Endogenous Motor Neuron Properties Contribute to a Program-Specific Phase of Activity in the Multifunctional Feeding Central Pattern Generator of *Aplysia*

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*Serrano GE, Martínez-Rubio C, Miller MW.* Endogenous motor neuron properties contribute to a program-specific phase of activity in the multifunctional feeding central pattern generator of *Aplysia*. *J Neurophysiol* 98: 29–42, 2007. First published March 28, 2007; doi:10.1152/jn.01062.2006. Multifunctional central pattern generators (CPGs) are circuits of neurons that can generate manifold actions from a single effector system. This study examined a bilateral pair of pharyngeal motor neurons, designated B67, that participate in the multifunctional feeding network of *Aplysia californica*. Fictive buccal motor programs (BMPs) were elicited with four distinct stimulus paradigms to assess the activity of B67 during ingestive versus egestive patterns. In both classes of programs, B67 fired during the phase of radula protraction and received a potent inhibitory postsynaptic potential (IPSP) during fictive radula retraction. When programs were ingestive, the retraction phase IPSP exhibited a depolarizing sag and was followed by a postinhibitory rebound (PIR) that could generate a postretraction phase of impulse activity. When programs were egestive, the depolarizing sag potential and PIR were both diminished or were not present. Examination of the membrane properties of B67 disclosed a cesium-sensitive depolarizing sag, a corresponding I$_n$-like current, and PIR in its responses to hyperpolarizing pulses. Direct IPSPs originating from the influential CPG retraction phase interneuron B64 were also found to activate the sag potential and PIR of B67. Dopamine, a modulator that can promote ingestive behavior in this system, enhanced the sag potential, I$_n$-like current, and PIR of B67. Finally, a pharyngeal muscle contraction followed the radula retraction phase of ingestive, but not egestive motor patterns. It is proposed that regulation of the intrinsic properties of this motor neuron can contribute to generating a program-specific phase of motor activity.

**INTRODUCTION**

The central pattern generator (CPG) neuronal networks that produce repetitive movements are frequently able to generate diverse motor programs (Gettig 1989; Harris-Warrick and Marder 1991; Marder and Calabrese 1996). Such flexibility is vital to the motor systems that control feeding behaviors of most organisms, where functionally antagonistic movement sequences are typically executed by a common peripheral apparatus (Dethier 1976; Stellar 1989; Travers and Norgren 1986). The CPGs that generate consummatory behaviors must therefore be capable of specifying either ingestive or egestive motor patterns, depending on exteroceptive and interoceptive sensory data, motivational variables, and learning (Gallistel 1980; García et al. 1974; Zeigler 1994).

The feeding behaviors of gastropod molluscs are generated by neuronal networks that have the capacity to produce ingestive and egestive motor programs (Croll and Davis 1981; Elliott and Susswein 2002; Kupfermann 1974a,b; Murphy 2001). Intensive study of the CPG that generates feeding in *Aplysia* has disclosed several features of its organization that enable it to specify functionally distinct motor programs (Hurwitz et al. 1997; Jing and Weiss 2001; Jing et al. 2003; Kupfermann and Weiss 2001; Morgan et al. 2002; Morton and Chiel 1993a,b; Nagahama et al. 1999; Nargeot et al. 1999a). *Aplysia* consummatory behaviors consist of sequential odontophore movements that cause the toothed and grooved radula to grasp material and transport it into or out of the buccal cavity (Audesirk and Audesirk 1985; Drushel et al. 1997; Howells 1936; Kupfermann 1974a). The feeding CPG is able to generate both ingestive and egestive behaviors by controlling the flexible phasing of radula opening and closing with respect to a relatively fixed sequence of protraction and retraction (Cropper et al. 2004; Hurwitz et al. 1996; Morton and Chiel 1993a,b). If the radula closes while protracting, it tends to move material out of the oral cavity (egestive behaviors). If it closes during retraction, it will reposition material toward the esophagus (ingestive behaviors). Many of the motor neurons that produce radula movements were previously identified (Church and Lloyd 1994; Church et al. 1991; Cohen et al. 1978; Evans et al. 1996; Hurwitz et al. 1996; Jordan et al. 1993) and many of the CPG interneurons that control their phasic activity are known (Gardner 1977; Hurwitz and Susswein 1996; Hurwitz et al. 1997; Plummer and Kirk 1990; Teyke et al. 1993).

The neural control of pharyngeal movements that contribute to the consummatory behaviors of *Aplysia* has received less scrutiny. Nagahama and Takata (1987) described a buccal motor neuron, the pharyngeal burster (PB) in *Aplysia kurodai*, and showed that this neuron fired out of phase with radula retraction during food-induced responses. In *Aplysia californica*, a motor neuron (B67) that is likely to correspond to the PB was reported to fire during the phase of radula opening/protration during ingestive buccal motor programs (Park et al. 1999, 2000). We previously observed that, in addition to firing in phase with radula protrusion, B67 often exhibited a postretraction phase of activity (Serrano and Miller 2006). This study was undertaken to determine whether the postretraction firing of B67 could be associated with specific parametric or
functional properties of the buccal motor programs that control radula movements. We also explored whether the intrinsic properties of this motor neuron and their modulation could contribute to its production of phasic pharyngeal contractions. No association was detected between expression of postretraction B67 firing and parametric properties of the buccal motor programs (BMPs). However, when programs were classified according to their fictive function, postretraction firing of B67 was found to occur during ingestive, but not egestive, motor programs. Finally, dopamine, a neuromodulator that can produce ingestive motor patterns, modified B67’s intrinsic properties in a fashion that could promote such phasic activity. These observations suggest that motor neuron modulation can act jointly with CPG circuits to generate behavior-specific phasic actions.

