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Rapid Dopaminergic Signaling by Interneurons That Contain Markers for Catecholamines and GABA in the Feeding Circuitry of *Aplysia*

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Díaz-Ríos, Manuel and Mark W. Miller. Rapid dopaminergic signaling by interneurons that contain markers for catecholamines and GABA in the feeding circuitry of *Aplysia. J Neurophysiol* 93: 2142–2156, 2005; doi:10.1152/jn.00003.2004. Consummatory feeding behaviors in *Aplysia californica* are controlled by a polymorphic central pattern generator (CPG) circuit. Previous investigations have demonstrated colocalization of markers for GABA and catecholamines within two interneurons, B20 and B65, that participate in configuring the functional output of this CPG. This study examined the contributions of GABA and dopamine (DA) to rapid synaptic signaling from B20 and B65 to follower cells that implement their specification of motor programs. Pharmacological tests did not substantiate the participation of GABA in the mediation of the excitatory postsynaptic potentials (EPSPs) from either B20 or B65. However, GABA and the $GABA_B$ receptor agonist baclofen were found to modify these signals in a target-specific manner. Several observations indicated that DA acts as the neurotransmitter mediating fast EPSPs from B20 to two radula closer motor neurons B8 and B16. In both motor neurons, application of DA produced depolarizing responses associated with decreased input resistance and increased excitation. B20-evoked EPSPs in both follower cells were occluded by exogenous dopamine and blocked by the DA antagonist sulpiride. While dopamine occlusion and sulpiride block of convergent signaling to B8 from B65 resembled that of B20, both of these actions were less potent on the rapid signaling from B65 to the multifunctional and widely acting interneuron B4/5. These findings indicate that dopamine mediates divergent (B20 to B16 and B8) and convergent (B20 and B65 to B8) rapid EPSPs from two influential CPG interneurons in which it is colocalized with GABA-like immunoreactivity.

INTRODUCTION

Cotransmission, or synaptic communication involving multiple neurotransmitters, appears to be a common property of neural signaling (Burnstock 1986; Chan-Palay and Palay 1984; Kupfermann 1991; Lundberg and Hökfelt 1986). Our present understanding of neurotransmitter colocalization and cotransmission has been broadened by studies using the large identified neurons that are found in invertebrate model systems (Adams and O'Shea 1983; Katz and Harris-Warrick 1989, 1990; Weiss et al. 1992). While the majority of these studies have focused on neurons with well-defined and accessible peripheral projections (Bishop et al. 1987; Brezina et al. 1994a,b; Vilim et al. 1996a,b; Whim et al. 1993), recent investigations have addressed the significance of cotransmission within the central pattern generator (CPG) circuits that regulate motor behavior (Koh et al. 2003; Marder 1999; Nusbaum et al. 2001; Thirumalai and Marder 2002).

As in many species, the feeding behavior of *Aplysia californica* consists of a highly variable appetitive phase that is followed by a more stereotyped consummatory phase (Kupfermann 1974a). Consummatory behaviors include multiple ingestive (such as biting and swallowing) and egestive (such as rejection) actions that are executed by intersecting sets of specialized organs and muscles. This motor system is governed by a multifunctional or polymorphic CPG network that is located primarily within the buccal and cerebral ganglia (Cropper et al. 2004; Kupfermann 1974b; Kupfermann and Weiss 2001; Morton and Chiel 1994). By regulating the phase relations of motor neuron firing, this CPG can determine the qualitative (ingestive vs. egestive) and quantitative features of the behavior that is produced by the system (Church and Lloyd 1994; Hurwitz et al. 1996; Jing and Weiss 2001, 2002; Morgan et al. 2002; Morton and Chiel 1993a,b).

B20 and B65 are two buccal interneurons that are capable of initiating coordinated buccal motor patterns (Kabotyanski et al. 1998; Teyke et al. 1993). Moreover, each is thought to play a critical role in determining the functional output of the feeding CPG. Recently, it was found that the motor programs elicited by B20 tend to be egestive and that this neuron contributes in a significant fashion to the production of egestive motor programs elicited by higher-order cerebral-buccal interneurons (CBIs) (Jing and Weiss 2001; Proekt et al. 2004). While B65 also evokes egestive BMPs (Due et al. 2004; Kabotyanski et al. 1998), its repeated firing can also achieve a transition of the buccal CPG toward ingestive motor programs (Kabotyanski et al. 1998).

B20 and B65 both exhibit catecholaminergic histofluorescence (Díaz-Rios et al. 2002; Kabotyanski et al. 1998; Teyke et al. 1993), and both contain tyrosine hydroxylase-like immunoreactivity (Díaz-Ríos et al. 2002). In addition to containing markers for catecholamines, B20 and B65 exhibit GABA-like immunoreactivity (Díaz-Ríos et al. 1999, 2002; Jing et al. 2003). As several neurons that receive direct excitatory synaptic input from these two influential interneurons are identified (Due et al. 2004; Jing and Weiss 2001; Kabotyanski et al. 1998; Teyke et al. 1993), the present study was undertaken to explore the possible roles of dopamine and GABA in their rapid synaptic signaling. Some of these observations have been reported in abstract form (Díaz-Ríos and Miller 2002; Díaz-Ríos et al. 2003).

