Transforming Tonic Firing Into a Rhythmic Output in the *Aplysia* Feeding System: Presynaptic Inhibition of a Command-Like Neuron by a CPG Element

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1The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center and 2Faculty of Life Sciences, Bar Ilan University, Ramat Gan, Israel; and 3Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York City, New York

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Hurwitz, Itay, Abraham J. Susswein, and Klaudiusz R. Weiss. Transforming tonic firing into a rhythmic output in the *Aplysia* feeding system: presynaptic inhibition of a command-like neuron by a CPG element. J Neurophysiol 93: 829–842, 2005. First published August 11, 2004; doi:10.1152/jn.00559.2004. Tonic stimuli can elicit rhythmic responses. The neural circuit underlying *Aplysia californica* consummatory feeding was used to examine how a maintained stimulus elicits repetitive, rhythmic movements. The command-like cerebral-buccal interneuron 2 (CBI-2) is excited by tonic food stimuli but initiates rhythmic consummatory responses by exciting only protraction-phase neurons, which then excite retraction-phase neurons after a delay. CBI-2 is inhibited during retraction, generally preventing it from exciting protraction-phase neurons during retraction. We have found that depolarizing CBI-2 during retraction overcomes the inhibition and causes CBI-2 to fire, potentially leading CBI-2 to excite protraction-phase neurons during retraction. However, CBI-2 synaptic outputs to protraction-phase neurons were blocked during retraction, thereby preventing excitation during retraction. The block was caused by presynaptic inhibition of CBI-2 by a key buccal ganglion retraction-phase interneuron, B64, which also causes postsynaptic inhibition of protraction-phase neurons. Pre- and postsynaptic inhibition could be separated. First, only presynaptic inhibition affected facilitation of excitatory postsynaptic potentials (EPSPs) from CBI-2 to its followers. Second, a newly identified neuron, B54, produced postsynaptic inhibition similar to that of B64 but did not cause presynaptic inhibition. Third, in some target neurons B64 produced only presynaptic and not postsynaptic inhibition. Blocking CBI-2 transmitter release in the buccal ganglia during retraction functions to prevent CBI-2 from driving protraction-phase neurons during retraction and regulates the facilitation of the CBI-2 induced EPSPs in protraction-phase neurons.

INTRODUCTION

A tonic stimulus can elicit rhythmic movements (Marder and Calabrese 1996; Pearson and Gordon 2000). Neural circuits giving rise to such movements must contain mechanisms for transforming a tonic stimulus into a rhythmic response. The present report uses the feeding behavior in the marine gastropod mollusk *Aplysia* to examine some of the mechanisms that transform a tonic stimulus into rhythmic, repetitive responses. *Aplysia* consummatory feeding behaviors are elicited by food touched to the lips or stimulating the interior of the mouth (Kupfermann 1974). Tonically maintained stimuli elicit repeated, rhythmic protraction and retraction movements of the toothed radula (Kupfermann 1974; Morton and Chiel 1993a,b). Buccal motor programs corresponding to protraction and retraction movements in the intact animal can be monitored from many neurons within the buccal ganglia as well as via extracellular recordings from buccal ganglia nerves (Church and Lloyd 1994; Hurwitz and Susswein 1996; Morton and Chiel 1993a,b). Buccal motor programs are organized by a central pattern generator (CPG) containing separate interneurons that drive the protraction and retraction phases (Hurwitz and Susswein 1996; Hurwitz et al. 1997; Plummer and Kirk 1990). CPG activity is initiated via command-like neurons. The most prominent such neuron, cerebral-buccal interneuron 2 (CBI-2), is excited by food stimulating the lips and monosynaptically excites protraction-phase interneurons, thereby initiating their activity (Hurwitz et al. 2003). Similar to the effects of tonic food stimulation on behavior, tonic depolarization of CBI-2 causes repeated, rhythmic buccal motor programs that cease when CBI-2 stimulation is terminated.

How are tonic stimuli to the lips or to CBI-2 converted into rhythmic buccal motor programs? Previous studies have identified two such mechanisms. One arises from reciprocal inhibition within the CPG. Activity in protraction-phase neurons initiates activity, after a delay, in retraction-phase neurons, which in turn inhibit protraction-phase neurons and thereby turn off protraction while retraction progresses. A second mechanism arises from a feedback loop from the CPG to the command-like CBI-2 neuron. CBI-2 is inhibited during the retraction phase of a buccal motor program, thereby reducing its likelihood to fire during the retraction phase (Hurwitz et al. 1999b; Rosen et al. 1991). Because of the recurrent inhibition from retraction-phase neurons, activity of CBI-2 in response to a tonic stimulus is itself phasic, and CBI-2 generally fires and excites protraction-phase interneurons only during the protraction phase. However, these two mechanisms are unlikely to be sufficient to ensure that tonic stimuli elicit a rhythmic buccal motor program. Strong inputs to CBI-2 that depolarize it sufficiently during the retraction phase will override the inhibition and elicit firing. CBI-2 firing during retraction would then excite and perhaps cause firing in protraction-phase neurons during the retraction phase.
This study examined the consequences of driving CBI-2 to fire during the retraction phase. We found that firing CBI-2 during retraction had no effects on buccal motor programs because a third mechanism is present that contributes to the conversion of a tonic stimulus to CBI-2 into a phasic response. In addition to inhibiting all of the protraction-phase neurons, the major retraction-phase interneuron B64 also causes powerful presynaptic inhibition of CBI-2, thereby blocking its effects in the buccal ganglia and preventing it from driving protraction-phase neurons during the retraction phase.

METHODS

The experimental subjects were *Aplysia californica* weighing 150–300 g provided by Marinus (Long Beach, CA) and by the National Resource for *Aplysia* at the University of Miami. Animals were maintained at 14–16°C in holding tanks containing aerated, filtered seawater. Before being dissected, animals were anesthetized and immobilized by injection with isotonic MgCl₂ (50% of body wt). The buccal and cerebral ganglia were removed with the cerebral-buccal connectives (CBCCs) intact. The cerebral and buccal ganglia were pinned to the floor of a recording chamber with the ventral surface of the cerebral ganglion and the caudal surface of the buccal ganglia facing up. The sheath overlying the surface of the ganglia was removed using ultrafine scissors.