METHODS

Subjects

Experiments were conducted on specimens of *Aplysia californica* (150–250 g) that were purchased from the *Aplysia* Resource Facility and Experimental Hatchery (University of Miami, Coral Gables, FL) or from Marinus Scientific (Garden Grove, CA). Animals were maintained in refrigerated aquaria (14–16°C) and fed dried seaweed twice per week.

Electrophysiology

Previous studies established that consummatory motor programs can be generated by stimulating individual interneurons within the buccal CPG (Hurwitz et al. 1997; Kabotyanski et al. 1998; Susswein et al. 1996; Teyke et al. 1993). The following four established protocols were used for generating buccal motor programs (Fig. 1A).

CARBACHOL. A Vaseline boundary was formed surrounding the cerebral ganglion, enabling restricted application of carbachol, a nonhydrolyzable cholinergic agonist (carbamylcholine; Sigma Chemical, St. Louis, MO). Carbachol (1 × 10^{-4} M to 1 × 10^{-3} M; 50 μl) was applied manually with a micropipette. This protocol was previously shown to excite specific cerebral-buccal interneurons that induce ingestive buccal motor programs (Susswein et al. 1996). After each test, the carbachol-containing artificial seawater (ASW) was withdrawn and replaced by at least three exchanges of drug-free solution.

DOPAMINE. A stock solution (1 mM) of dopamine [DA (5.6 hydroxytryptamine); Sigma Chemical] was prepared from powder before each experiment. It was further diluted in ASW to a concentration of 1 × 10^{-4} M immediately before bath application to the entire preparation (buccal plus cerebral ganglia). This protocol was shown to increase the occurrence of buccal motor programs and to bias their functional configuration toward ingestive patterns (Kabotyanski et al. 2000). In a previous study (Serrano and Miller 2006) dose–response experiments performed in the presence and absence of ascorbic acid showed that DA efficacy was not appreciably affected by oxidation using this protocol.

B65. The intrinsic buccal ganglion interneuron B65 is located within the confluence of the esophageal nerve and buccal nerve 1 (Fig. 1; Teyke et al. 1993) and Byrne 1988; Teyke et al. 1993), by stimulating specific cerebral-buccal interneurons (CBIs; Hurwitz et al. 1999, 2003; Rosen et al. 1991; Sánchez and Kirk 2000) and by stimulating certain nerves of the buccal and cerebral ganglia (Chiel et al. 1986; Lechner et al. 2000; Nargeot et al. 1997; Plummer and Kirk 1990). Moreover, application of neuromodulators can activate motor programs (Kabotyanski et al. 2000; Susswein et al. 1996; Teyke et al. 1993). The following four established protocols were used for generating buccal motor programs (Fig. 1A).

**FIG. 1.** Methods used in this study. A1: schematic diagram of four protocols used to generate buccal motor programs (BMPs). Labels B–E correspond to the recordings shown in subsequent panels. Dashed line signifies Vaseline border between the buccal ganglia (top) and cerebral ganglion (bottom, only the anterior portion drawn) that was used to confine carbachol application. Dopamine was applied to the entire preparation. Intracellular stimulation of the buccal interneuron B65 (white circle) was achieved by passing depolarizing current through a microelectrode. Esophageal nerve (Esoph n.) was stimulated by a suction electrode. Membrane potential of B67 was monitored with an intracellular microelectrode and the activity of the radula nerve (R n.) was recorded with a suction electrode. A2: B67-pharynx neuromuscular system. Diagram (modified from Nagahama and Takata 1987) is highly schematic and is not drawn to scale or perspective. B67 projects a large axon by the ipsilateral buccal nerve 1 (Bn1) to the pharynx musculature that joins the dorsal buccal food canal to the esophagus. Inset: contraction from a portion of the pharynx (Px) excised while retaining its innervation by buccal nerve 1. Contraction was produced by a burst of impulses in B67 generated by injecting a depolarizing current pulse (I) into its soma. B: BMP produced with application of carbachol (1 × 10^{-4}; 50 μl) to the cerebral ganglion. In this and subsequent panels, the activity level of large units in an extracellular R n. recording was used to determine the period of fictive radula closure. Phases of radula protrusion (light gray bar) and retraction (dark gray bar) are indicated below the recordings. In BMPs produced with carbachol and dopamine (C), the coincidence of radula closure and retraction was indicative of ingestive motor patterns. In BMPs generated by stimulation of B65 (D) and the esophageal nerve (E), radula nerve activity occurred predominantly during the protrusion phase, signifying egestive motor patterns. Although all of the motor programs exhibited some postretraction rebound and synaptic activity (shaded areas in all recordings), only the ingestive-like programs exhibited postretraction firing (B and C).
see Díaz-Ríos et al. 2002; Kabotyanski et al. 1998). When stimulated, it has the capacity of generating a series of coordinated buccal programs that are initially biased toward the egestive pattern, but that can undergo a transition toward ingestion (Due et al. 2004; Kabotyanski et al. 1998). In this study, stimulation of B65 was brief and infrequent, resulting in predominantly egestive buccal motor programs.

ESOPHAGEAL NERVE. Stimulation of the esophageal nerve (E n.) produces egestive buccal motor patterns (Chiel et al. 1986; Morton et al. 1991; Proekt et al. 2004; Susswein and Byrne 1988). The entire esophageal nerve, proximal to its division into the posterior (E n.1) and anterior (E n. 2) branches (Nurgeot et al. 1997; Schwarz and Susswein 1986), was drawn into a polyethylene tube (Fig. 1A1) and stimulated with 3-ms, 2-Hz (30- to 90-s) pulses.

