). E: a decrease in the EPSPs amplitudes was observed when interval-length was increased. E1: the 3rd EPSPs recorded in B34 were 10, 6, 5, and 4.5 mV in amplitude to intervals of 3, 10, 40, and 60 s, respectively. E2: similarly, the 3rd EPSPs recorded in B63 were 13, 8, 4, and 3 mV in amplitude to intervals of 3, 10, 40, and 60 s, respectively.

After the facilitation was initiated, it could be maintained for close to a minute, thereby affecting the ability of successive bursts of activity in CBI-2 to become progressively more effective in initiating activity in protraction phase neurons. The relationship was examined between the facilitation of the EPSPs that CBI-2 elicits in B63 and B34 and the latency of the subsequent initiation of firing in these neurons (n = 3). A conditioning train (1.5 s at 20 Hz) of action potentials was triggered in CBI-2 (Fig. 9, left). After a variable delay (3, 10, 40, and 60 s) a test train of lower frequency was triggered in CBI-2. The size of the synaptic potentials elicited by the test train was inversely related to the duration of the delay between the conditioning and the test train (see Fig. 9, A–D, right, and E, 1 and 2, which illustrate the 1st 3 EPSPs elicited by CBI-2 at higher gain and with an expanded time base). The size of the EPSPs elicited by CBI-2 declined as the delay between the conditioning and test trains increased. Importantly, the latency to initiate spiking also increased as the duration of delay was increased. These data indicate that the size of the facilitation of the EPSPs that CBI-2 elicits in a key protraction phase interneuron contributes to a reduction in the latency for the onset of spikes in its buccal followers and may thus advance the initiation and perhaps accelerate the rate of buccal motor programs.

The background rate of firing in CBI-2 also contributed to the amplitude of the EPSPs elicited by CBI-2 during a burst. Tonic firing at a relatively high rate maintained the facilitation for longer periods than did lower firing frequencies (n = 4). To explore this effect systematically, CBI-2 was excited with a high-frequency burst under different conditions of tonic background firing (Fig. 10). Decreasing the background firing rate of CBI-2 from nearly 3 Hz (Fig. 10A) to 2 Hz (Fig. 10B) decreased the decay time of facilitation from >8 s to <4 s. Furthermore, interrupting the tonic firing in CBI-2 immediately after the burst for ~8 s decreased the EPSPs amplitude below the baseline value (Fig. 10C). These data indicate that following a burst, the firing frequency of CBI-2 is critical for maintaining the facilitation. The data also indicate that under the conditions of this experiment, in which CBI-2 was firing individual

**FIG. 9.** Facilitation of the EPSPs elicited by firing of CBI-2 can last for tens of seconds. The intervals between 2 trains of spikes elicited in CBI-2 affect both the amplitude of subsequent EPSPs as well as the latency for firing of protraction interneurons B34 and B63. The 1st stimulus train was delivered for 2 s at 15 Hz, and the 2nd train was set to 7 Hz and lasted until the CBI-2 follower neurons began to fire. A: when the interval between the 1st and 2nd train was set to 3 s, the onset of firing in the follower cells occurred at 2.5 s for B34, and at 3 s for B63. B: at a 10-s interval, the B34 latency was 4.2 s and the B63 latency was 4.8 s. C: at a 40-s interval between the 2 trains, the latency for firing in B34 was 4.9 s and the B63 latency was 5.7 s. D: at a 60-s interval, both B34 and B63 latencies were ~6.5 s. Longer intervals lead to a similar latency (not shown). E: a decrease in the EPSPs amplitudes was observed when interval-length was increased. E1: the 3rd EPSPs recorded in B34 were 10, 6, 5, and 4.5 mV in amplitude to intervals of 3, 10, 40, and 60 s, respectively. E2: similarly, the 3rd EPSPs recorded in B63 were 13, 8, 4, and 3 mV in amplitude to intervals of 3, 10, 40, and 60 s, respectively.

**FIG. 10.** The decay of facilitation elicited by firing of CBI-2 is dependent on the background firing rate of CBI-2. A: CBI-2 was stimulated to produce a background firing frequency of 3 Hz. On this background, a 15-Hz train of firing was elicited, and this elicited a profound facilitation. The EPSP amplitude declined to baseline values during the subsequent 9 s. B: the facilitated EPSPs declined to baseline values within ~4 s when the background firing frequency was decreased to 2 Hz. thereby affecting the ability of successive bursts of activity in CBI-2 to become progressively more effective in initiating activity in protraction phase neurons. The relationship was examined between the facilitation of the EPSPs that CBI-2 elicits in B63 and B34 and the latency for the subsequent initiation of firing in these neurons (n = 3). A conditioning train (1.5 s at 20 Hz) of action potentials was triggered in CBI-2 (Fig. 9, left). After a variable delay (3, 10, 40, and 60 s) a test train of lower frequency was triggered in CBI-2. The size of the synaptic potentials elicited by the test train was inversely related to the duration of the delay between the conditioning and the test train (see Fig. 9, A–D, right, and E, 1 and 2, which illustrate the 1st 3 EPSPs elicited by CBI-2 at higher gain and with an expanded time base). The size of the EPSPs elicited by CBI-2 declined as the delay between the conditioning and test trains increased. Importantly, the latency to initiate spiking also increased as the duration of delay was increased. These data indicate that the size of the facilitation of the EPSPs that CBI-2 elicits in a key protraction phase interneuron contributes to a reduction in the latency for the onset of spikes in its buccal followers and may thus advance the initiation and perhaps accelerate the rate of buccal motor programs.