Cerebral and buccal ganglia circuitry

Tonic depolarization of the command-like CBI-2 neurons induces repeated buccal motor programs (BMPs) that are organized by the buccal ganglion CPG (Church and Lloyd 1994; Hurwitz et al. 1999a,b; Perrins and Weiss 1998; Rosen et al. 1991). The ability of CBI-2 to elicit BMPs arises in part from the large, facilitating fast excitatory postsynaptic potentials (EPSPs) that CBI-2 firing induces in the protraction-phase interneurons that are part of the CPG (Hurwitz et al. 2003; Sanchez and Kirk 2000). CBI-2 also induces slow EPSPs in the protraction-phase interneurons (Hurwitz et al. 1999a). Buccal ganglia protraction-phase neurons are excited by CBI-2. The present study examined a number of protraction-phase neurons that are excited by CBI-2, such as B31/B32, B34, B61/B62, and B63 (Fig. 1A). Previous studies have indicated that activity in most of the protraction-phase neurons is not necessary to elicit a buccal motor program because preventing them from firing does not abolish the ability of the ganglion to express a program (Hurwitz et al. 2003). Exceptions are the electrically coupled B31/B32 and B63 neurons, which are always active during buccal motor programs. In addition, hyperpolarizing them can block the expression of a program. The ability of CBI-2 to initiate buccal motor programs is largely explained by its strong, facilitating excitation of B63 (Hurwitz et al. 2003).

The retraction phase of a buccal motor program is triggered by firing in neuron B64 (Hurwitz and Susswein 1996). Additional interneurons have important functions in retraction (Plummer and Kirk 1990), but they are not always active during the expression of a buccal motor program (Nargeot et al. 2002), whereas B64 firing is an invariant feature of a buccal motor program. B64 activity is initiated as a result of activity in the protraction-phase neurons via their effects on an unidentified element of the CPG that has been called the “z” cell (Baxter et al. 1997; Hurwitz and Susswein 1996). The existence of the z cell has been inferred by modeling studies (Baxter et al. 1997) as well as via its effects on B64. B64 displays a prominent, endogenous plateau potential: a brief depolarization induces a burst of spikes that long outlasts the stimulus. Firing in B64 produces a large inhibitory postsynaptic potential (IPSP) in protraction-phase neurons and depolarizes and caused firing in other retraction-phase neurons (Hurwitz and Susswein 1996).

Recording apparatus and bathing solutions

Preparations were bathed in artificial seawater [ASW, which contained (in mM): 460 NaCl, 10 KCl, 11 CaCl₂, 55 MgCl₂, and 5 NaHCO₃] at pH = 7.64. In some experiments, the buccal and cerebral ganglia were placed in a solution containing an increased concentration of divalent cations [HiDi saline containing (in mM) 311 NaCl, 9 KCl, 33 CaCl₂, 132 MgCl₂, and 5 NaHCO₃] to reduce polysynaptic activity of coupled neurons and follower neurons (Hurwitz et al. 2000). Except for experiments using voltage clamping, intracellular recordings were obtained from isolated ganglia preparations maintained at room temperature (18–22°C). Most experiments were performed under continuous fluid exchange, using a peristaltic pump at a rate of 10g volume/min.

Intracellular recordings were made from CBI-2 as well as from buccal interneurons B64, B54, B63, and B34, and the buccal protraction-muscle (12) motoneurons B31/B32 and B61/B62. Other neurons that were often recorded include radula closure motor neuron B8 and B4/B5. The neurons were identified by previously established morphological and/or physiological criteria (Gardner and Kandel 1977; Hurwitz and Susswein 1996; Hurwitz et al. 1994, 1996, 1999b; Jahan-Parwar et al. 1983; Morton and Chiel 1993a,b; Rosen et al. 1991; Susswein and Byrne 1988).

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 tonically fired at 7–20 Hz for a period that did not exceed 3 min. Repeated stimulus sets to CBI-2 were separated by 10-min intervals.

Intracellular recording, and stimulation

For intracellular recording and stimulation, neurons were impaled with single-barreled microelectrodes that were made of thin-walled glass tubing that was filled with 1.9 M potassium acetate and 0.1 M potassium chloride. The electrodes were pulled so that their impedances ranged from 10 to 15 MΩ, and following beveling, they had final resistances of 6–10 MΩ. The activity of up to four neurons was monitored via intracellular recording using conventional electrometers. The extracellular activity in up to two nerves was also monitored. A Grass stimulator (S88) controlled the intracellular stimuli delivered to the neurons.

Voltage clamping

Voltage clamping was performed at 17°C using 1–5 MΩ electrodes filled with 1 M KCl. Experiments were performed in an 0.5-ml chamber. The currents and voltages were recorded with an Axoclamp 2 (Axon Instruments) current/voltage clamp that was controlled by a computer running the Clampex component of pClamp 8.0 (Axon Instruments). Data were digitized and recorded using this program, via a Digidata 1200A digitizer (Axon Instruments). Preliminary data analyses used the Clampfit component of pClamp 8.0.