Experiments were conducted at room temperature (19 –21°C). The normal ASW contained the following (in mM): 460 NaCl, 10 KCl, 55 MgCl2, 11 CaCl2, and 10 HEPES. In some experiments, an ASW solution with elevated concentrations of divalent cations (2.2 × [Ca2+] and 2 × [Mg2+]; Liao and Walters 2002) was used to attenuate polysynaptic activity. Preparations were superfused with the ASW solution at a rate of 0.5 ml/min using a gravity-fed multichannel system (Model VM4; ALA Scientific Instruments). Intracellular microelectrodes filled with 2 M KCl (10–20 MΩ) were used for recording. An independent microelectrode (5–10 MΩ) was used for injecting current into B67. Voltage clamp of B67 was performed with the AxoScope software (Version 9.0). Voltage-clamp experiments were conducted in raised divalent solutions containing tetrodotoxin (TTX, 5 μM) and tetraethylammonium (TEA, 20 mM). Extracellular signals were recorded with polyethylene suction electrodes and AC-coupled amplifiers (Model 1700; AM Systems). The typical configuration consisted of two cut-end recordings from buccal nerve 1 (B n.1; Fig. 1A) and the radula nerve (R n.), monitors of radula retraction and closure, respectively (Morton and Chiel 1993a,b).

Contractions were measured from a portion of the pharyngeal muscle forming the transition between the dorsal buccal food canal and the esophagus (Fig. 1A2). A segment of this muscle [designated Px by Nagahama and Takata (1987) and I12 by Park et al. (1999)] was excised near the termination of buccal nerve 1 (B n.1) by which it remained attached to the buccal ganglion. Contractions were monitored with an isotonic motion transducer (Harvard Apparatus). One end of the muscle was pinned to the Sylgard floor of the recording chamber and the other end was connected to the rotating lever arm of the transducer by a stainless steel hook and a nylon filament. All nerves except the ipsilateral B n.1 were severed.

Data analysis

Buccal motor pattern classification was based on the method of Morgan et al. (2002; see also Jing et al. 2004). Large unit activity recorded from the radula nerve was used to determine the phase of radula closure. Ingestive-like programs were defined as those in which the ratio of the average firing frequency during protraction to its average firing frequency during retraction was <0.65. Egestive-like programs were defined as those in which the ratio of the average firing frequency during protraction to its average firing frequency during retraction was >2.0. Programs that did not fulfill either criterion were classified as intermediate.

Statistical tests (Student’s t-test, two-tailed) were performed by comparing measurements obtained before drug application to those attained at the peak of the response. Multiple group comparisons were performed with the one-way ANOVA followed by Tukey–Kramer multiple pairwise comparisons. A value of P < 0.05 was established as the criterion for significance.

RESULTS

B67 motor patterns produced by the multifunctional buccal CPG

It was reported previously that B67 fires during the phase of buccal motor programs that corresponds to protraction of the radula–odontophore complex (Park et al. 1999, 2000; Serrano and Miller 2006). We also observed that it can exhibit a second later phase of activity during some spontaneous BMPs (Serrano and Miller 2006). To determine whether the postretraction firing of B67 could be associated with specific parametric or functional features of BMPs, its activity was evaluated with four distinct stimulation paradigms (Fig. 1; see METHODS). B67 was recruited into buccal motor programs elicited with the four stimulation paradigms tested in this study (Fig. 1, B–E, top recordings; see Park et al. 1999, 2000). In all programs, B67 exhibited intense firing during the phase of fictive radula protraction (Fig. 1, B–E, gray bars below records) and was silent during the phase of radula retraction (Fig. 1, B–E, black bars below records). The protraction-to-retraction transition was marked by a large inhibitory postsynaptic potential (IPSP) that caused the membrane potential of B67 to shift abruptly from its depolarized state to a level substantially more hyperpolarized than the resting Vm. In some programs, the retraction phase was followed by a second phase of firing (Fig. 1, B and C, shaded areas).

The temporal parameters of the B67 protraction–retraction sequence exhibited considerable variability (Fig. 1, B–E). To facilitate our objective of identifying determinants of B67’s postretraction firing, we examined the extent to which such variability could be attributed to stimulus mode (Fig. 2, A and B) versus putative function (Fig. 2, C and D). Summary data showed that duration of the protraction phase of programs did not differ in a significant fashion according to stimulus type [carbachol: 7.6 ± 1.9 s, mean ± SE; dopamine: 6.9 ± 1.0 s; B65 stimulation: 8.3 ± 1.4 s; esophageal nerve stimulation: 5.6 ± 0.4 s; ANOVA: F(3,12) = 0.39; P = 0.76; Fig. 2A]. Summary measures of retraction phase durations, however, did exhibit significant overall variation [carbachol: 2.6 ± 0.7 s; dopamine: 7.0 ± 0.5 s; B65 stimulation: 5.4 ± 0.4 s; esophageal nerve stimulation: 2.5 ± 0.3 s; ANOVA: F(3,12) = 27.05, P = 0.001]. Pairwise post hoc comparisons determined that the retraction-phase durations of programs elicited with DA and B65 stimulation were longer than those elicited with carbachol and esophageal nerve stimulation (Fig. 2B). In sum, whereas protraction-phase durations did not vary in a significant fashion according to stimulus mode (Fig. 2A), retraction durations partitioned into two distinct classes (Fig. 2B).