The background rate of firing in CBI-2 also contributed to the amplitude of the EPSPs elicited by CBI-2 during a burst. Tonic firing at a relatively high rate maintained the facilitation for longer periods than did lower firing frequencies (n = 4). To explore this effect systematically, CBI-2 was excited with a high-frequency burst under different conditions of tonic background firing (Fig. 10). Decreasing the background firing rate of CBI-2 from nearly 3 Hz (Fig. 10A) to 2 Hz (Fig. 10B) decreased the decay time of facilitation from >8 s to <4 s. Furthermore, interrupting the tonic firing in CBI-2 immediately after the burst for ~8 s decreased the EPSPs amplitude below the baseline value (Fig. 10C). These data indicate that following a burst, the firing frequency of CBI-2 is critical for maintaining the facilitation. The data also indicate that under the conditions of this experiment, in which CBI-2 was firing individual
spikes, the facilitation decays within several seconds (see Fig. 10), whereas in other conditions, when CBI-2 is firing in bursts, the facilitation lasts for almost a minute (see Fig. 9).

We also examined systematically the effect of triggering CBI-2 with different firing frequencies on the relative length of the protraction and retraction phases of a BMP (Fig. 11). To generate a BMP, CBI-2 firing frequencies were set to 7 Hz because lower frequencies did not initiate even a single motor pattern within a minute. CBI-2 was stimulated to fire at frequencies of 7, 10, 15, 20, 25, and 30 Hz, until the completion of a single BMP. The experiment was performed with intervals of 1 min between stimulus trains. Data were discarded from the first four BMP cycles elicited because data from these trials were quite variable. Data that were collected for subsequent analysis were from the fourth and later BMP cycles, when the BMPs became highly reproducible and regular. Each of the frequencies was tested three times in each animal in a random order to ensure a stable baseline, and the three tests were averaged. The buccal ganglia were not desheathed to reduce the potential for damage. The protraction phase was monitored via extracellular recording of the I2 nerve (I2N), and the time interval between cessation of I2N activity and the cessation of RN activity was used as a monitor of the duration of the retraction phase.

Several parameters of the BMP were sensitive to increasing the firing frequency of CBI-2 (Fig. 11A). Specifically, at a frequency of 7 Hz, the latency to the onset of protraction and the duration of the protraction phase were both relatively long. In the example shown in Fig. 11A, the latency for the onset of the protraction phase was >10 s, and the duration of the protraction phase reached 15 s (Fig. 11A1). As the stimulation frequencies were progressively increased, the latencies for the onset of the protraction phase decreased and reached values of <1 s (Fig. 11A, 2-6). The duration of the protraction phase also decreased to <7 s. The duration of a BMP also decreased (from ~20 to ~10 s), even though the duration of the retraction phase appeared resistant to changes in frequency (see the table in Fig. 11B for the calculated averages). The mean values

<table>
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<tr>
<th>CBI-2 firing rate (Hz)</th>
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<th>15</th>
<th>20</th>
<th>25</th>
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<tr>
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<tr>
<td>Protraction-Duration</td>
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<tr>
<td>Average</td>
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<td>15.8</td>
<td>16.0</td>
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</tr>
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<tr>
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<td>BMP-Duration (Prot. + Ret)</td>
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Note the particularly large effect of high frequencies on the latency for the onset of protraction. Note also the large effect of 30 Hz on the duration of the protraction phase. As the stimulation frequency is increased, the latency for the onset of protraction decreases, and the duration of the protraction phase decreases. This is also true for the duration of the BMP. However, the duration of the retraction phase appears resistant to changes in frequency. These effects are statistically significant (Fig. 11C) and are consistent with the idea that the CBI-2 neurons have a direct excitatory influence on the motor neurons of the protraction phase.
of four parameters at the six frequencies of stimulation are presented in Fig. 11B.

In view of the variability between different preparations, data for statistical analyses were expressed as percentages of the results obtained in response to a 7-Hz stimulation. Data from all seven preparations were then combined. The latency for the onset of activity in the I2N showed a significant difference \[F(5,36) = 17.8; P < 0.0001\] among the six stimulus frequencies (Fig. 11C1). In addition, there was a significant difference in the duration of protraction phase \[F(5,36) = 4; P < 0.01\] among the six stimulus frequencies (Fig. 11C2). There was also a significant difference in the duration of BMP cycles \[F(5,36) = 3.7, P < 0.01;\] Fig. 11C3). There was no difference in the length of the retraction phase in the different CBI-2 firing frequencies \[F(5,36) = 0.65, P > 0.05;\] all tests are 1-way ANOVAs; data not shown.

**Hexamethonium blocks fast synaptic potentials elicited by CBI-2 and delays the onset of firing of its followers**

To examine further the properties and functions of the fast synaptic potentials from CBI-2 to the buccal ganglia CPG, we sought to identify an antagonist of a transmitter that elicits these EPSPs. Hexamethonium, a cholinergic antagonist that has been extensively used to block fast cholinergic EPSPs and end junction potentials (EJPs) in *Aplysia* (Cohen et al. 1978; Gardner and Kandel 1977; Hurwitz et al. 2000), was found to be an effective blocker of the fast EPSPs that CBI-2 elicited in its followers, suggesting that CBI-2 is a cholinergic neuron. Figures 12 and 13 demonstrate the block of the fast EPSPs that CBI-2 elicited in various neurons, including B31/B32, B63, B61/62, and B34. In the example illustrated in Fig. 12, the follower neurons of CBI-2 (B31/B32, B63, and B61/62) were slightly hyperpolarized to preventing spiking of CBI-2 followers and to increase the amplitude of the EPSPs. In this condition, a train of action potentials that was triggered in CBI-2 elicited one for one EPSPs in B34 (Fig. 12A). A complete elimination of the fast EPSPs was observed during bath application of \(10^{-3}\) M hexamethonium (Fig. 12B). The action of hexamethonium was reversible if the washout began shortly after the fast component of the EPSPs was blocked (Fig. 12C). If, however, hexamethonium was left in the bath for >20 min, only a partial recovery occurred even after a 1 h of washout (not shown).