METHODS

Subjects

Experiments were conducted on specimens of *Aplysia californica* (150–250 g) that were purchased from the *Aplysia* Resource Facility

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and Experimental Hatchery (University of Miami, Coral Gables, FL) or from Marinus (Long Beach, CA). Animals were maintained in refrigerated aquaria (14–16°C) and fed dried seaweed twice per week.

Electrophysiology and pharmacology

Neurons were identified in preparations consisting of the paired buccal and cerebral ganglia. Extracellular signals were recorded with polyethylene suction electrodes and AC-coupled amplifiers (Model 1700, AM Systems). The typical configuration consisted of two *en passant* recordings from the cerebral-buccal connectives and one cut-end recording from a major branch of the radula nerve. Intracellular microelectrodes filled with 2 M KCl (10–20 MΩ) were used to record from known synaptic follower cells of neurons B20 (Teyke et al. 1993) and B65 (Kabotyanski et al. 1998). A second microelectrode was used to locate the presynaptic neuron. After presynaptic cell identification, another intracellular electrode (5–10 MΩ) was introduced for passing current into the postsynaptic neuron. Experiments were conducted at room temperature (19–21°C) in artificial seawater (ASW) containing elevated concentrations of divalent cations ($2.2 \times [Ca^{2+}]$ and $2 \times [Mg^{2+}]$) (Liao and Walters 2002) to attenuate polysynaptic activity.

Solutions of drugs were prepared from powder in high-divalent ASW immediately before their application. Methylergonovine maleate salt, ergonovine (ergometrine), chlorpromazine hydrochloride, fluphenazine dihydrochloride, clozapine, (±)-butaclamol, *S*-(−)-raclopride, (±)-baclofen, saclofen, picrotoxin, and bicuculline were obtained from Sigma Chemical Co. (St. Louis, MO). Piperidine-4-sulfonic acid (piperidine-4S) and (±)-nipecotic acid were purchased from Research Biochemicals International (Natick, MA). All experiments shown in this study were performed with application of agonists and antagonists at a concentration of 1 mM. This concentration was chosen due to its reliable production of effects that were reversible. Moreover, as there is evidence for receptors with higher sensitivity in this system (see DISCUSSION), elevated concentrations were chosen to increase the likelihood that observations reflected actions at receptors within the synapse, where high levels of DA are likely to mediate rapid signaling. Preparations were superfused with the ASW solution at a rate of 0.5 ml/min using a gravity-fed multi-channel system (ALA Scientific Instruments, Model BM4). Responses were assessed 2, 5, and 10 min after perfusion switches. Baclofen, GABA, and dopamine were also applied through a puffer electrode (0.5- to 1-s pulses) at a concentration of 1 mM using a Picospritzer II (General Valve, Fairfield, NJ) pressure ejection system. For these tests, the superfusion was briefly interrupted and the neurotransmitter or agonist was mixed with a small amount of dextran fluorescein (Molecular Probes, Eugene, OR) dye to aid visualization of the puff. The pipette tip was typically placed close to the soma and initial segment of the target neuron. The ASW superfusion was reinitiated immediately after application to ensure rapid removal of the ejected solution. Application of dextran fluorescein alone did not produce any detectable effects.

All results reported in this study were observed in a minimum of three specimens. Experiments conducted on the left and right buccal hemiganglia were pooled. Measurements are reported as the means ± SE. Due to the presence of long-lasting dopaminergic (see DISCUSSION) and GABAergic (Díaz-Ríos and Miller 2002, Díaz-Ríos et al. 2003) actions in this system, statistical tests (Student's *t*-test; 2-tailed) were performed by comparing measurements obtained prior to drug application to those attained at the peak of the response. A value of $P < 0.05$ was established as the criterion for significance.

Neurobiotin injections

After identification of specific neurons based on location, size, pigmentation, electrophysiological features, and synaptic connectivity, one KCl microelectrode was withdrawn and replaced with one containing Neurobiotin. Injections were modified from the methods described by Delgado et al. (2000). The microelectrode tips were filled with 4% Neurobiotin (Vector Laboratories, Burlingame, CA) dissolved in 0.5 M KCl and 50 mM Tris (pH 7.6). The electrode shafts were filled with 2 M KCl, resulting in resistances ranging from 15 to 30 MΩ. Depolarizing current pulses (1–2 nA; 0.5 s; 1 Hz; 10–30 min) were used to eject the Neurobiotin. This procedure did not appear to affect the resting potential or spontaneous electrical activity of the injected neuron. The preparations were usually left at room temperature for 2–3 h to allow material to diffuse from the injection site (cell body) into small and distant processes. They were then repinned if necessary, and fixed in 4% paraformaldehyde (1–4 h). The fixed ganglia were transferred to microcentrifuge tubes, and washed five times (20 min each) with a phosphate buffer containing 1% Triton X-100 and 0.1 mM sodium azide (PTA solution). They were then incubated in $Rhodamine_{600}$ Avidin D (Vector Labs) diluted (1:200–1:3,000) in PTA (24–48 h, room temperature). Tissues were washed five times with PTA and viewed on a Nikon Eclipse TE200 fluorescence microscope. Images were captured using the ACT-1 software package (Nikon) and processed using Photoshop (Version 6.0) and Corel Draw (Version 9.0).