Variability was also observed in the putative function of the fictive motor programs produced with the four stimuli (Fig. 1, B–E). A functional classification of motor programs (see METHODS) was therefore implemented using radula nerve activity (Fig. 1, B–E, bottom records) as an indicator of radula closure with respect to the protraction–retraction cycle (Fig. 2C; see METHODS). Large unit radula nerve activity was predominantly associated with the retraction phase of programs elicited by carbachol (Fig. 1B) and dopamine (Fig. 1C), stimuli that were shown to produce ingestive motor patterns (Kabotyanski et al. 2000; Susswein et al. 1996). Pooling all data (n = 4 specimens), of 135 programs produced with carbachol, 124 (91.8%) were classified as ingestive (χ2 = 100.27, df = 2; P < 0.0001).
Of 116 programs observed in the presence of dopamine (n = 4 specimens), 81 (69.8%) were classified as ingestive ($\chi^2 = 30.49; df = 2; P < 0.0001$; Fig. 2C). In contrast, large unit radula nerve activity occurred predominantly during the protraction phase of programs elicited with stimulation of B65 (Fig. 1D) and the esophageal nerve (Fig. 1E; see Due et al. 2004; Kabotyanski et al. 1998; Morton et al. 1991). Of 32 programs produced with B65 stimulation (n = 4 specimens), 16 (50%) were classified as egestive ($\chi^2 = 6.44; df = 2; P < 0.05$) and of 71 programs produced by esophageal nerve stimulation (n = 4 specimens), 64 (90.1%) were classified as egestive ($\chi^2 = 49.15; df = 2; P < 0.001$; Fig. 2C). In sum, although the BMPs produced by all of the stimulus paradigms were heterogeneous, in each case they were biased in a significant fashion toward either ingestive or egestive patterns.

The partitioning of BMP functional tendencies (Fig. 2C) did not correspond to the profiles of temporal parameters of the programs produced by the four stimulus protocols (Fig. 2, A and B). We therefore examined whether B67’s phase durations varied according to fictive program function (Fig. 2D). For this comparison, all 354 programs were pooled, regardless of stimulus type, and classified as ingestive, egestive, or intermediate using the criteria described in METHODS. When examined in this manner, protraction duration did not differ in a significant fashion between ingestive (8.0 ± 0.9 s) and egestive (7.0 ± 1.1 s) programs ($t = 2.18; df = 12; P = 0.049$). The retraction-phase durations also did not differ significantly when pooled ingestive (4.6 ± 0.7 s) and egestive (3.5 ± 0.6 s) programs were compared ($t = 2.14; df = 14; P = 0.25$). These findings indicate that the temporal parameters of BMPs classified as ingestive did not differ from those classified as egestive when the programs generated with all four stimulus types were sorted according to putative function using the algorithm implemented in this study.

Although differences were not detected between the phase durations of ingestive versus egestive motor programs (Fig. 2D), we observed that B67 sometimes exhibited depolarizing drifts, or sags, during its retraction phase and subsequent postretraction firing (Fig. 1, B–E, shaded segments of recordings). We therefore explored whether these features were specifically associated with particular stimulus paradigms (Fig. 3) or fictive function (Fig. 4). When the four stimulus paradigms were compared, sags were present in the programs produced with carbachol application (Fig. 1B) and dopamine (Fig. 1C). In the programs elicited with stimulation of B65 (Fig. 1D) and the esophageal nerve (Fig. 1E), the membrane potential during the retraction phase was either stable or drifted toward slightly more hyperpolarized levels. Measurement of sag magnitudes, defined as the difference between the initial peak and the final level of the retraction phase hyperpolarization (Fig. 3A, inset), revealed that they differed as a function of stimulus type [$F(3,11) = 11.51; P < 0.05$; Fig. 3A]. Large sags occurred in programs elicited with carbachol (4.5 ± 1.0 mV) and dopamine (3.5 ± 0.9 mV). In programs generated with stimulation of B65 the sag was small (0.2 ± 0.7 mV) and with esophageal nerve stimulation, sag measurements could have negative values (−0.5 ± 0.3 mV) as a result of the slowly developing hyperpolarization described earlier. All four direct post hoc comparisons indicated significant differences between members of these two groups (Fig. 3A).
The postretraction behavior of B67 paralleled the occurrence of the sag potential (Fig. 3, B and C). The largest numbers of impulses were observed in programs produced with carbachol (13.3 ± 4.0; Fig. 1B) and dopamine (18.4 ± 2.3; Fig. 1C). Postretraction firing was considerably reduced, and often did not occur (see Fig. 1, D and E), in programs generated with B65 (2.5 ± 1.8) or esophageal nerve stimulation [0.7 ± 0.2; F(3,12) = 11.96, P < 0.001; P < 0.05 in all four post hoc comparisons; Fig. 3B]. Finally, in those BMPs that did exhibit postretraction firing, the time that elapsed from the termination of the retraction phase IPSP to the initial postinhibitory impulse (Fig. 3C inset) was briefer in programs produced with carbachol (0.34 ± 0.04 s) and dopamine (0.45 ± 0.07 s) than it was with esophageal nerve stimulation (1.49 ± 0.13 ms) and B65 (1.45 ± 0.24 ms) stimulation [F(3,12) = 19.43; P < 0.001; P < 0.05 in all four post hoc comparisons, Fig. 3C].

The enhanced sag potentials and postinhibitory rebound (PIR) observed in B67 during programs elicited with carbachol and DA did not correspond to differences in the duration of the retraction-phase inhibition elicited with the four stimuli (compare Fig. 3, A–C with Fig. 2B). One additional parameter of this inhibition that could be directly compared across the stimulus paradigms was the magnitude of the retraction-phase IPSPs. When measured at their peaks, no differences were observed in the absolute V_m attained by the IPSP in programs produced by the four stimulus paradigms [F(3,15) = 0.52; P = 0.67; Fig. 3D]. Thus the presence of a sag and PIR in B67 was not associated with differences in either the duration or the magnitude of its retraction-phase inhibition.