![Fig. 12. EPSPs evoked in B61, B63, and B31 by firing of CBI-2 are blocked by the cholinergic antagonist hexamethonium. A: firing of CBI-2 for 1.5 s at a frequency of 11 Hz evoked a train of facilitated EPSPs that was observed in B61, B63, and B31. B: hexamethonium \((10^{-3}\) M\) caused a decrease in the amplitude or completely blocked the EPSPs. C: the effects of hexamethonium were partially reversed after 10 min of washing in artificial seawater (ASW).](image)

![Fig. 13. Fast EPSPs evoked in B34 by firing of CBI-2 are blocked by the cholinergic antagonist hexamethonium. Note that the recording is in HiDi. A: firing of CBI-2 for 1.5 s at a frequency of 14 Hz evokes a train of facilitated EPSPs in B34. B: the amplitude of the fast EPSPs was decreased in \(10^{-3}\) M hexamethonium. C: a higher concentration of hexamethonium \((10^{-4}\) M\) reduced the summed EPSPs amplitude still further. D: in \(10^{-3}\) M hexamethonium, the fast EPSP was completely blocked. E: the effects of hexamethonium were partially reversed after 10 min of washout in HiDi (post Hex).](image)

The EPSPs elicited by CBI-2 in B34 were examined, to test the response to different concentrations of hexamethonium. In this experiment, ASW was replaced with HiDi to prevent polysynaptic activity. This allowed us to record from neuron B34 at −45 mV. Under these conditions, the slow component of the EPSP is readily observed. A train of action potentials triggered in CBI-2 elicited one-for-one EPSPs in B34 (Fig. 13A). Application of \(10^{-3}\) M hexamethonium caused a reduction in the amplitude of the fast EPSPs (Fig. 13B) by 25%. Increasing the concentration of hexamethonium to \(10^{-4}\) M reduced the EPSPs by 70% (Fig. 13C), and the fast EPSPs were abolished at a concentration of \(10^{-3}\) M hexamethonium (Fig. 13D). The action of hexamethonium was reversible (Fig. 13E).

Figures 12 and 13 also demonstrate that hexamethonium preferentially blocks the fast component of the EPSP while...
leaving essentially intact the slow component that CBI-2 elicited in its followers.

CBI-2 elicits both fast and slow EPSPs in its followers, and in principle, the ability of CBI-2 to initiate BMPs could depend on either or both types of EPSP. Because hexamethonium preferentially blocks the fast EPSPs, we attempted to use hexamethonium to characterize the contribution of fast synaptic potentials to the initiation of BMPs. In every case (n = 3 for B61/B62 and B63, n = 4 for B31/B32, and n = 7 for B34), a partial block of the fast EPSPs by a brief (<1 min) perfusion with 10^-4 M hexamethonium delayed the onset of activity in the protraction phase interneurons (Fig. 14, A–D). However, in the presence of hexamethonium, the slow build-up of the depolarization elicited by CBI-2 stimulation in its followers was also reduced. In part, this reduction could be explained by the lack of summed fast EPSPs and in part because the slow EPSP may be partially voltage-dependent and the lack of the fast EPSPs reduces its depolarization-dependent amplification.

To quantify the data on the delay of activity in CPG elements as a result of treatment with hexamethonium, the data were expressed as a percentage of the result obtained when stimulation of CBI-2 was performed in ASW (Fig. 14E). An analysis of these data showed that there were significant increases in the latency to trigger the first action potential in B31/B32 [P < 0.05; n(3) = 5.7], B34 [P < 0.05; n(6) = 2.29], and B63 [P < 0.05; n(2) = 4.91; 2-tailed paired t-test; Fig. 14]. For B63, the onset was delayed from 5.2 to 7.7 s, for B34, the onset was delayed from 8.6 to 13.5 s, and for B31, the onset was delayed from 4.4 to 5.8 s.

**Hexamethonium attenuates fast synaptic potentials elicited by B34 and B63 and differentially delays the onset of firing in their followers**

The preceding data indicate that CBI-2 may be a cholinergic neuron because the fast EPSPs it elicits in the protraction phase neurons of the buccal ganglia were blocked by hexamethonium. We found that the buccal ganglion protraction-phase neurons are also likely to be cholinergic because hexamethonium was also found to block the interconnections between protraction phase interneurons (Fig. 15). Hexamethonium (10^-4 M) attenuated the connection from B63 to B31/B32 by 70% and blocked the connection from B34 to B31/B32. It is important to note that the EPSP from B63 to B31/B32 was previously shown to be a mixed electrical/chemical synapse, and the residual EPSP displayed in the presence of hexamethonium is likely to be the electrical component of this connection. The effects of hexamethonium were fully reversed when it was washed out.