RESULTS

Rapid excitatory signaling from B20 to B16 and B8

B20 is a small elongated bipolar neuron located in the medial region of the ventral surface of each buccal hemiganglion (Fig. 1*A1*) (see also Díaz-Ríos et al. 2002; Teyke et al. 1993). Previous investigations have demonstrated direct excitatory synaptic signaling from B20 to B16 and B8 (Fig. 1, *A2* and *B*), two buccal motor neurons that participate in closure of the food-grasping radula (Jing and Weiss 2001; Teyke et al. 1993). B16 is a large motor neuron located in the medial region of the ventral motor neuron cluster (Fig. 1*A1*) (see Cohen et al. 1978; Kreiner et al. 1987). Its axon projects across the hemiganglion in the lateral direction to exit via buccal nerve 3 and innervate the ipsilateral I5 (also known as the accessory radula closer or ARC) muscle (Cohen et al. 1978) and the I4 muscle (Jordan et al. 1993). The cell body of B8 (actually 2 neurons designated B8a and B8b that are indistinguishable by presently known physiological criteria) is located in the most lateral region of the ventral motor neuron cluster (Fig. 1*A1*) (see Church and Lloyd 1991, 1994; Morton and Chiel 1993b; Rosen et al. 2000). Its axon traverses the hemiganglion, exits via the radula nerve, and innervates the ipsilateral I4 and I6 muscles.

Simultaneous recording from B16 and B8 while firing B20 in high-divalent ASW confirmed that each motor neuron received a fast excitatory postsynaptic potential (EPSP) with a brief latency (4–5 ms) and rapid time to peak (~30 ms; Fig. 1*B*). The amplitude of the EPSP recorded in the ipsilateral B8 cell body (3.9 ± 0.23 mV, $n = 5$) was larger than that recorded in the ipsilateral B16 (1.7 ± 0.26 mV, $n = 5$). The relation between postsynaptic membrane potential and the amplitude of the B20-evoked fast EPSP was examined in B16 and B8 by setting the two postsynaptic cells to hyperpolarized levels with current injected through a second microelectrode (Fig. 1*C, 1* and *2*). The peak amplitudes of the EPSPs evoked in both motor neurons were increased at hyperpolarized membrane potentials and they exhibited similar extrapolated reversal potentials (between −5 and −15 mV; $n = 3$ for each cell, Fig. 1*C3*).