Because the sag potentials and PIR in B67 were predominantly observed with the stimulus paradigms that produced ingestive BMPs (compare Fig. 3 with Fig. 2C), they were next compared according to putative program function (Fig. 4). When all 354 programs produced by the four stimuli were pooled, those classified as ingestive had larger sags (ingestive: 3.6 ± 0.1 mV; egestive: 0.2 ± 0.4 mV; t = 6.10; df = 16; P < 0.001; Fig. 4A), more postretraction impulses (ingestive: 12.6 ± 2.4; egestive: 0.6 ± 0.1; t = 3.70; df = 15; P < 0.05; Fig. 4B), and briefer delays to the first postinhibitory impulse (ingestive: 0.43 ± 0.03 s; egestive: 1.56 ± 0.09 s; t = 14.04; df = 18; P < 0.05; Fig. 4C) than those classified as egestive. Again, the absolute magnitude of the inhibition imposed on B67 during the retraction phase (ingestive: −63.6 ± 2.0; egestive: −63.0 ± 1.3) did not vary (t = 2.36; df = 7; P = 0.09; Fig. 4D).

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activity became gradually more associated with retraction, the number of postretraction impulses (vertical lines drawn from each point) became progressively increased.

Together, these findings showed that the sag potential and PIR observed during B67’s retraction and postretraction phases, respectively, varied in a systematic fashion according to putative motor program function. This variation was not associated with differences in either the duration or the magnitude of the retraction-phase inhibition. The presence of B67’s sag and PIR in ingestive BMPs elicited with multiple modes of stimulation, each of which produced functionally heterogeneous programs, supports the conclusion that their expression was specifically associated with the ingestive configuration of the multifunctional buccal network.

**Endogenous properties of B67**

In a previous investigation, B67 was found to produce an endogenous TTX-resistant burst-forming driver potential in response to depolarizing stimuli (Serrano and Miller 2006). The presence of a sag potential and PIR in B67’s activity during motor programs prompted us to examine its responses to hyperpolarizing stimuli. Voltage deflections produced by long (5-s) hyperpolarizing pulses to membrane potentials more negative than approximately −55 mV exhibited a slowly developing depolarizing drift, or “sag potential” (Fig. 6A; see Arbas and Calabrese 1987; Marder and Calabrese 1996). On termination of the current pulse, B67 displayed a rebound depolarization (Fig. 6A, left, arrow).

The sag potentials and PIR observed in the responses of B67 to hyperpolarizing current pulses suggested the presence of a hyperpolarization-activated inward current, $I_h$ (Pape 1996; Robinson and Siegelbaum 2003). This was explored using the $I_h$ blocker cesium (20 mM; Fig. 6A, right). In the presence of cesium, the input resistance of B67 was substantially reduced, making it necessary to inject a larger current pulse to hyperpolarize the membrane to levels equal to control values. When such current pulses were tested, however, the corresponding voltage deflections exhibited a slowly developing hyperpolarization rather than a sag potential (cf. Straub and Benjamin 2001). Moreover, no PIR was detected on termination of the pulse.

The responses of B67 to hyperpolarizing pulses were also examined under voltage clamp (Fig. 6B). Pulses to potentials more hyperpolarized than approximately −60 mV produced slowly activating inward currents (Fig. 6B, left). The magnitude and rise time of these currents increased as the membrane potential was stepped to increasingly more hyperpolarized levels over a range of −60 to −120 mV. In the presence of cesium (20 mM), these slowly developing currents were blocked (Fig. 6B, right). Although these observations support the presence of an $I_h$-like current in B67, the contribution of such a current to the sag potential and PIR observed during motor programs will require further investigation (see Discussion).

**Endogenous properties of B67 are modulated by dopamine**

Dopamine is a major modulator of molluscan feeding systems (Quinlan et al. 1997; Teyke et al. 1993; Trimble and Barker 1984; Wieland and Gelperin 1983) that is thought to
stimulus paradigms: esophageal nerve stimulation; application of carbachol to the cerebral ganglion; application of dopamine to the buccal ganglion; B6 stimulation. Number of postretraction impulses (upward vertical lines drawn from each point) increased as high-frequency radula nerve firing became more associated with retraction and less with protraction. Dashed line depicts equality of values on the 2 axes. When the ratio of retraction phase firing to protraction phase firing reached its highest values (shaded region), the number of postretraction impulses was reduced. B: application of DA to a preparation that was exhibiting egestive BMPs produced a gradual transition toward fictive ingestion. Successive programs are connected by dashed lines with arrows pointing toward the subsequent BMP. Number of postretraction impulses (vertical lines drawn from each point) was progressively increased as radula closure became increasingly associated with retraction.

FIG. 6. Responses of B67 to hyperpolarizing stimuli. A: injection of a long (5-s) hyperpolarizing current (I) pulse stepped the membrane potential of B67 from −40 mV, its resting value, to −70 mV (left). Hyperpolarization exhibited an early peak and sag, i.e., a slow return toward the resting $V_m$. On termination of the pulse, the membrane potential exhibited a transient postinhibitory rebound (PIR, arrow). Experiment was conducted in a raised divalent solution (see METHODS). When 20 mM cesium (Cs⁺) was added to the bathing medium (right), a larger amount of current was required to step the $V_m$ from the resting potential to −70 mV, indicating that the input resistance of B67 was decreased by Cs⁺. Voltage deflections produced by such pulses exhibited a slowly developing hyperpolarization rather than the sag potential, and no PIR was detected on termination of the pulse. B: under voltage clamp (left), pulses to potentials (V) more hyperpolarized than approximately −60 mV (from a holding potential of −40 mV) produced slowly activating inward currents (I). Magnitude and rise time of these currents increased as the membrane potential was stepped to progressively more hyperpolarized levels over a range of −60 to −120 mV. When Cs⁺ was added to the medium (right), the slowly developing inward currents were blocked.