Previous data showed that B63 elicits monosynaptic EPSPs in B31/B32 and also initiates a BMP, which consists of a protraction and, after a delay, a retraction. The finding that B63 is a cholinergic neuron prompted us to examine whether B63 can drive either protraction or retraction when cholinergic transmission is blocked with hexamethonium.

Firing B63 at a low rate elicited a BMP in which firing in the B31/B32 axon was initiated after ~6 s, and retraction was initiated after ~21 s (Fig. 16A). In the presence of hexamethonium (10^-4 M), firing in the B31/B32 axon was delayed and began after ~16 s, but the time of onset of retraction was minimally affected (Fig. 16B). The action of hexamethonium was partially reversible (Fig. 16C). This experiment demonstrates that hexamethonium slows but does not block the protraction phase while having a minimal effect on retraction. Thus B63 activity is likely to activate receptors that are not sensitive to hexamethonium, which set into play a delayed protraction and a normally timed retraction. Experiments similar to those in Fig. 16 were performed in seven preparations.

**FIG. 14.** The latency for the onset of the protraction phase is sensitive to hexamethonium. A: CBI-2 was fired at a frequency that drives the protraction phase neurons. In ASW, the latency for firing B34 was <2 s (see ▼). After hexamethonium (10^-4 M) application, the latency increased. The effects of hexamethonium were partially reversible. B–D: similar effects of hexamethonium application were observed on the latency for firing B31, B61, and B63. Note that the time bar for A and B represents 2 s, but for C and D, it represents 5 s. E: summary of the data from experiments similar to that in A–D in a number of preparations. For each preparation, several runs were performed, and the data were averaged and normalized to the results obtained before the hexamethonium was applied. A total of 3 B63, 7 B34, and 4 B31 preparations were tested. Data were combined and tested for statistical significance, which revealed that hexamethonium application significantly affected the latency for firing in each of the protraction neurons tested [for B63, P < 0.05; n(2) = 4.91; for B34, P < 0.05; n(6) = 2.29, and for B31, P < 0.02; n(3) = 5.7; 2-tailed paired t-test].
The activity of CBI-1 affects the pattern of a BMP via its direct effects on the protraction phase neurons in the buccal ganglia. We found that when both the cerebral and buccal ganglia were bathed in HiDi \((n = 5)\), firing CBI-1 for 2 s evoked a 2-mV depolarization in B63, but not in B34, when the membrane potentials of the cells were held at \(-65\) mV (Fig. 18A). However, when B63 and B34 were held at \(-50\) mV, CBI-1 firing led to a stronger depolarization and firing in B63.

**CBI-1 and CBI-3 modulate BMPs**

The frequency of CBI-2 firing was shown to affect the length of the protraction phase, and the onset of retraction (see Fig. 11). In part, this is likely to arise from the facilitation of the EPSPs elicited by CBI-2 in B63 and an increase in B63 firing. However, an increased firing frequency in CBI-2 can also have additional effects. Previous data (Rosen et al. 1991) have shown that activity in CBI-2 causes firing in additional CBIs, which in turn could affect the buccal ganglion CPG. These CBIs are also activated by food stimuli in parallel to their activation of CBI-2. The ability of CBI-2 to recruit other CBIs is likely to be dependent on the firing frequency of CBI-2. We examined the effects of CBI-2 on the protraction-phase neurons with and without the additional activity of two other CBIs, CBI-1 and CBI-3.

To test whether firing CBI-1 along with CBI-2 could affect the length of the protraction phase, and the transition to retraction, we initiated BMPs by firing CBI-1 at a relatively low frequency (12 Hz until the end of a single BMP). Superimposed on this activity, CBI-1 was stimulated at different frequencies, beginning at 5 s after the start of the CBI-2 stimulus. Increasing the firing frequency of CBI-1 systematically decreased the BMP duration (Fig. 17A).

To quantify the effects of CBI-1 activity on BMPs elicited by CBI-2, in five preparations, CBI-1 was stimulated at five different frequencies (0, 5, 10, 20, and 30 Hz), during programs initiated by CBI-2. The different frequencies were applied in a random order with a rest of 60 s between trials. Stimulation of CBI-1 began 5 s after the start of CBI-2 stimulation and was continued until the end of the elicited motor program. The data were averaged and then expressed as a percentage of the results in the absence of CBI-1 activity. There were significant differences in the overall duration of the BMP \([F(4,20) = 19.8; P < 0.0001]\) as well as in the duration of the protraction phase \([F(4,20) = 39.8; P < 0.0001]; \text{Fig. 17, B and C}\). However, there was no significant difference in the duration of the retraction phase \([F(4,20) = 0.4; P > 0.5] ; 1\)-way analyses of variance; not shown]. These data indicate that an increase in CBI-2 frequency may affect the pattern of a BMP in part via effects on CBI-1 as well as via the increased facilitation.

The activity of CBI-1 affects the pattern of a BMP via its direct effects on the protraction phase neurons in the buccal ganglia. We found that when both the cerebral and buccal ganglia were bathed in HiDi \((n = 5)\), firing CBI-1 for 2 s evoked a 2-mV depolarization in B63, but not in B34, when the membrane potentials of the cells were held at \(-65\) mV (Fig. 18A). However, when B63 and B34 were held at \(-50\) mV, CBI-1 firing led to a stronger depolarization and firing in B63.