RESULTS

In *Aplysia*, a CPG in the buccal ganglia drives repeated cycles of radula protraction and retraction (Figs. 1, B and C, and 2A). The buccal CPG is composed of mutually inhibitory protraction and retraction-phase interneurons (Figs. 1, B and C, and 2A). There is little or no overlap in the firing of protraction phase (e.g., B31/ B32, B34, B61/B62, and B63), and retraction-phase (e.g., B64) interneurons and motor neurons (Hurwitz et al. 1997). A small population of additional neurons (e.g., B4/B5, the B8s) may shift the degree to which they are active in the two phases (Fig. 1D, I and 2). In the isolated buccal ganglia, activation of the CPG causes organized BMPs, which correspond to protraction and
retraction movements in the intact animal (Hurwitz et al. 1996, 1997). CBI-2 elicits monosynaptic facilitating EPSPs in protraction-phase neurons, thereby initiating the protraction phase of a BMP (Hurwitz et al. 2003). After a delay of several seconds, retraction-phase interneurons are activated (Hurwitz et al. 1997). The firing pattern of CBI-2 is similar to that of the protraction-phase interneurons: it fires in phase with buccal ganglia protraction-phase neurons (although CBI-2 activity slightly precedes buccal ganglia activity), and the firing is suppressed during retraction phase of a BMP in part via buccal to cerebral interneurons (Hurwitz et al. 1999b; Rosen et al. 1991). The inhibition of CBI-2 during retraction would be functionally adaptive because firing of CBI-2 during retraction might cause protraction-phase interneurons and motor neurons to fire during the retraction phase. The simultaneous firing of the protraction and retraction muscles would interfere with the retraction movement.

Similar motor programs are generated by phasic and tonic firing of CBI-2

Although CBI-2 is inhibited during the retraction phase, the inhibition can be overcome by depolarizing stimuli. We examined the possible consequences on BMPs of firing CBI-2 during the retraction phase (Fig. 2). Firing in CBI-2 was regulated so that it fired at a constant rate during both the protraction and retraction phases. In this experiment (n = 5), the protraction phase was monitored by intracellular recordings from protraction-phase interneuron B63 as well as by extracellular recordings from the I2 nerve, which contains axons of the protraction-phase interneuron B63 as well as by extracellular recordings from the I2 nerve, which contains axons of the protraction-phase interneurons B31/B32 and B61/B62 that drive contractions of the major protraction muscle, I2 (Hurwitz et al. 1994, 1996, 2000). The retraction phase was monitored via intracellular recordings from neuron B4, which fires during retraction, as well as via intracellular
recordings from motor neuron B8 and extracellular recordings from the radula nerve. B8 and radula nerve activity are seen during both protraction and retraction. The activity that is maintained after the end of protraction was utilized for monitoring retraction phases. BMPs were initiated via continuous stimulation of CBI-2 at 17 Hz with brief current pulses that evoked only a single spike in CBI-2.

When CBI-2 was injected with 18-nA current pulses (Fig. 2A1), spikes were initiated in CBI-2. The spikes elicited summat- 
ing and facilitating monosynaptic EPSPs in B63. When the amplitude of the EPSPs exceeded threshold, B63 began to fire and BMPs were elicited as has been described previously (Hurwitz et al. 2003). The 18-nA current pulses that were sufficient to initiate spikes in CBI-2 prior to and during the protraction phase did not initiate spikes during the retraction phase (Fig. 2A2) presumably because spikes were blocked by the phasic inhibitory input that CBI-2 received during retraction.

Raising the amplitude of the current pulses in CBI-2 from 18 to 25 nA (Fig. 2B1) overcame the inhibition that CBI-2 received during retraction and elicited spikes during both phases of the BMP (Fig. 2B2). However, the patterns of activity in the buccal ganglia were remarkably similar to those elicited when CBI-2 was fired only during protraction (compare Fig. 2, A1 and B1), in spite of the additional firing of CBI-2 during retraction. In addition, no EPSPs were observed in B63 when CBI-2 was fired during the retraction phase.

Previous experiments have shown that CBI-2 elicits facilitating EPSPs in a number of protraction-phase neurons (see Fig. 1C), including B34, B63, B31, and B61 (Hurwitz et al. 2003; Sanchez and Kirk 2000). We examined the ability of CBI-2 to elicit EPSPs in a variety of protraction-phase neurons at different phases of a BMP (n = 26). Figure 3 illustrates that a train of spikes in CBI-2 elicited facilitating EPSPs in B63, B63, and B31 (see fast sweep in Fig. 3B). These eventually summed, causing a BMP (Fig. 3A). However, a train at the same frequency (14 Hz) delivered during the retraction phase completely failed to elicit detectable EPSPs (see the fast sweep in Fig. 3C). Later trains, which occurred during the late portion of the retraction phase or following the end of the retraction phase, elicited EPSPs of progressively increasing amplitude within the bursts and between them (Fig. 3C).

These data indicate that when CBI-2 is fired early in the retraction phase, some mechanism completely suppresses CBI-2 elicited EPSPs in protraction-phase neurons. The suppression gradually declines through the later portion of the retraction phase and subsequent to retraction. The suppression of CBI-2 elicited EPSPs is likely to explain why CBI-2 firing during retraction has little or no effect on BMPs. This suppres-
tion could be caused by either presynaptic or postsynaptic inhibition.

**Postsynaptic inhibition cannot suppress CBI-2 elicited EPSPs**

Protraction-phase neurons, including B34, B31/B32, B63, and B61/B62, are strongly inhibited during the retraction phase by a large IPSP mediated via a conductance increase (Hurwitz et al. 1994, 1997). In principle, this IPSP could account for the suppression of EPSPs from CBI-2 to protraction-phase neurons during retraction. If the conductance increase was sufficiently large, it could shut completely the CBI-2-elicited EPSP. We tested this possibility by examining the conductance increases elicited in the protraction-phase neurons during the retraction phase. In this experiment, EPSPs in protraction neurons were simulated by intracellular depolarizing current pulses. Firing CBI-2 was used to elicit BMPs ($n = 11$). CBI-2 stimulation was terminated just after the onset of the retraction phase, and sometime later two identical current pulses were injected into a variety of protraction-phase neurons. One pulse was delivered during the retraction phase, and the other was delivered a number of seconds after the retraction phase had ended. The voltage change during the retraction phase was decreased by somewhat $\sim 50\%$ with respect to the voltage change after the retraction phase (Fig. 4). These data indicate that the conductance change caused by the IPSP that underlies retraction would shunt a CBI-2-elicited EPSP in the protraction-phase neurons, thereby lowering its amplitude, but the shunting would be insufficient to block the EPSP completely.