Synaptic signaling from CPG interneurons to B67

Many key interneurons that constitute the buccal CPG were previously identified (Gardner 1977; Hurwitz et al. 1996a, 1997; Jing and Weiss 2002; Plummer and Kirk 1990; Susswein and Byrne 1988; Teyke et al. 1993). These CPG elements are typically classified according to their influence on odontophore
and radula movements, such as protraction, retraction, and closure. Having established the activity pattern of the pharyngeal motor neuron B67 during BMPs, it was possible to assess the participation of these interneurons in the control of its activity. Previously, we established that one source of direct excitatory synaptic input during the protraction phase was B65 (Serrano and Miller 2006), a buccal interneuron that promotes motor programs, but is not essential for their expression (Due et al. 2004; Kabotyanski et al. 1998; see Fig. 1D). In this study, we explored whether B67 received synaptic input from the B63, B31/32 kernel of interneurons that is thought to be obligatory for the generation of all BMPs (Hurwitz et al. 1996, 1997; Susswein and Byrne 1988). The presence of synchronous rapid excitatory postsynaptic potentials (EPSPs) in B67 and B31/32 (Fig. 8A), probably originating from B63 (Hurwitz et al. 1997), suggested that B67 is driven by the core elements that generate the protraction phase of buccal motor programs.

Many motor neurons that fire during the protraction phase of buccal motor programs receive potent synaptic inhibition from the influential retraction-phase interneuron B64 (Hurwitz and Susswein 1996). During motor programs, the inhibition of B67 throughout the retraction phase corresponded well with the duration of B64 firing (Fig. 8B). In the presence of raised divalent cations, direct inhibitory synaptic potentials were observed in B67 when B64 was fired with injection of depolarizing current (Fig. 9A). The peak of this inhibition ($-65.4 \pm 3.4$ mV, $n = 3$) was comparable to levels achieved during motor programs (cf. Figs. 2D and 3D). During repetitive B64 firing, its synaptic inhibition of B67 exhibited a substantial depolarizing sag and PIR. As the duration of B64 firing was increased (Fig. 9, A1 and A2), the postinhibitory rebound that followed its inhibition of B67 became progressively larger, eventually surpassing threshold (Fig. 9, A3 and B). Plotting all measures of PIR as a function of the durations of the B64-induced inhibition that preceded them revealed a highly significant coefficient of determination ($r^2 = 0.87$; $df = 17$, $P < 0.01$; Fig. 9B).

Together, these findings show that B67 receives synaptic input from CPG elements that promote radula protraction during buccal motor programs (see also Serrano and Miller 2006). They also suggest that the synaptic inhibition received during the retraction phase can activate the endogenous sag
potential and PIR that contribute to B67’s postretraction firing (see DISCUSSION).

Pharyngeal movements produced by B67 during buccal motor programs

The data presented to this point indicated that the duration (Fig. 2D) and maximal amplitude (Fig. 4D) of the synaptic inhibition received by B67 did not differ between ingestive-like and egestive-like motor programs. The programs did partition into the behavioral classes, however, when attributes of the sag (Figs. 3A and 4A) and PIR (Figs. 3, B and C and 4, B and C) were compared across stimulus paradigms. Moreover, dopamine, a modulator that promotes ingestive motor programs (Kabotyanski et al. 2000), enhanced these endogenous properties in a manner that could account for such differences. In view of its reported targets (Park et al. 1999, 2000) and previous demonstrations of the function of the PB neuron in Aplysia kurodai (Takata and Nagahama 1988), we reasoned that B67 could contribute to postretraction pharyngeal movements that occur during ingestive behaviors (Drushel et al. 1997; Neustadter et al. 2002; Takata and Nagahama 1988; Ye et al. 2006; see DISCUSSION). This hypothesis was tested by recording B67 activity and contractions of pharyngeal muscle during egestive and ingestive motor programs (Fig. 10). In egestive programs generated with esophageal nerve stimulation (Fig. 10A), B67 fired during the protraction phase and a large pharyngeal contraction was observed. It did not exhibit a second phase of firing and no postretraction pharyngeal contraction was detected. During ingestive motor programs produced with carbachol, however, B67 produced a postretraction burst of impulses and a corresponding contraction of the pharyngeal muscle (Fig. 10B). Because this second contraction was never observed in the absence of B67 firing, we propose that it reflects the program-specific expression of postretraction activity of this motor neuron (see DISCUSSION).

DISCUSSION

Intensive study has increased our understanding of the buccal motor programs that coordinate movements of the Aplysia odontophore–radula complex (Hurwitz and Susswein 1996; Hurwitz et al. 1997; Kupfermann 1974b; Plummer and Kirk 1990; Susswein and Byrne 1988). These movements include the successive phases of radula protraction and retraction that occur in a relatively fixed sequence during both ingestive and egestive behaviors (Church and Lloyd 1994; Cropper et al. 2004; Morton and Chiel 1993a,b). This study showed that the buccal CPG could generate an additional phase of activity in a
pharyngeal motor neuron, B67, and that this phase of motor activity was preferentially expressed when the multifunctional buccal CPG specified ingestive motor programs. Our observations indicate that this conditional expression of postretraction activity can occur, at least in part, by modulation of the endogenous properties of B67 (Fig. 10B). The association of pharyngeal activity with ingestive motor programs is proposed to reflect the adaptive value of promoting movement of material from the buccal cavity to the esophagus during ingestive behaviors and preventing such movement during egestive behaviors (following text).