**FIG. 15.** EPSPs elicited in B31 by firing protraction-phase interneurons B63 and B34 are hexamethonium sensitive. Note that this experiment was performed in HiDi. A, left: firing of B63 for one second at a frequency of 10 Hz evoked a train of EPSPs in B31. A, middle: the EPSPs amplitude was attenuated in hexamethonium \((5 \times 10^{-4} \text{M})\). Note that the residual component of the EPSP is a previously described electrical PSP. A, right: the block of the chemical EPSP caused by hexamethonium was reversed when the preparation was washed in HiDi. B: similar results were obtained when EPSPs in B31 were elicited by firing B34. B, left: firing B34 for 1 s at frequency of 10 Hz evoked a train of facilitated EPSPs in B31. B, middle: the EPSPs were fully abolished in hexamethonium \((5 \times 10^{-4} \text{M})\). B, right: the effect of hexamethonium was reversed following a washout in HiDi.

**FIG. 16.** Hexamethonium delays the onset of B31 firing but leaves intact the onset of the retraction phase during B63-elicited BMPs. A: in this experiment, B63 was stimulated to fire at a low frequency. This drives a cycle of BMP with \(-30\) ms duration. The B31 axon fires \(-6\) s after the start of the stimulation. In this and in all recordings from B31, spikes in the B31 axon fail to invade the soma and are recorded as fast depolarizations of \(-10\) mV that are superimposed on a sustained depolarization of B3. The axon spikes occur on a background of EPSPs from B63 and from other neurons. Activity in B63 and in B31 is terminated and B4 is depolarized and fires, at the start of the retraction phase. B: hexamethonium \((10^{-4} \text{M})\) delayed the onset of firing in B31 \((f)\) in response to B63 stimulation to \(-16\) s. However, the latency for onset of retraction phase was unchanged. C: the hexamethonium effect was reversible. Note that in hexamethonium, B63 still elicited electrical EPSPs in B31 that are \(-60\%\) smaller than the mixed chemical-electrical EPSPs elicited in the absence of hexamethonium.
In a higher gain recording (Fig. 18B), a small, summated inhibitory postsynaptic potential (IPSP) of \(-1 \text{ mV}\) was detected in B34 and a small, summated EPSP of \(0.5 \text{ mV}\) was detected in B31. These PSPs lasted for \(1 \text{ s}\). To investigate the differential effects of CBI-1 activity on B63 and B34, B34 was depolarized with brief suprathreshold pulses with and without stimulating CBI-1, and the effect of this stimulus on EPSPs in B31/B32 was examined. CBI-1 activity caused a suppression of B34 firing (Fig. 18C) and a cessation of the B34-elicited EPSPs in B31/B32. In place of the excitation from B34, a barrage of EPSPs was seen in the B31/B32 neuron from another source, (presumably B63 because the EPSPs are similar to those elicited by firing of B63). These data indicate that recruiting CBI-1 leads to a modulation of BMPs by amplifying the activity of B63 while inhibiting B34. The differential targeting of protraction phase neurons is likely to affect the motor output because neurons such as B34 excite some of the motorneurons (see Hurwitz et al. 1997 for its excitatory effects on B61/B62 and B8). Other CBIs may also modulate the form of a BMP, as shown by a previous study that demonstrated the role of CBI-3 in shaping CBI-2 programs (Morgan et al. 2002). Thus these data are consistent with the hypothesis that recruiting CBI-1 affects the protraction phase via the direct effects of CBI-1 in the buccal ganglia.

We also examined the effects of stimulating CBI-3, a second CBI neuron excited by CBI-2. These experiments \((n = 4)\) were done in HiDi. In contrast to the effects of firing CBI-1, firing of CBI-3 elicited fast excitatory EPSPs of low amplitude (0.5 mV) in B34 with no comparable fast EPSPs in B31/B32 or B63 (not shown). Firing CBI-3 amplified the effects caused by weak depolarizing currents injection into B34. Pulses that were below threshold for inducing firing in B34 in the absence of CBI-3 activity induced firing during and somewhat after CBI-3 stimulation (Fig. 19A). A similar amplification of subthreshold pulses was not observed in B63 (not shown). However, a further investigation of the effects of CBI-3 on B63 showed that suprathreshold stimuli to B63 were weakly amplified in that CBI-3 caused a twofold increase in the firing frequency (Fig. 19B).
To examine in greater detail the differential effects of CBI-3 activity on B63 and B34, CBI-3 was depolarized sufficiently, so that firing was seen in both the B31 axon and in B34 (Fig. 19, left). Hyperpolarizing B34, and thereby preventing it from firing, led to a large decrease in the depolarization, from a value of 40 to <20 mV, recorded in B31, and a cessation of its axon firing (Fig. 19C, middle). The effect of B34 hyperpolarization was easily reversed (Fig. 19C, right). B34 cannot alone drive a depolarization of B31 as large as that seen in Fig. 19C (Hurwitz et al. 1997), and this depolarization is likely to be via the effects of B34 on B63, which is able to elicit strong excitation of B31. These data indicate that both CBI-1 and -3 act on the protraction phase neurons that are followers of CBI-2. This action presumably shapes the form of the BMPs elicited by CBI-2. These effects are likely to be components of the modulation of BMPs elicited by CBI-2.

**DISCUSSION**

We found that the buccal motor programs driven by the CBI neurons that were examined are similar to those observed when CPG neurons in the buccal ganglia are directly stimulated (Figs. 1, 5, 6, 7, 16, and 17). This is because the ability of the CBIs to drive motor programs stems in part from monosynaptic excitatory connections onto key protraction-phase interneurons (Figs. 2, 3, 4, 8, 18, and 19), which can themselves initiate programs. One of the cells examined, CBI-2, causes firing of the protraction-phase neurons only after the direct connections undergo a strong facilitation (Fig. 8). Thus the facilitation is an integral feature contributing to the ability of CBI-2 to recruit the CPG neurons and to initiate buccal motor programs.