To examine quantitatively whether the retraction causes a conductance change that is sufficient to shunt CBI-2-induced EPSPs, protraction-phase neuron B31/B32 was voltage clamped (Fig. 5). The neuron was held at $-60\, \text{mV}$, its resting potential. Either 4- or 8-s sweeps were observed at a frequency of once per 30 s. Voltage steps were applied in $\pm 10\, \text{mV}$ increments, until 0 mV, and the steps were maintained for most of the sweep. Many records showed spontaneous intermittent slow inward deflections followed by outward deflections. In current-clamp conditions, the inward currents would underlie depolarizations of B31/B32 during protraction, and the outward currents would underlie the retraction phase. The currents underlying the BMPs were seen at a variety of voltages, allowing us to determine the voltage dependence of the currents underlying the retraction phase. Currents recorded in traces showing a BMP were subtracted from currents measured in the same cell, at the same voltage, in the absence of a BMP (BMPs were prevented by treating ganglia with tetrodotoxin), allowing us to estimate the currents that are exclusively attrib-

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**FIG. 3.** CBI-2 elicited EPSPs are not detected during retraction phase of CBI-2-generated BMP. **A:** in protraction-phase neurons B34, B63, and B31 firing of CBI-2 at a fixed rate for 10 s elicited facilitating fast EPSPs that generate a biphasic single cycle of BMP. By contrast, shorter trains of CBI-2 firing during the retraction phase do not cause detectable EPSPs. **B:** a fast sweep of the 1st part of the train marked as $B$ in $A$ demonstrates a train of facilitated EPSPs that increase their amplitude from $-1$ to $>5\, \text{mV}$ in B34 and B63. **C:** in contrast, a train of similar duration and rate failed to elicit detectable EPSPs during retraction phase. The amplitude of EPSPs progressively increased during the repeated trains.

**FIG. 4.** The postsynaptic change in resistance during retraction does not block depolarization in response to a current pulse. Injection of 2 depolarizing current pulses into B34 (A) and B63 (B) during and following the retraction phase demonstrates changes in resistance. During retraction, B31 shows the same hyperpolarization seen in B34 and B63, the input resistance of B34 and B63 both were decreased by $\sim 50\%$ during the retraction phase.
utable to the presence of a BMP. The current-voltage relationship for the retraction phase was then plotted, and a linear regression best fit was calculated. The best fit linear regression showed that the currents underlying the retraction phase have a net reversal potential of $-61 \text{ mV}$, and the change in input resistance caused by the retraction phase is $1.01 \text{ M\Omega}$ (Fig. 5B). The input resistance of B31/B32 at rest was calculated from conductances recorded in response to voltage steps in B31/B32 at voltages in which the current-voltage relationship is ohmic. The measured passive input resistance was $2.71 \text{ M\Omega}$. The $1.01\text{-M\Omega}$ change in input resistance during the retraction phase in B31/B32 would have reduced the input resistance to 1.70, which is a 37.25% reduction in the input resistance. Thus current adequate to cause a 20-mV voltage change at rest would cause a 13-mV voltage change during the retraction phase of a buccal motor program. These data are consistent with those in Fig. 4 and indicate that the shunting of the CBI-2-induced EPSP by the increase in conductance during retraction would be adequate to reduce the amplitude of an EPSP from CBI-2 but would be too small to block completely the CBI-2-induced EPSP. Thus another mechanism must account for the complete block of the EPSP.

**Complete block of CBI-2-elicited EPSPs is related to firing of B64**

A number of retraction-phase interneurons have been identified (Hurvitz and Susswein 1996; Nargeot et al. 1999; Plummer and Kirk 1990). The most prominent of these, B64, is sufficient to induce the retraction phase (Hurvitz and Susswein 1996). In spontaneous or elicited BMPs, a burst of spikes in B64 signals the start of the retraction phase. In addition, if B64 is stimulated and begins to fire prematurely this firing leads to the termination of protraction and an immediate, premature onset of the retraction phase (Hurvitz and Susswein 1996). Because firing in B64 is a fixed marker of the start of retraction, we examined whether the firing of B64 is temporally related to the block of the CBI-2-elicited EPSPs in protraction-phase neurons.

During CBI-2-initiated BMPs, the termination of the protraction phase was correlated with firing in B64 as well as with the block of CBI-2-elicited EPSPs in the protraction-phase neurons (Fig. 6A). In one experiment, stimulating B64 to fire during the protraction phase caused a premature termination of the protraction phase (Fig. 6B) as was described previously (Hurvitz and Susswein 1996). This stimulus also caused a premature initiation of the retraction phase (see the 1st and the 2nd cycles of BMP in Fig. 6B). Furthermore, firing B64 during the protraction, before it would have been activated without experimenter intervention, led to three cycles of BMP in the recorded period instead of two (CBI-2 firing was maintained for 50 s, compare Fig. 6, A and B). In addition, the premature onset of firing in B64 also blocked the CBI-2-elicited EPSPs in the protraction-phase neurons ($n = 7$). This experiment indicates that firing of B64 causes the block of the CBI-2-elicited EPSPs in protraction-phase neurons in addition to initiating the retraction phase.

The block of CBI-2-initiated EPSPs caused by firing B64 could be mediated via direct effects of B64, or via indirect effects, by B64 recruiting other retraction-phase neurons, which in turn could block the EPSPs. If the effect of firing B64 was via intervening neurons, the block of CBI-2-induced EPSPs should be eliminated, and the EPSPs should be restored when the buccal ganglia are bathed in HiDi saline (see METHODS), which elevates the firing threshold of neurons and therefore makes it more difficult to recruit potential followers of B64.