Postretraction activity in Aplysia buccal motor programs

The majority of motor neurons that participate in buccal motor programs can be classified in the context of radula movements, specifically protraction, retraction, opening, or closure (Church and Lloyd 1994; Cohen et al. 1978; Morton and Chiel 1993b). The firing patterns of these motor neurons during BMPs, coupled with an understanding of the muscles that they innervate, enables their classification into these four functional categories (Church and Lloyd 1994; Evans et al. 1996; Hurwitz et al. 1997). Moreover, the interneurons that have been identified as members of the buccal CPG are also classified according to these functional subunits or modules (Hurwitz and Susswein 1996; Hurwitz et al. 1997; Kabotyanski et al. 1998; Jing and Weiss 2002; Jing et al. 2004; Plummer and Kirk 1990). In this study, a pharyngeal motor neuron was found to be controlled by the same CPG elements that generate radula protraction (see Nagahama and Takata 1988; Park et al. 1999, 2000). However, this neuron could display the unusual feature of a second phase of activity that followed retraction.

Postretraction activity in the buccal motor programs of Aplysia was previously observed. Of particular relevance to the present study, Nagahama and Takata (1987) demonstrated that the pharyngeal burster neuron of Aplysia kurodai burst out of phase with radula retraction motor neurons, and proposed that it functioned to promote movement of food through the pharynx. In feeding specimens of Aplysia californica, Neustadter et al. (2002) reported a major postretraction burst of activity in buccal nerve 1 and speculated that it reflected activation of pharyngeal movements that transport material into the most anterior portion of the esophagus (see also Ye et al. 2006).

In reduced preparations, Church and Lloyd (1994) identified several motor neurons that fired postretraction bursts of impulses in fictive ingestive motor programs generated with stimulation of cerebral-buccal interneuron 2 (CBI-2). These included B48, a motor neuron that innervates radula opener muscles (see also Evans et al. 1996). It was proposed that radula opening after the peak of retraction would facilitate passage of food into the esophagus and prevent it from being returned to the buccal cavity (Evans et al. 1996). Notably, this phase of activity was not observed when the same neurons were recorded during egestive motor patterns (Church and Lloyd 1994).

Finally, a postretraction phase of firing was also demonstrated in B52, a multifunctional participant in the buccal CPG (Evans et al. 1999; Nargeot et al. 2002; Plummer and Kirk 1990; Shetreat-Klein and Cropper 2004). The presence of widespread inhibitory synaptic connections from B52 to motor neurons and interneurons led to the proposal that this phase of its firing contributes to terminating programs (Baxter et al. 1997; Nargeot et al. 2002; Plummer and Kirk 1990). Interestingly, B52 projects an axon into the contralateral buccal nerve 1, the nerve by which B67 innervates the pharynx. The possibility that B52 exerts postretraction motor or modulatory actions in addition to its influential interneuronal role has not yet been explored.

Modulation of endogenous motor neuron properties

The degree to which B67’s endogenous properties contribute to its postretraction firing is uncertain because B67 clearly...
receives excitatory synaptic signals during this phase of motor programs (see Fig. 1). However, modulation of intrinsic motor neuron properties can provide effective and highly specific control over CPG circuits (Harris-Warrick and Marder 1991; Kiehn and Katz 1999). Previously, we showed that dopamine enhanced the bursting activity of B67 and demonstrated a DA-induced prolongation of B67’s TTX-resistant burst-forming driver potential (Serrano and Miller 2006). In this study, dopamine was found to modulate two additional intrinsic properties of B67, an I_h-like current and PIR. It is presently not possible to determine the degree to which the PIR of B67 is a direct consequence of the I_h, or the extent to which the enhancement of PIR by DA is a direct consequence of its enhancement of this current (see Calabrese and Feldman 1999; Friesen 1994). Additional currents that operate near the resting V_{re}, including those that contribute to generation of B67’s bursting properties, are potential targets that can influence motor network phase transitions (Angstadt et al. 2005; Jones and Thompson 2001; Matsushima et al. 1993).

It is becoming increasingly clear that the contributions of dopamine to buccal motor program function are diverse and that they are mediated by multiple receptor types. At least two influential interneurons, B65 and B20, use DA as a mediator of rapid excitatory synaptic signals (Díaz-Ríos and Miller 2005; Due et al. 2004). These neurons fire during the protraction phase of BMPs and can promote egestive programs by their direct dopaminergic excitation of radula closure motor neurons (Díaz-Ríos and Miller 2005; Due et al. 2004; Kabotyanski et al. 1998; Proekt et al. 2004; Teyke et al. 1993). Dopamine also mediates rapid PSPs evoked in specific buccal interneurons when the esophageal nerve is stimulated (Nargeot et al. 1999b). Although the rapid EPSPs mediated by B65 and B20 are blocked by sulpiride, but not methylergonovine (Díaz-Ríos and Miller 2005; Due et al. 2004; Teyke et al. 1993), the PSPs originating from esophageal nerve stimulation, some of which are inhibitory, are sensitive to methylergonovine (Nargeot et al. 1999b).