The CBIs were found to excite monosynaptically only pro-
ttraction phase interneurons and motorneurons, such as B31/ B32, B34, B61/B62, and B63, and not retraction-phase interneurons, such as B64. Additional studies (Jing and Weiss 2001, 2002) have shown that the CBIs also excite other protraction phase neurons, such as B20 and B40. Previous studies have shown that BMPs initiated by stimulating peripheral nerves or identified neurons, as well as feeding responses to food, always begin with a protraction phase, and this is followed by retraction (Kupfermann 1974; Susswein and Byrne 1988). The sequencing of motor programs seems to arise in part from the direct recruitment of protraction phase neurons by the CBIs, with the subsequent recruitment of retraction-phase neurons via circuitry within the buccal ganglia. In *Aplysia*, all of the consummatory behaviors that have been identified, as well as all of the BMPs that are generally observed, are characterized by the protraction phase preceding the retraction. However, in *Helisoma*, there are programs that can begin with retraction (Quinlan and Murphy 1996).

We also found that the different CBIs examined differentially connect with the various protraction-phase neurons (Fig. 20). The three protraction-phase neurons examined in detail, B31/B32, B34, and B63, are all able to drive motor programs when depolarized (Hurwitz et al. 1994, 1997; Susswein and Byrne 1988) but are not equal targets for inputs from the CBIs. Some CBIs cause depolarization and firing of all of these neurons, whereas others differentially excite and inhibit the different interneurons. The differential activation of protraction phase neurons changes the form of a BMP (Figs. 11 and 17). Previous studies have shown that the differential activity of different CBIs can act as a mechanism for selecting specific types of BMPs (Jing and Weiss 2001, 2002; Morgan et al. 2002). In addition, firing CBIs at different frequencies also changes the form of a BMP, suggesting that modulating the firing frequency may also allow the CBIs to play a role in shaping a specific consummatory behavior.

It is important to note that there is little information on the firing frequencies of the CBIs in behaving animals. However, previous studies (Rosen et al. 1991) have characterized the firing frequency in response to natural stimuli such as food on the lips as well as characterizing the frequencies during buccal motor programs. The stimulus frequencies used in our experiments on the CBI cells are well within those that have been found previously to drive BMPs or to be characteristic of the activity of these neurons during BMPs. According to Rosen et al. (1991), the firing rate of CBI-2 in response to inner lips stimulation by seaweed is >10 Hz. Because such stimuli activate both the left and the right CBI-2, the effective input to buccal ganglia may be stronger than that represented by the rates of stimulation that we used with a unilateral stimulation of CBI-2. In addition, close examination of Fig. 8 in Rosen et al. (1991) shows that CBI-1 fires at frequencies in excess of 25 Hz. Thus the various effects of the CBIs that are described in this paper are likely to occur in behaving animals.

**Command-like and Modulatory Functions of the CBIs**

CBI-2 has some of the properties of a command neuron. Command neurons are defined by being both necessary and sufficient to induce a specific behavior (Kupfermann and Weiss 1978). To be necessary and sufficient, the command neuron must be recruited by stimuli that cause the behavior, firing the command neuron should induce the behavior, and removing the command neuron should block the behavior (Kupfermann and Weiss 1978). CBI-2 is excited by food stimulating the lips (Rosen et al. 1991), the natural stimulus that initiates biting, one of the consummatory behaviors that are generated by the buccal ganglia CPG. Stimulating CBI-2 initiates motor programs, and previous data using chronic recording techniques have shown that the motor programs elicited by the protraction-phase interneurons that are recruited by CBI-2 are correlates of consummatory behaviors, such as biting, swallowing, and rejection (Hurwitz et al. 1996). Thus firing CBI-2 is sufficient for initiating a consummatory behavior, perhaps biting (see Morgan et al. 2002). There are no data directly testing whether CBI-2 is necessary for behavior. However, our data as well as that of others (Hurwitz et al. 1999b; Morgan et al. 2002; Rosen et al. 1991), suggest that CBI-2 is unlikely to act alone in initiating a consummatory response and is therefore unlikely to fulfill the criterion of necessity. In addition to exciting CBI-2, food stimuli also excite other CBIs as does the firing of CBI-2 (Rosen et al. 1991). Furthermore, the specific combination of CBIs that are activated biases the CPG to produce ingestion- or egestion-like behaviors (Jing and Weiss 2001, 2002; Morgan et al. 2002). Thus it is unlikely that CBI-2 acts as a command neuron eliciting a unique motor program, but rather CBI-2 is likely to act as an important element in a system that both initiates programs and also biases the CPG toward one of a number of different motor patterns.

CBI-2 recruits a number of protraction phase neurons whose activity can initiate BMPs (Fig. 20A). However, our data indicate that the recruitment of B63 is particularly important.
because this neuron fires early in CBI-2-initiated programs and the EPSPs elicited by CBI-2 in B63 are larger than are those elicited in B31/B32. In addition, hyperpolarization of B63 blocks the ability of CBI-2 to initiate a BMP. Although B31/B32 and B63 and electrically coupled and hyperpolarizing B63 will therefore also hyperpolarize B31/B32, it is unlikely that the block of CBI-2-induced motor program is via the coupling to B31/B32. First, the coupling from B63 to B31/B32 is smaller than is the coupling in the opposite direction (Hurwitz et al. 1997; Susswein et al. 2002). Second, the input resistance of B31/B32 is very low, and large currents are therefore required to affect its activity (Susswein and Byrne 1988; Susswein et al. 2002). The currents injected into B63 were smaller than those required to block activity of B31/B32.