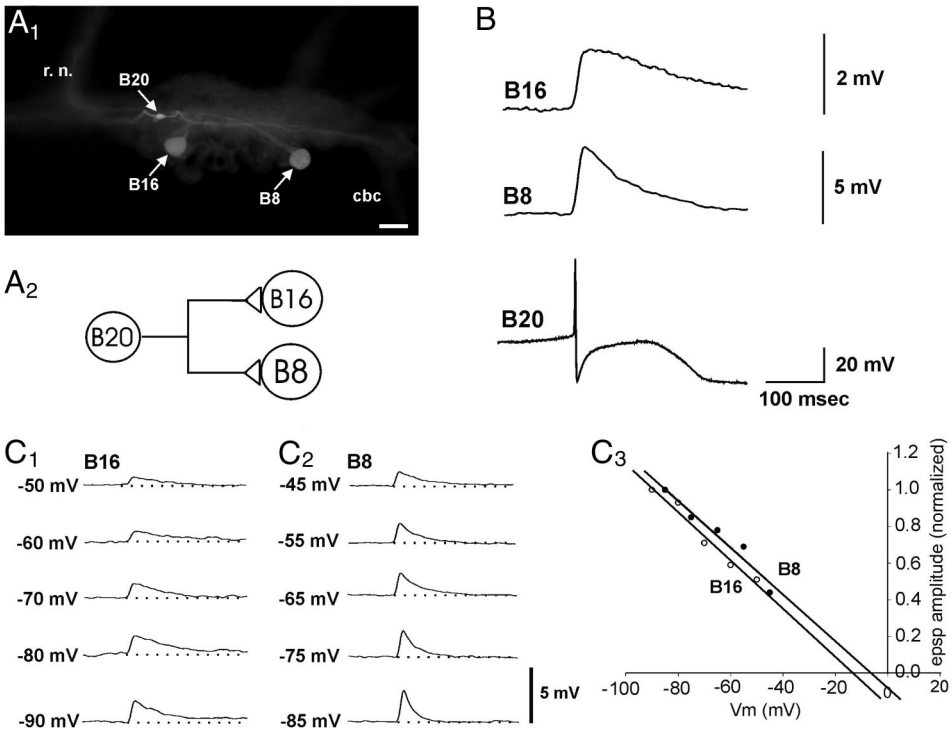

FIG. 1. Divergent signaling from B20 to B16 and B8. *A1*: neurobiotin fills of the GABA-immunoreactive and catecholamine containing interneuron B20 and its 2 follower motor neurons B16 and B8. Rostral surface of the left buccal hemiganglion. B20 is a small bipolar interneuron that projects a fiber to both cerebral-buccal connectives (cbc). B16 is a large motor neuron that projects a fiber to innervate the accessory radula closer (ARC) muscle via the ipsilateral buccal nerve 3. B8 (actually a pair of cells designated B8a and B8b) is a laterally positioned ventral motor neuron that projects a large caliber axon to the radula closer muscle I4 via the radula nerve (r n.). Calibration bar = 100 μm. *A2*: schematic depicts direct divergent excitatory synaptic signaling from B20 to B16 and B8. Axons from the 3 cells come into close proximity in the central core of the ganglion where they give rise to multiple processes that are likely to be the sites of synaptic transmission. Signaling is unidirectional in both cases, i.e., B20 does not receive direct synaptic input from either B8 or B16. *B*: excitatory postsynaptic potentials (EPSPs) evoked in B16 (*top*; V_m = −44 mV) and B8 (*middle*: V_m = −56 mV) by an impulse produced in B20. Experiment was conducted in a raised divalent artificial seawater (ASW) solution that attenuates polysynaptic signaling. *C*: potential dependence of fast synaptic signaling from B20 to B8 and B16. *C, 1* and *2*: follower neurons were hyperpolarized to indicated levels using sustained current from a 2nd microelectrode. B20 was fired by passing depolarizing current through the recording electrode. In both B16 and B8, the fast EPSP increased in amplitude when the membrane potential of the postsynaptic cell was set at hyperpolarized levels. *C3*: plot of the fast EPSP amplitude as a function of postsynaptic membrane potential in B16 (○) and B8 (●). When amplitudes were normalized by setting the maximal EPSP = 1.0, the fast EPSPs in the 2 motor neurons were found to be similarly influenced by membrane potential. The extrapolated reversal potential (x intercept) was −8 mV for B8 and −11 mV for B16.

RAPID SYNAPTIC SIGNALING OF B20: TESTING THE ROLE OF GABA. Excitatory GABAergic signaling contributes to the generation of feeding motor programs in pulmonate and pteropod mollusks (Arshavsky et al. 1993; Bravarenko et al. 2001; Norekian 1999; Norekian and Satterlie 1993; Richmond et al. 1993). The known ability of GABA to produce excitatory responses on *Aplysia* neurons (Yarowsky and Carpenter 1977, 1978), coupled with the presence of GABA-like immunoreactivity in B20 (Díaz-Ríos et al. 2002), therefore prompted us to examine the possible contribution of GABA to the signaling of this cell to the radula closer motor neurons B16 and B8.

Application of GABA produced differential effects on the resting membrane potentials of the two motor neurons (Fig. 2). No changes in the membrane potential of B16 were detected with application of GABA to its cell body and initial segment (Fig. 2*A; n* = 4). To ensure that the absence of a response was not due to the membrane potential (V_m) of B16 being close to the GABA reversal potential, additional tests were made with the V_m adjusted to various levels (−40 to −80 mV), but no responses were observed (not shown). Puffed application of GABA (1 mM) to the soma of B8 produced a biphasic response consisting of an initial hyperpolarization followed by a long-lasting depolarization (Fig. 2*B1; n* = 3). A picrotoxin-sensitive hyperpolarizing response to GABA was recently demonstrated in B8, and inhibitory GABAergic PSPs were shown to originate from an identified interneuron, B40 (Jing et al. 2003; see DISCUSSION). The long-lasting depolarization was further explored using baclofen, an agonist of $GABA_B$ type receptors in vertebrates. Bath application of baclofen produced a monophasic depolarization of the B8 membrane potential (Fig. 2*B2; n* = 3). This depolarization was often irregular and gradual in onset, but it was sustained for the duration of baclofen exposure. It was reversed when washed with normal ASW (Fig. 2*B2,* ↑).

GABA also produced distinct effects on the input resistance of the two follower cells of B20. Consistent with the absence of detectable GABAergic effects on the membrane potential of B16, measurements of the input resistance of this cell (*n* = 3) were not affected by GABA (Fig. 2*C1*). In B8, however, GABA produced an increase in the input resistance (Fig. 2*C2; n* = 4). As these experiments required considerable time to execute, they were conducted with bath application of GABA and measurements were made during the late depolarizing phase of its response (see Fig. 2*B1*).

In contrast to the stable voltage deflections produced in B16 with hyperpolarizing current pulses, those elicited in B8 exhibited a prominent time-dependent decline or "sag" (compare