In addition to these rapid synaptic signals, dopamine acts in a modulatory fashion to regulate the synaptic and intrinsic properties of several buccal neurons (Kabotyanski et al. 2000; Serrano and Miller 2006). Moreover, dopaminergic receptors that are pharmacologically distinct from those mediating rapid PSPs participate in the reinforcement pathways of classical and operant conditioning paradigms (Brembs et al. 2002; Nargeot et al. 1999b; Reyes et al. 2005). Modulatory DA receptors can typically be activated with concentrations (micromolar) that are substantially lower than those required to activate synaptic DA receptors (millimolar). Moreover, modulatory DA receptors exhibit a distinct pharmacology and are less prone to desensitization than those mediating rapid signals. Previously, we demonstrated that the dopaminergic interneuron B65 produced sulpiride-sensitive rapid EPSPs in B67 and that firing B65 also produced prolonged modulation of B67’s activity (Serrano and Miller 2006). We proposed that DA released from B65 could produce both rapid and long-lasting signals in B67 and that the prolonged signals were associated with the modulatory capabilities of B65 and DA to bias the entire buccal system toward ingestive programs (Kabotyanski et al. 1998, 2000). This study suggests that, in addition to actions described previously, dopamine can produce increased sag potentials and PIR in B67.

It is striking that a single neuromodulator, dopamine, produces multiple effects that can act in a synergistic fashion to increase the activity of B67. The ability of a particular modulator to exert concurrent regulation of multiple cellular and circuit properties provides efficient and effective control over motor systems (see for example Brezina and Weiss 1997a,b; Fort et al. 2004; Katz and Frost 1995; Marder and Weimann 1992). Although prolongation of the driver potential will increase the number of impulses per burst, the actions on I_h and PIR are predicted to increase burst frequency and to contribute to postretraction firing during BMPs. These, and possibly additional modulatory actions of DA, also increase the rhythmicity of B67 bursting and promote synchrony between the bilateral B67 pair (Serrano and Miller 2006).

Aminergic modulation of motor neuron properties has been intensively studied in the pyloric network of the crustacean stomatogastric (STG) system, where the endogenous currents of motor neurons are regulated in a highly specific fashion (Flamm and Harris-Warrick 1986; Harris-Warrick et al. 1995; Kiehn and Harris-Warrick 1992). In that system, the motor neurons have direct synaptic participation in the CPG circuit, so factors that modify their intrinsic properties will influence overall circuit function. Similarly, serotonergic modulation of endogenous motor neuron properties in the swim circuit of the leech is thought to increase their influence on the CPG (Angstadt et al. 2005; Mangan et al. 1994). Of particular relevance to the present study, intrinsic properties of motor neurons that participate in radula retraction in the pond snail Lymnaea stagnalis are regulated by serotonin (Straub and Benjamin 2001). As in the crustacean STG and the leech swim circuits, the motor neurons that are modulated in Lymnaea are thought to exert substantial influence on pattern generation, in this instance by their electrotonic coupling to CPG elements (Staras et al. 1998). In the case of the motor neuron studied here, B67, there is presently no evidence for access to CPG elements either by synaptic signaling or electrical coupling. Its modulation may thus be implemented without affecting other properties of the buccal CPG.

Functional considerations and comparisons to related systems

In the majority of molluscan feeding systems previously studied, consummatory buccal motor programs are composed of three major phases (see Brierly et al. 1997; Elliott and Benjamin 1985; Elliott and Susswein 2002; Quinlan and Murphy 1996). Murphy (2001) proposed a “composite universal tripartite CPG model” for the control of rasping-like molluscan feeding in which he proposed the existence of homologous neurons for many CPG elements. In support of this model, evidence was presented for the presence of neurons in Lymnaea stagnalis and Helisoma trivolvus corresponding to the three Aplysia interneurons examined in this study; the protraction phase interneurons B63 and B65 and the retraction phase interneuron B64. It was suggested that CPG elements corresponding to the third phase of his model, termed “Subunit 3 hyper-retraction,” were yet to be identified in Aplysia.

In the present model of the feeding network of Lymnaea, six types of CPG elements were identified (Brierly et al. 1997). Two of these interneurons, termed N3p and N3t, were shown to be active during the postretraction phase that was termed...
“swallowing” (Brierly et al. 1997). In *Aplysia californica*, “swallowing” is depicted as a prolongation of the retraction phase, also referred to as hyperretraction, during which both retraction and closure are enhanced (Cropper et al. 1998; Evans and Cropper 1998; Jing et al. 2004). As described earlier, postretractive firing of specific neurons was described in several studies, but it has been difficult to reconcile this phase of activity with the two-phase model of radula movements (see Cropper et al. 2004; Shetreat-Klein and Cropper 2004). Our findings indicate that at least one function of postretractive activity is to produce contractions of pharyngeal muscles. Interestingly, Weiss et al. (1986) proposed that the postretractive period corresponds to a phase of postretraction activity. Our results are consistent with this hypothesis because pharyngeal contractions may be predicted to exert forces that will act to displace the radula in the anterior direction.

In proposing a universal tripartite model for molluscan feeding, Murphy (2001) contended that the presence of a third phase in the CPG in *Aplysia* warranted further investigation because of the presence of such an organization in closely related species. However, he recognized the possibility that “phase 3 effects could be explained by mechanisms not requiring phase 3 interneurons.” Our results support the generalizable insight that synaptic and modulatory CPG actions can operate in a concerted fashion with the endogenous properties of motor neurons to generate phasic activity for which no interneurons are required. Moreover, we found that expression of such activity could occur in a program-specific fashion. Whether dedicated buccal CPG elements also contribute directly to this conditional activity remains to be explored.

**REFERENCES**