To test whether the inhibitory effects of B64 stimulation may be mediated via intervening neurons, various protraction-phase neurons were examined during the combined firing of B64 and CBI-2, or during alternated firing of B64 and CBI-2, while both the buccal and cerebral ganglia were bathed in HiDi saline. To increase the size of the EPSPs, the various protraction...
tation from burst to burst is constant (Fig. 7A). Firing of B64 2 s before firing CBI-2 did not affect the CBI-2-elicited EPSPs (Fig. 7B). By contrast, firing of B64 and CBI-2 simultaneously completely blocked CBI-2-elicited EPSPs (Fig. 7C). This experiment indicates that B64 probably inhibits CBI-2-elicited EPSPs directly because the inhibition is seen in HiDi saline. It also suggests that B64 may produce presynaptic inhibition of the CBI-2 processes because the block is critically dependent on the simultaneous activity of CBI-2 and B64. Data similar to those in Fig. 6 were also obtained when recordings were made from additional protraction-phase neurons, such as B31/B32, B61, and B63 (n = 11).

Block of CBI-2-induced EPSPs is caused by presynaptic inhibition

A conclusive demonstration that B64 acts on CBI-2 processes would require recordings from the CBI-2 processes within the neuropile of the buccal ganglia. Attempts to perform such recordings were not successful in our hands (n = 11), (but see Sanchez and Kirk 2001). We therefore gathered indirect evidence supporting the hypothesis that B64 directly inhibits CBI-2 neurites and thereby prevents them from releasing transmitter.

SIMULTANEOUS FIRING OF B64 AND CBI-2 BLOCKS FACILITATION BUILD-UP OF CBI-2-ELICITED EPSPS

Previous data indicated that the facilitation of the EPSPs induced in protraction-phase neurons by CBI-2 is a presynaptic process (Sanchez and Kirk 2000). This facilitation is strongly affected by small changes in the firing frequency of CBI-2 (Hurwitz et al. 2003). If the block of the EPSPs induced by CBI-2 arises via presynaptic inhibition, which causes a complete block of transmitter release, the facilitation should be affected by the block in a manner similar to that caused by a complete interruption of CBI-2 firing. By contrast, if the block of the EPSPs arises as a result of a postsynaptic mechanism, the facilitation should be unaffected by the block.

To examine the effects of firing B64 on the facilitation, CBI-2 was stimulated in bursts so that the bursts of EPSPs recorded in B34 reached a steady-state facilitation (n = 3). These experiments were performed in a HiDi solution, and B34 was held at −80 mV. In these conditions, firing CBI-2 in trains drives repeated bursts of EPSPs with summated amplitudes that reach −35 mV at the end of a burst (Fig. 8A).

On the background of trains of current pulses applied to CBI-2 alone, B64 and CBI-2 were stimulated in tandem four times. The simultaneous stimulation of B64 and of CBI-2 blocked the EPSPs in B34 (Fig. 8B). Following the four stimuli with combined trains of stimuli to both CBI-2 and B64, firing of CBI-2 alone again elicited EPSPs in B34. However, the amplitude of these EPSPs was decrease with respect to that seen prior to the firing of B64. The amplitude of the EPSPs returned to the values seen before the B64 stimulation only after three bursts in which CBI-2 was stimulated alone (18 s after the end of the B64 stimulus).

Skipping four trains of CBI-2 spikes produced effects that were similar to those produced by the simultaneous stimulation of B64 and CBI-2. When CBI-2 stimulation was resumed, the amplitude of the summated EPSPs was reduced with full recovery of summated EPSPs amplitudes being observed only by the third train after the pause (Fig. 8C). These results
Dissociation of inhibition and block of EPSPs. B54 produces postsynaptic inhibition of the protraction-phase neurons as well as a block of the EPSPs from CBI-2. The data above suggest that these are separable processes. Additional evidence in support of this suggestion could be obtained if a second neuron could be found that causes postsynaptic inhibition of the protraction-phase neurons, similar to that caused by B64, but that does not also block the CBI-2-induced EPSPs.

We have found such a neuron, which was not previously identified and which we now name B54 (see Fig. 1A). B54 has a soma ~150% larger than that of B64. It is located medial to B64 (n = 9). We have not examined systematically the role of B54 in CBI-2 generated BMPs. B54 is similar to B64 in its ability to hyperpolarize protraction-phase neurons, including B34 (Fig. 9). In addition, the increase in the conductance of protraction-phase neurons elicited by firing B54 is similar to that caused by B64 (not shown, n = 5). B54 can be distinguished from B64 because a brief depolarization of B64 elicits a plateau potential that long outlasts the stimulus (Hurwitz and Susswein 1996), whereas B54 does not display this property. By contrast to B64, firing in B54 did not block CBI-2-elicited EPSP (Fig. 9C), although it did cause a 50% decrease in the amplitude of the EPSPs as would be predicted if it shunted the EPSPs via a conductance increase IPSPs. This inhibition can be seen by comparing the EPSP amplitudes resulting from combined bursts of CBI-2 and B54 (Fig. 9C) to the EPSPs amplitudes resulting from driving CBI-2 alone (Fig. 9B). These data are consistent with the interpretation that postsynaptic inhibition is separable from the block of CBI-2 induced EPSPs and provide further evidence that the EPSP block arises from presynaptic inhibition.