Our data also suggest that CBI-1 and CBI-3 are components of the system that initiates and selects between BMPs (Fig. 20B). However, these neurons have more of a modulatory and less of a command function than does CBI-2 in that they bias the CPG to less of a command function than does CBI-2 in that they bias the CPG to fire in a specific pattern, by their differential effects on different protraction-phase interneurons, and they are less effective in initiating buccal motor programs (Rosen et al. 1991). Their behavioral function is likely to depend on which combination of CBIs and CPG elements are active at a given time.

One characteristic of motor programs elicited by stimulating CBI-2 is that the retraction phase is relatively brief (<5 s), and its length is not varied by changes in the CBI-2 firing frequency. Increasing the firing frequency of CBI-2 affects the retraction phase only by reducing the length of the protraction phase, and thereby advancing the onset of retraction (Fig. 11). It is important to note that the duration of the retraction phase is changed by a variety of experimental procedures, which give rise to retraction phases of ≤8 s in length. The variability in the length of the retraction phase may arise from the activity of additional CBIs that were not examined in this study (Morgan et al. 2002) as well as by the recruitment of additional protraction-phase neurons whose activity is not essential in organizing the protraction-retraction sequence but that may function in shaping the sequence for different behavioral functions (Jing and Weiss 2001, 2002; Nargeot et al. 1997).

DIFFERENTIAL SELECTION OF PROTRACTION PHASE NEURONS. CBI-2 initiates BMPs in part via its monosynaptic excitation of B31/B32, B34, and B63. However, it is possible that motor programs elicited by stimulation of other neurons, or via natural stimuli initiating consummatory behaviors, differentially act on other CPG elements.

Stimulation of CBI-2 recruits other CBIs that affect the buccal ganglia CPG (Morgan et al. 2002; Rosen et al. 1991). Such recruitment is unlikely to be responsible for the effects seen with brief stimulation of CBI-2 because we have found that the recruitment of other CBIs is via a recurrent pathway from one of the buccal ganglia projection neurons back to the cerebral ganglion (unpublished observation). Thus other CBIs would not fire until after the buccal ganglia CPG is recruited. In addition, in many experiments, the use of HiDi saline would have prevented the recruitment of additional CBIs. However, when CBI-2 is stimulated for a longer period in the absence of HiDi, some of the effects seen are likely to be caused by recruiting other CBIs, such as CBI-1 and -3. The modulatory effects of these neurons on some of the protraction interneurons in the buccal ganglia are documented in this paper, as well as in Jing and Weiss (2001, 2002).

The other CBIs examined differed from CBI-2 in their effects on the protraction-phase neurons. CBI-1 was similar to CBI-2 in that it excited B63, but it differed from CBI-2 in that it inhibited B34 (Fig. 18). By contrast, CBI-3 more strongly excited B34 than B63 (Fig. 19). Its ability to induce a BMP is likely to be via its excitation of B34, which in turn excites B63 and B31/B32 (Fig. 20B).

It is important to note that additional protraction-phase interneurons have been identified (e.g., B20, B40, and B65) (see Jing and Weiss 2001; Kabotyansky et al. 1998; Teyke et al. 1993), and activity of these neurons can bias programs to be more ingestion or rejection-like. The connections of some of the CBIs to these neurons were not examined in the present research. However, Jing and Weiss (2001, 2002) have shown that CBIs affect these neurons as well.

FUNCTION OF FACILITATION. We have shown that the fast EPSPs from CBI-2 to the protraction-phase interneurons undergo a prominent facilitation (Figs. 2, 3, 4, 9, and 10). This is consistent with previous findings (Hurwitz et al. 1999a), which showed that the output of CBI-2 to B63, as well as the output to protraction phase motor neurons B61/B62 and to interneuron B34 (Sanchez and Kirk 2000), undergo facilitation. Our data extend the previous findings by showing that facilitation affects both the fast and slow components of the EPSP, by showing that facilitation in part underlies the ability of CBI-2 to initiate motor programs, and by examining systematically the effects of different firing frequencies as well as by examining a number of properties of the EPSPs. We confirmed that the facilitation elicited by a brief high-frequency burst of activity in CBI-2 is maintained for ≤1 min as was demonstrated in a previous study (Sanchez and Kirk 2000). However, the previous work did not distinguish between the effects of the fast and slow components of the EPSP. In addition, tonic firing preceding a burst of activity was now shown to produce a less profound facilitation lasting only several seconds, an issue that was not previously explored. There are functional differences in the effects of the quickly and slowly decaying components of facilitation. The quickly decaying component of facilitation is likely to affect only a single cycle of a program (Fig. 10) because it decays during the retraction phase. By contrast, the slowly decaying component of facilitation will affect multiple cycles of BMPs (Fig. 9). Our data also indicate that the ability of CBI-2 to command a BMP is functionally related to the facilitation because firing in the protraction phase neurons is elicited only after the EPSPs are facilitated.