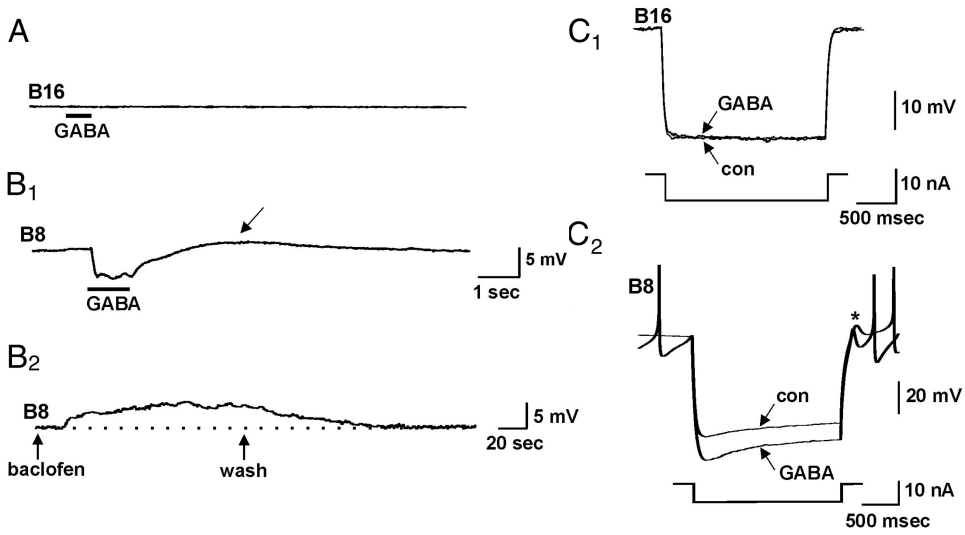

FIG. 2. Effects of GABAergic agonists on synaptic follower cells of B20. *A*: pulsed application of GABA (1 mM) to the cell body region of B16 (V_m = −52 mV) did not produce a detectable response. *B1*: pulse (1 s) of GABA (1 mM) applied by pressure ejection from a micropipette directed toward the B8 cell body (V_m = −46 mV) produced a rapid hyperpolarization followed by a long-lasting depolarization (↓). *B2*: bath application of baclofen (1 mM) produced a depolarization of B8 that was slow in onset (resting potential = −46 mV denoted by · · ·). The baclofen response persisted until it was reversed by wash with normal artificial seawater (ASW; wash). Note different time calibrations in *B, 1* and *2*. *C*: effects of GABA on the input resistance of 2 follower cells of interneuron B20. *C1*: B16, under control conditions a hyperpolarizing current pulse (8 nA, 2 s, *bottom record*) produced a voltage deflection of ~35 mV (con). In the presence of GABA (1 mM), an identical current pulse (superimposed on control pulse) produced a hyperpolarizing deflection (GABA) that did not differ appreciably from the control pulse. *C2*: B8, under control conditions a 2-s hyperpolarizing current pulse (8 nA; *bottom record*) produced a voltage deflection of ~60 mV (con). In the presence of GABA (1 mM), an identical current pulse (superimposed on control pulse) produced a larger hyperpolarizing deflection (GABA). The GABA recording was offset to compensate for a depolarization (~5 mV) produced by GABA (see *B1*). Note spontaneous action potential prior to the current pulse. Both voltage deflections exhibited time-dependent sags and postinhibitory depolarization (*). The latency to the 1st action potential after the pulse was shorter in the presence of GABA.

Fig. 2*C, 2* and *1*). The GABA-induced increase in the B8 input resistance was nearly equivalent throughout the pulse. Conspicuous effects on the magnitude or kinetics of the sag itself were not noted.

All GABAergic antagonists that were tested, including agents that block GABA-mediated inhibitory postsynaptic potentials (IPSPs) in *Aplysia* (picrotoxin and bicucculine) (Jing et al. 2003), a $GABA_B$ antagonist (saclofen), and compounds that block GABAergic EPSPs in the feeding system of *Clione limacina* (piperidine-4-sulfonic acid and 5-aminovaleric acid) (Norekian 1999) did not produce detectable effects on the EPSPs produced by B20 in B16 or B8 (all tested at concentrations ≤1 mM). Moreover, nipecotic acid (1 mM), an inhibitor of GABA reuptake that augments signaling at GABAergic synapses in mollusks (see Jing et al. 2003; Norekian 1999) did not affect the EPSPs from B20 to either of its followers.

Recent studies have shown that application of exogenous GABA can occlude GABAergic PSPs in this circuit (Jing et al. 2003; Wu et al. 2003). The actions of GABAergic agonists were therefore evaluated on the EPSPs produced by B20 in B16 and B8. Bath application of GABA produced a partial (38.7 ± 5.1%, n = 5) reduction of the amplitude of the EPSP evoked by B20 in B16 (t = 9.93, $P < 0.05$, Fig. 3*A1*). Exogenous baclofen produced a comparable reduction (47.2 ± 7.1% decrease, n = 4) of the B20-to-B16 EPSP (t = 8.66, $P <$ 0.05, Fig. 3*A2*). In contrast, application of GABA enhanced (102.5 ± 8.2% increase; n = 5) the amplitude of the EPSP evoked by B20 in B8 (t = 12.70, $P < 0.05$, Fig. 3*B1*). Baclofen (1 mM) also augmented (56.4 ± 7.5% increase; n = 4) the B20-to-B8 EPSP (t = 14.66, $P < 0.05$, Fig. 3*B2*).

In sum, our pharmacological tests do not substantiate the participation of GABA in the mediation of the fast EPSPs in B16 and B8 that originate from B20. They do, however, indicate the presence of $GABA_B$-like receptors that could influence the strength of these EPSPs in a coordinated and target-specific fashion (see also Díaz-Ríos et al. 2002, 2003).

RAPID SYNAPTIC SIGNALING OF B20: TESTING THE ROLE OF DOPAMINE. Dopamine promotes motor programs in the feeding systems of several mollusks (Kyriakides and McCrohan 1989; Quinlan et al. 1997; Trimble and Barker 1984; Wieland and Gelperin 1983), including *Aplysia* (Kabotyanski et al. 2000; Teyke et al. 1993). Previous experiments suggested participation of dopamine in the generation of buccal motor programs elicited by interneuron B20 but left unresolved its involvement in its fast synaptic signaling (Teyke et al. 1993). As our findings did not support the role of GABA in this capacity (see preceding text), experiments were conducted to test the participation of dopamine in the mediation of the rapid EPSPs from B20 to B16 and B8.

Exposure to exogenous DA produced depolarizations of both motor neurons. A brief (0.5 s) pulsed application to the soma region of B16 elicited complex responses, consisting of a rapid depolarization (time to peak, ~100 ms), an irregular secondary phase, and a smaller sustained late depolarization (Fig. 4*A; n* = 4). When the membrane potential of B16 was set to hyperpolarized levels prior to DA application, the three components of its DA response were all increased in amplitude (Fig. 4, *A* and *C*).