Because B54 appears to exert only postsynaptic actions, one would predict that the simultaneous firing of CBI-2 and B54 would affect the size of summatting EPSPs but not the facilitation of the CBI-2-elicited EPSPs as does the simultaneous firing of CBI-2 and B64. This prediction was confirmed in five experiments (Fig. 10). In the absence of either B54 or B64 stimulation, three trains of stimuli to CBI-2 elicited a gradual build-up in the amplitude of the EPSPs recorded in B34 (Fig. 10A). When CBI-2 and B54 were stimulated together, the amplitude of the EPSPs elicited by stimulating CBI-2 was decreased (compare the 1st and 2nd trains in Figs. 10B to the 1st and 2nd trains in A). However, when B54 was not stimulated during the third train, the EPSPs elicited by CBI-2 were fully facilitated, as they would have been if there had been no B54 stimulation in the previous trains (compare last trains of EPSPs in Fig. 10, A and B). By contrast, when CBI-2 and B64 were stimulated in tandem during the first two trains, the CBI-2-elicited EPSPs were not seen, and in their place, the characteristic B64-elicited IPSPs were observed (Fig. 10C, 1st 2 trains). When B64 was not stimulated during the third train, the EPSPs elicited by CBI-2 were expressed. However, in place of full recovery of facilitation during the third train, like the one observed when B54 was not stimulated, skipping the third train of firing in B64 caused a reduction in the amplitude of CBI-2-elicited EPSPs with respect to the amplitude seen when B64 was not stimulated. Furthermore, during the third train of EPSPs, when B64 was not stimulated, the EPSPs elicited by CBI-2 were comparable to those seen during the first train of CBI-2 stimulation alone (compare the last train of EPSPs in Fig. 10C to the 1st train in A). Similar results were also obtained when another protraction-phase neuron, B63, was monitored in place of B34 (not shown, n = 2). These data indicate that the postsynaptic inhibition of protraction-phase neurons by B54 and B64 reduces the amplitude of the EPSPs elicited by CBI-2. However, only the presynaptic inhibition elicited by B64 blocks synaptic release from CBI-2 terminals and thereby prevents the gradual buildup of EPSP facilitation.

**Fig. 7.** Combined, but not alternated firing of B64 and CBI-2 eliminates CBI-2-elicited EPSPs. CBI-2 was stimulated at a rate of 10 trains/min, each train with a 1-s duration at 16 Hz. This protocol leads to a progressive increase in the amplitude of the summatmed EPSPs over 1 or 2 min, until reaching steady-state values. The figures shown are after the EPSPs in B34 have reached steadystate values. The bars indicate the maximum amplitudes of the summatmed EPSP. B34 was hyperpolarized to approximately −80 mV throughout the experiment via constant current injection. High divalent (HiDi) saline was replacing artificial seawater (ASW). A: the EPSP produced in B34 by stimulating CBI-2 in the absence of activity in B64. B: when B64 was stimulated several seconds preceding CBI-2 stimulation, CBI-2-elicited EPSPs were unaffected. C: by contrast, when B64 fired simultaneously with CBI-2, the EPSPs were blocked. The recording of CBI-2 is also indicative of the timings of CBI-2 activity in A and B.
B64 can also elicit purely presynaptic effects. The data above provide strong evidence that the pre- and postsynaptic inhibitions produced by B64 are separable processes, and the effects of each can be characterized. Further evidence that B64 can produce both pre- and postsynaptic inhibition could be marshaled if an additional neuron was found that displayed only presynaptic inhibition when B64 is fired with no concomitant postsynaptic inhibition.

B8 was found to be such a neuron \( (n = 9) \). CBI-2 elicited a slow EPSP in B8. The slow EPSP was also present in HiDi saline, indicating that the connection is likely to be monosynaptic (Fig. 11A). In addition, firing of B64 did not inhibit B8 as it did the protraction-phase neurons but rather elicited a weak conductance decrease (Fig. 11B—compare the responses to current injections just before and after firing of B64 with those during B64 firing, which also elicits IPSPs in B61).

A conductance decrease should increase the amplitude of CBI-2-elicited slow EPSP in B8 if firing of CBI-2 precedes the firing in B64. Such an increase was seen when B64 was fired during the slow EPSPs elicited by CBI-2 (Fig. 11C). In this experiment, a series of five CBI-2 bursts were elicited, and B64 was fired during the slow EPSPs elicited by the second and third burst. In the absence of B64 firing, CBI-2 elicited six spikes. By contrast, when B64 was also fired, CBI-2 elicited eight spikes. There is a significant delay between firing of CBI-2 and the expression of the slow EPSP in B8, which permits the expression of B64 effects on the ongoing EPSP (i.e., B64 was fired during the slow EPSP rather than during CBI-2 firing). However, when B64 was fired simultaneously with CBI-2, it completely blocked the slow EPSP in B8 (Fig. 11C2). Thus firing CBI-2 alone evoked five to six action potentials in B8. When CBI-2 and B64 were fired together, no spikes were seen in B8 (compare the responses to the 1st and the last trains of CBI-2 firing with that of the 2nd train). These data conclusively illustrate that the presynaptic and postsynaptic effects of firing B64 are separable. Firing B64 in tandem with CBI-2 causes presynaptic inhibition, whereas firing of B64 in tandem with the slow EPSP elicited by CBI-2 causes postsynaptic excitation.

B54 causes inhibitory postsynaptic potentials (IPSPs) in B34 that decrease the amplitude but do not block CBI-2 elicited EPSPs. A: brief depolarization of B54-evoked spikes, which elicited a train of IPSPs that hyperpolarized B34 by several millivolts. B: when BCI-2 spikes were triggered during firing of B54, they elicited facilitating EPSPs in B34. C: firing a train of spikes in CBI-2 in the absence of B54 spikes evoked facilitating EPSPs in B34 that were larger in amplitude by 50%. This experiment was performed in HiDi saline while the neurons were held at their resting potential (approximately −60 mV).
DISCUSSION

Neural circuits in many animals are designed to transform a tonic input into rhythmic output. Investigating the mechanisms underlying the transformation of a tonic input into a rhythmic output in the Aplysia buccal motor system provides insight into the general question of how to design a circuit to convert a tonic input into a rhythmic output.