Our data suggest that facilitation may have a number of functions in the regulation of BMPs. First, our data have shown that the initial EPSPs from CBI-2 to protraction phase interneurons are very small, and must be facilitated, before they can induce firing in follower cells. The need for sufficient facilitation of the EPSPs from CBI-2 to the protraction interneurons is likely to cause a delay in the initiation of a BMP in response to CBI-2 activity as well as in response to food. Such a delay is also a property of the protraction interneurons themselves, which often respond with a latency of a few seconds to stimuli that induce a buccal motor program (Susswein and Byrne 1988; Susswein et al. 2002). During the time gap between a stimulus that initiates a BMP and the response, the protraction-
phase neurons are exquisitely sensitive to additional inputs, which may advance, delay, or even block the expression of a BMP (Susswein et al. 2002). Thus the system has a time window between a sufficient stimulus and the response, in which it is not yet fully committed to the response and in which the response characteristics can be changed. The need for facilitation may contribute to the ability to delay a full commitment to respond.

Second, our data (see Fig. 1), as well as previous data (Morgan et al. 2000; Susswein et al. 1996), have shown that activation of CBIs-driven BMPs often occurs with a delay, followed by a gradual build-up in the amplitude and frequency of BMPs. This build-up is highly reminiscent of the food-induced arousal seen in intact behaving animals in which the first responses to food are initiated with a long latency and a weak amplitude and subsequent responses occur at a higher frequency and amplitude (Kupfermann 1974; Susswein et al. 1978). Our data demonstrate that the facilitating EPSPs elicited in B63 and in B34 are correlated with a shortening in the latency (Fig. 9), suggesting that the facilitation may serve as a mechanism contributing to the progressive decrease in the latency. However, it is important to note that the facilitation cannot account for the maintenance of the increased frequency and amplitude of BMPs because food-induced arousal can be maintained for many minutes (Susswein et al. 1978), whereas CB1-2-induced facilitation decays in ~1 min (Sanchez and Kirk 2000). Thus CB1-2-induced facilitation could contribute to a warm-up of feeding lasting for no longer than 1 min, and other mechanisms must be involved in the longer-lasting maintenance of facilitation.

Third, different levels of facilitation that stem from different frequencies of CB1-2 firing may produce BMPs of different waveforms with higher frequencies associated with a shortened protraction phase (see Fig. 11). A reduction in the protraction-phase duration, with respect to retraction, is characteristic of ingestion, particularly swallowing (Hurwitz et al. 1996; Nargeot et al. 1997) because in ingestion, retraction is the power phase. Previous studies have shown that firing CB1-2 initially causes rejection-like behaviors, which later become more ingestion-like (Morgan et al. 2002). This change in the nature of the BMPs has been attributed in part to the recruitment of other mechanisms contributing to the progressive decrease in the latency. However, it is important to note that the facilitation cannot account for the maintenance of the increased frequency and amplitude of BMPs because food-induced arousal can be maintained for many minutes (Susswein et al. 1978), whereas CB1-2-induced facilitation decays in ~1 min (Sanchez and Kirk 2000). Thus CB1-2-induced facilitation could contribute to a warm-up of feeding lasting for no longer than 1 min, and other mechanisms must be involved in the longer-lasting maintenance of facilitation.

It is important to note that the possible functions in the preceding text are not mutually exclusive, and changes in CB1-2 firing frequency may participate in all of these processes.

CHOLINERGIC TRANSMISSION BY CBIS AND BY PROTRACTION INTERNEURONS. Our data suggests that acetylcholine (ACh) is the transmitter underlying the fast EPSPs from CB1-2 to the buccal ganglion protraction interneurons as well as underlying the fast EPSPs caused by the firing of the protraction-phase interneurons. Previous studies (Hurwitz et al. 2000) have shown that ACh is also the transmitter released by protraction-phase motor neurons B31/B32 and B61/B62 onto muscle I2, the major muscle that effects protraction. Additional data supporting the notion that these neurons are cholinergic stem from the finding that muscarinic antagonists block the slow components of the EPSPs. However, additional experiments will be needed to demonstrate conclusively that these neurons are cholinergic. By contrast, B20 and B65, two additional interneurons that are active during the protraction phase, utilize dopamine as their transmitter (Kabotyaniski et al. 1998; Tekey et al. 1993). Thus the protraction phase neurons are heterogeneous with respect to the transmitter used.

In *Lymnaea*, one of the protraction phase neurons, the SO (slow oscillator) neuron, is cholinergic (Yeoman et al. 1993). The transmitter used by other protraction-phase neurons has not been identified. By contrast, interneurons underlying retraction in *Lymnaea* (N2 cells) and Helisoma (S2 cells) utilize glutamate as their transmitter (Brierly et al. 1997; Quinlan and Murphy 1996). The transmitter utilized by the retraction-phase interneurons in *Aplysia* has not yet been identified. However, by the B52 neurons, which fire at the end of retraction and terminate it, utilize histamine as their transmitter (Baxter et al. 1997; Evans et al. 1999).

*Aplysia* feeding is regulated by changes in environment, by motivational variables, and by learning (for review, see Elliott and Susswein 2002). These variables often have widespread effects on many aspects of behavior. Our data have shown that receptors sensitive to hexamethonium are found in a number of hierarchically arranged sites within the *Aplysia* feeding motor system. The use of a common receptor at these sites potentially allows the common regulation of many sites in tandem by an exogenous input. However, it is also likely that mechanisms exist that can selectively regulate a specific site in the hierarchy, can modulate selectively the recruitment of different CBIs or CPG elements, or can modulate the facilitation selectively.

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