Unlike the complex responses observed in B16, pulsed application of DA to the soma and initial segment of B8 produced a monophasic response that was slow in onset (time to peak ~4 s) and long-lasting (Fig. 4*B; n* = 5) (see also Due et al. 2004). When B8 was at its resting potential, the response

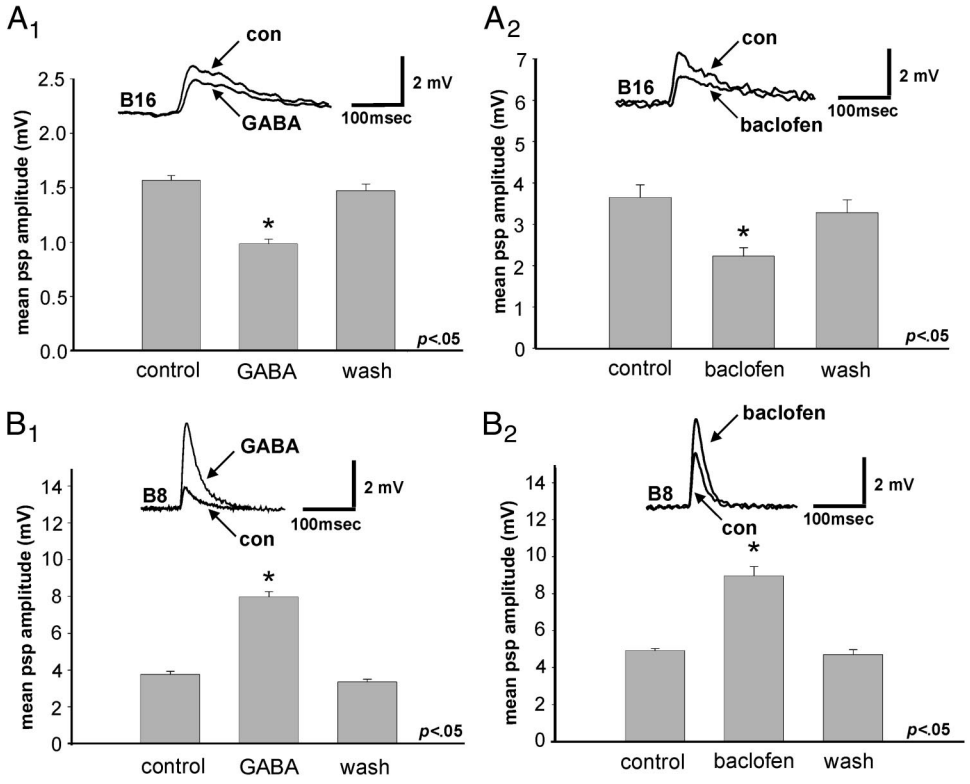

FIG. 3. Differential GABAergic actions on synaptic signaling from B20 to B16 and B8. *A*: application of GABA (*A1*) or baclofen (*A2*) reduced synaptic transmission from B20 to B16. *Insets*: representative excitatory postsynaptic potentials recorded under control conditions (con) and in the presence of 1 mM GABA (*A1*, GABA) and 1 mM baclofen (*A2*, baclofen). Graphs summarize data from experiments such as shown in the *insets*. *A1*: exogenous GABA produced a significant ($t = 9.93$, $P < 0.05$) decrease (38.7 ± 5.1% reduction; $n = 5$) in the amplitude of the EPSP produced by B20 in B16. *A2*: exogenous baclofen produced a significant ($t = 8.66$, $P < 0.05$) decrease (47.2 ± 7.1% reduction, $n = 4$) in the EPSP produced by B20 in B16. *B*: exogenous application of GABA (*B1*) or baclofen (*B2*) augmented synaptic transmission from B20 to B8. *Insets*: representative EPSPs recorded under control conditions (con) and in the presence of 1 mM GABA (*B1*, GABA) and 1 mM baclofen (*B2*, baclofen). Bar graphs summarize data from experiments such as shown in the *insets*. *B1*: exogenous GABA produced a significant ($t = 12.70$, $P < 0.05$) augmentation (102.5 ± 8.2% increase; $n = 5$) in the amplitude of the EPSP produced by B20 in B8. *B2*: exogenous baclofen produced a significant ($t = 14.66$, $P < 0.05$) augmentation (56.4 ± 7.5% increase; $n = 4$) in the EPSP produced by B20 in B8.

to DA could exceed threshold, producing repetitive impulses (Fig. 4*B, top*). In contrast to the responses evoked in B16, the DA response in B8 was decreased when the membrane potential was set to hyperpolarized levels (Fig. 4, *B* and *C; n* = 4).