The data presented in this paper, in addition to those shown previously, indicate that at least three mechanisms contribute in parallel to the transformation of a tonic input to CBI-2 into a phasic motor output. First, the buccal CPG that is responsible for generating protraction-retraction sequences consists of separate, mutually inhibitory protraction-phase and retraction-phase interneurons (Hurwitz and Susswein 1996; Hurwitz et al. 1997). CBI-2 monosynaptically recruits the protraction-phase neurons but not the retraction-phase neurons (Hurwitz et al., 2003). The protraction-phase neurons in turn recruit retraction-phase neurons, with a delay, via a presently unidentified element that has tentatively been named the z cell (Baxter et al. 1997). Activation of the retraction-phase neurons leads to inhibition of protraction-phase neurons (Hurwitz and Susswein 1996; Hurwitz et al. 1997). Thus CBI-2 induces biphasic activity by activating protraction, which itself then activates retraction and thereby shuts itself off. Second, phasic feedback from the buccal CPG to the cerebral ganglia postsynaptically inhibits CBI-2 (Hurwitz et al. 1999b; Rosen et al. 1991), leading to a rhythmic pattern of CBI-2 firing (Rosen et al. 1991). CBI-2 will then fire and excite the protraction-phase neurons in the buccal ganglia during one phase of a cycle. Constraining the firing of CBI-2 to the protraction phase restricts the recruitment of buccal ganglion protraction-phase neurons by CBI-2 to the appropriate phase of a motor program and inhibits the firing of CBI-2 during the inappropriate phase. Last, we have now shown that if the CBI-2 neuron is sufficiently excited and therefore fires during the retraction phase in spite of the inhibition...
seen during this period, such firing does not elicit excitation of protraction-phase neurons in the buccal ganglia. The lack of excitation is a result of presynaptic inhibition from the major retraction element of the CPG, B64, onto the output of CBI-2 within the buccal ganglia.

Evidence in favor of presynaptic inhibition

Our evidence that B64 blocks CBI-2 output via presynaptic inhibition is indirect. Presynaptic inhibition is most directly demonstrated via intracellular recordings from the presynaptic terminals (e.g., Baxter and Bittner 1991) and via quantal analysis (see Boyd and Martin 1956), which compares the quantal number and the quantal content of a synapse while it is inhibited to the values before inhibition and then shows that the quantal number, rather than quantal content, is reduced by presynaptic inhibition (e.g., Boyd and Martin 1956). In our system, a quantal analysis would be unlikely to provide useful information because the synapse from CBI-2 to protraction-phase neurons is completely blocked during the retraction rather than merely being reduced. When a synaptic connection is completely absent, it would unrealistic to estimate changes in the quantum number or the quantal content. In place of a quantal analysis, we have marshaled a great deal of indirect support for the contention that B64 presynaptically inhibits the terminals of CBI-2 on protraction phase. The indirect measures of presynaptic inhibition are similar to those used previously in other systems that display presynaptic inhibition (Dudel and Kuffler 1961; Peng and Frank 1989). First, we have shown that overriding the inhibition of CBI-2 during retraction and causing it to fire does not cause excitation of the protraction-phase neurons during the retraction phase and therefore does not affect the BMPs recorded in the buccal ganglia (Figs. 2 and 3). Although retraction is characterized by postsynaptic inhibition of protraction-phase neurons, particularly by B64, the lack of any excitation during retraction could not be explained by postsynaptic inhibition of the protraction-phase neurons because such inhibition would attenuate the amplitude of the EPSP caused by CBI-2 by 37% (Fig. 5) but would not eliminate it completely (Fig. 4) as was observed. In other systems, a reduction of a synaptic potential beyond that which can be explained by postsynaptic inhibition has been used as evidence of presynaptic inhibition (Frost et al. 2003). Second, we have shown that the block of excitation from CBI-2 to protraction-phase neurons is correlated precisely with firing in B64 as would be expected if the block was exerted via a phasic presynaptic inhibition (Fig. 7). The need for a precisely timed inhibitory input immediately preceding the input that is inhibited is a widely seen feature of presynaptic inhibition that is caused by directly gated receptors (for review, see MacDermott et al. 1999). Third, we have shown that block of excitation from CBI-2 to protraction-phase neurons causes changes in synaptic facilitation (a presynaptic process) identical to those seen when CBI-2 does not fire, whereas postsynaptic inhibition does not affect synaptic facilitation in this synapse (Fig. 8). Modulation of facilitation has been widely used as a monitor of presynaptic inhibition (Baxter and Bittner 1991). Fourth, we have identified a neuron, B54 (Figs. 1 and 9), that produces postsynaptic inhibition of the CBI-2-initiated EPSPs similar to that produced by B64 but does not produce presynaptic inhibition, thereby demonstrating the separate effects of pre-
postsynaptic inhibition of the connection from CBI-2 to protraction-phase neurons (Figs. 9 and 10). Last, we have found that for some neurons, B64 produces only a presynaptic inhibition of the EPSP from CBI-2 with no postsynaptic inhibition at all (Fig. 11), further emphasizing that pre- and postsynaptic inhibition are separable processes.

**MECHANISM OF PRESYNAPTIC INHIBITION.** Presynaptic inhibition can be caused via directly gated channels (for review, see MacDermott et al. 1999) as well as via second-messenger-mediated channels (Fossier et al. 1994). A number of cellular mechanisms have been proposed to explain presynaptic inhibition. These mechanisms lead to a reduction in Ca\(^{2+}\) entry into the presynaptic terminal or a decrease in the efficacy of Ca\(^{2+}\) in producing transmitter release. Presynaptic inhibition is often associated with a reduction in the size of the presynaptic action potentials, which may be correlated with depolarization of the presynaptic terminals (e.g., Pearson and Goodman 1981) or with hyperpolarization of the terminals (Dudel and Kuffler 1961; Kretz et al. 1986b). An increase in conductance in the terminal, independent of whether it causes a depolarization or hyperpolarization, would shunt the currents in the terminal and thereby reduce the amplitude of presynaptic spikes (Baxter and Bittner 1991) or perhaps block their invasion into the terminal. In addition, depolarization itself reduces the size of action potentials and also causes partial inactivation of the presynaptic terminal (Burrows and Matheson 1994). Presynaptic inhibition may also be caused by an inhibition of the voltage-dependent Ca\(^{2+}\) currents (Kretz et al. 1986b; Wu and Saggau 1997) by which Ca\(^{2+}\) for synaptic release enters the cell or by a direct inhibition of transmitter release (Parnas et al. 2000).

Presynaptic inhibition of the CBI-2 synaptic output by B64 leads to a complete block of the EPSP rather than to its reduction. Mechanisms that reduce the size of the presynaptic action potential, without eliminating it, could not account for a complete block of synaptic transmission. Depolarization of the terminal large enough to inactivate it completely, and thereby block spikes would probably also affect facilitation of the synapse differently from that observed and are therefore unlikely to explain presynaptic inhibition in our system. Blocking Ca\(^{2+}\) entry via the regulation of Ca\(^{2+}\) channels is likely to be mediated via second-messengers, the operation of which would be too slow to account for the precise timing that is seen in the effects of B64 on CBI-2-induced EPSPs and are therefore also unlikely to operate in our system. The most likely hypothesis to explain presynaptic inhibition in our system is block of spike invasion into the terminals.

**TRANSMITTER IDENTIFICATION OF B64.** Additional evidence that B64 causes presynaptic inhibition of CBI-2 could be gathered by showing that exogenous application of the B64 transmitter produces inhibition of CBI-2 similar to that produced by firing B64. However, the B64 transmitter has not yet been identified. In *Aplysia*, histamine release by identified neurons has been shown to cause presynaptic inhibition in both the cerebral (Chiel et al. 1988) and the abdominal ganglion (Kretz et al. 1986a). A number of buccal ganglia neurons use histamine as their transmitter (Evans et al. 1999). Studies that have localized transmitters to neurons in the buccal ganglia are therefore uninformative. The most likely hypothesis to explain presynaptic inhibition in our system is block of spike invasion into the terminals.

**DIFFERENTIAL CONTROL OF FACILITATION.** A second possible function of the presynaptic inhibition produced by B64 may be to regulate the strong facilitation of the synapses from CBI-2 to the protraction-phase neurons. Previous data (Hurwitz et al. 2003; Sanchez and Kirk 2000), as well as data in this paper (Figs. 7 and 8), have shown that these synapses undergo frequency-dependent changes in amplitude, leading to a large facilitation. The facilitation in the CBI-2-induced EPSP is a central feature in the ability of CBI-2 to induce a buccal motor program because the amplitude of the EPSPs preceding the protraction phase may grow from 0 to >10 mV (Hurwitz et al. 2003). A decrease in the facilitation may significantly delay or block a BMP (Hurwitz et al. 2003). In addition, small changes in the background firing frequency of CBI-2 can profoundly affect the amplitude of the PSPs during a subsequent burst of activity in CBI-2 (Hurwitz et al. 2003). As in most systems (Katz and Miledi 1967), facilitation of the CBI-2 to protraction neuron synapses is a presynaptic process (Sanchez and Kirk 2000). Presynaptic inhibition assures that the possible occasional firing of CBI-2 during the retraction phase will not cause an undesired modification in the amplitude of the PSPs during the subsequent protraction phase. Our data support the possi-
bility that presynaptic inhibition has this function because the effect of presynaptic inhibition on facilitation is equivalent to that of a complete cessation of CBI-2 firing, whereas postsynaptic inhibition does not affect facilitation (Fig. 9).

REGULATING PEPTIDE RELEASE. CBI-2 contains a number of peptide co-transmitters that can initiate or modulate buccal motor programs (Morgan et al. 2000). The release of peptide co-transmitters, as well as their effects, is strongly affected by variations in firing pattern and frequency (Vilim et al. 2000). The presynaptic inhibition of CBI-2 may contribute to achieving a functionally appropriate release of peptide co-transmitters.

DEVELOPMENTAL CONSTRAINTS. Biological features can be explained by ontogenetic or phylogenetic constraints of a system (Gould 2002). Presynaptic inhibition of CBI-2 by B64 could also be explained in this way. B64 postsynaptically inhibits protraction-phase neurons in the buccal ganglia, while exciting retraction-phase neurons (Hurwitz and Susswein 1997). During development, appropriate synapses must be made between B64 and the protraction- and retraction-phase neurons. Because CBI-2 fires during protraction, its neurites within the buccal ganglia may share molecular markers with protraction-phase neurons. Such markers may lead to the development of inhibitory connections from B64.

Comparison to other systems

In most systems, presynaptic inhibition reduces the amplitude of synaptic potentials rather than blocking them completely. By contrast, B64 blocks the synaptic output. Presynaptic block of a synapse is an effective mechanism for preventing a synapse from functioning at an inappropriate time or to an inappropriate stimulus. In the crayfish lateral giant initiated tail flip, presynaptic inhibition blocks the sensory inputs that initiate the response, so that a tail flip will not itself initiate a second tail flip, leading to habituation of the response (Krasne and Bryan 1973). Presynaptic inhibition also phasically blocks the output of crab neuron MCN1 in the stomatogastric ganglion but not in the commissural ganglion, thereby creating phasic firing in one ganglion, and tonic firing in the other. In addition, MCN1 activity initiates both the gastric mill and pyloric rhythms. However, MCN1 is presynaptically inhibited during one phase of the gastric mill rhythm, thereby decreasing the recruitment of the pyloric rhythm. Thus presynaptic inhibition participates in the coordination of two rhythmic behaviors (Bartos and Nusbaum 1997).

Cerebral to buccal interneurons similar to the CBIs in Aplysia have been described in a number of different gastropods (Elliott and Susswein 2002). Similar to the CBIs, these neurons also receive tonic input and act on a buccal ganglion CPG that generates a phasic response. Both tonically and phasically firing neurons have been identified in Pleurobranchaea (Gillette et al. 1982; Kovac et al. 1982, 1983a,b 1986) as well as in Limax (Delaney and Gelperin 1990a,b). In Lymnaea, a single phasically firing CBI-like neuron has been identified (McCrohan and Kyriakides 1989). The possibility that presynaptic inhibition contributes to the conversion of a tonic input to a phasic output in these systems has not been examined.

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