The possible role of dopamine in mediating synaptic signaling from B20 was further tested by examining its effect on the input resistance and excitability of B16 and B8 (Fig. 5). As these protocols required bath application of DA (see preceding

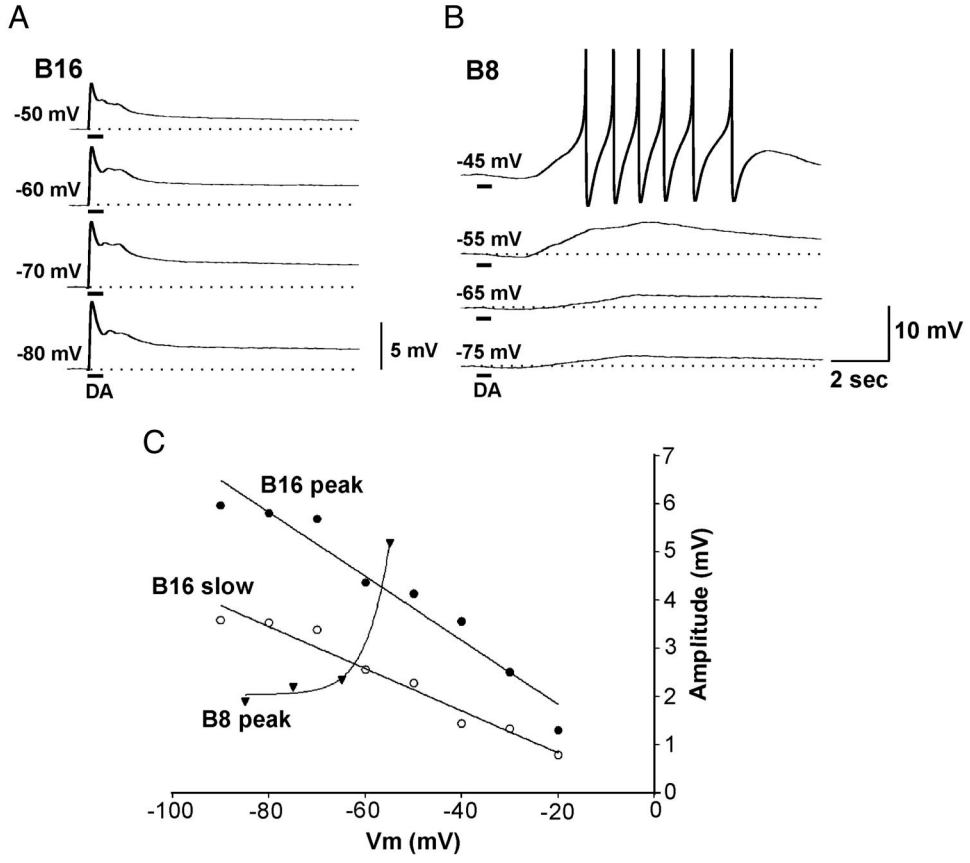

FIG. 4. Responses of B8 and B16 to focal application of dopamine (DA). DA (1 mM) was ejected from a micropipette directed toward the soma of each motor neuron. *A*: responses in B16 were rapid in onset and consisted of a complex early component followed by a sustained depolarization. All components of the B16 response were increased in amplitude when its membrane potential was preset at hyperpolarized levels. *B*: the response in B8 consisted of a slow excitatory depolarization that diminished when the B8 membrane was preset at hyperpolarized levels. *C*: plot of the DA response amplitude as a function of postsynaptic membrane potential in B16 and B8. In B16, the rapid (●) and delayed (○) responses were similarly influenced by membrane potential (extrapolated × intercepts = +7 and +3 mV, respectively). In contrast, the response of B8 to DA (▼), was diminished at hyperpolarized postsynaptic membrane potentials in a nonlinear fashion.

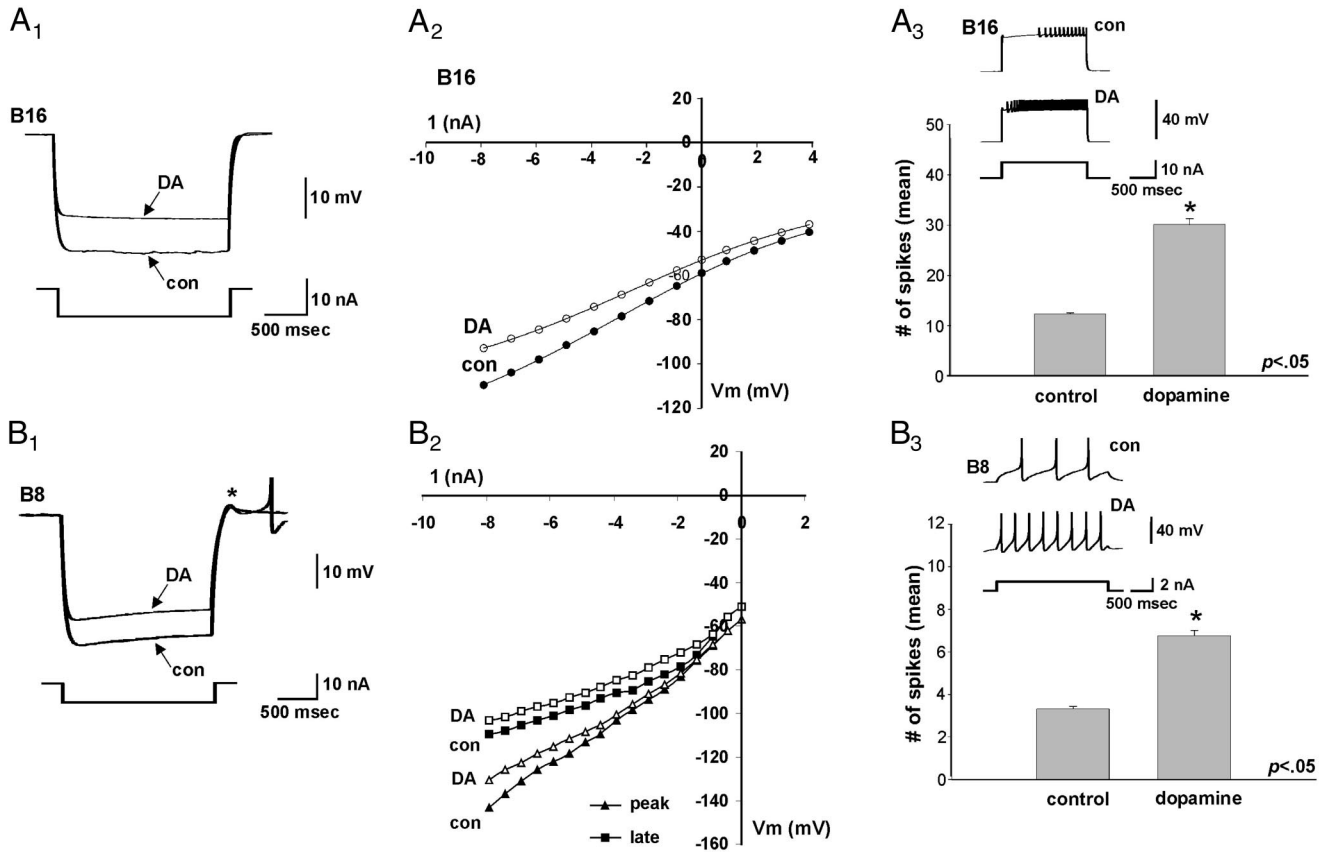

FIG. 5. Dopamine produces decreased input resistance and increased excitatory responsiveness of both follower cells of interneuron B20. *A1*: B16, under control conditions a hyperpolarizing current pulse (8 nA, 2 s; *bottom record*) produced a voltage deflection of ~35 mV (labeled con). In the presence of dopamine (1 mM), an identical current pulse (*bottom record,* superimposed on control pulse) produced a smaller hyperpolarizing deflection (labeled DA). The DA record was offset to compensate for the DA-induced depolarization (~5 mV; compare *y* intercepts in *A2*) to enable direct comparison of the 2 recordings. Note that DA did not produce spontaneous firing in B16. *A2*: current-voltage relationship generated from an experiment such as *A1*. *A3*: dopamine increased the excitatory responses to depolarizing current in B16. *Inset*: under control conditions the amplitude of a 2-s depolarizing current pulse was adjusted (8 nA, *bottom record*) to evoke 10–15 impulses in B16 (con, $V_m = -55$ mV). In the presence of dopamine (1 mM), an identical pulse (*bottom record*) evoked a greater number of impulses (30 in the record shown, DA, $V_m = -51$ mV). Graph: in 4 experiments such as shown in *inset,* the number of impulses evoked by depolarizing pulses was significantly ($t = 14.60$; $P < 0.05$) increased from 12.2 ± 2.4 to 31.6 ± 6.3 ($n = 7$) in the presence of dopamine [error bars (SE) drawn in 1 direction only]. *B1*: B8, under control conditions a 2-s hyperpolarizing current pulse (8 nA, *bottom record*) was injected via an independent electrode into B8, producing a voltage deflection of ~55 mV (con). In the presence of dopamine (1 mM), an identical current pulse (*bottom record,* superimposed on control pulse) produced a smaller hyperpolarizing deflection (DA). The dopamine recording was offset to compensate for the depolarization (~5 mV) produced by DA (see also *B2*). The control voltage deflection exhibited a prominent time-dependent sag and a transient postinhibitory depolarization (*). In the presence of dopamine, the pulse was followed by short-latency postinhibitory firing. *B2*: current-voltage relationship generated from an experiment such as *B1*. When measured either at the peak of hyperpolarizing voltage deflections (con, ▲), or immediately prior to its termination (con, ■), dopamine produced a decrease in the input resistance (DA, △ and □). *B3*: dopamine increased the excitatory response to depolarizing current pulses in B8. *Inset*: under control conditions, the amplitude of a 2-s depolarizing current pulse was adjusted (1.5 nA, *bottom record*) to evoke 2–4 impulses in B8 (con, *top trace;* $V_m = -46$ mV). In the presence of dopamine (1 mM), an identical pulse (DA) evoked a greater number of impulses (8 in the record shown, *middle trace;* $V_m = -41$ mV). Graph: data from four experiments such as *B1,* revealed a significant ($t = 14.26$, $P < 0.05$) increase from 3.6 ± 1.5 to 6.8 ± 1.2 ($n = 6$) in the number of impulses evoked in B8 in the presence of dopamine.

text), observed effects are likely to reflect the summed and weighted contributions of all dopamine receptors with electrical influence on the soma. Moreover, the slow kinetics of this mode of agonist application would be expected to diminish the contribution of those DA receptors that display desensitization (see Ascher 1972). Finally, bath application of DA could produce release of other neuroactive substances from sources within the ganglion. These considerations notwithstanding, DA produced decreases in the membrane input resistance of B16 (Fig. 5*A, 1* and *2; n* = 4) and B8 (Fig. 5*B, 1* and *2; n* = 4).

Due to the presence of a sag in the responses of B8 to hyperpolarizing pulses (Fig. 2*C2*), two voltage measurements were performed for each current pulse. The first was obtained at the peak amplitude of the voltage deflection and the second value was measured immediately prior to termination of the pulse. DA produced reductions of input resistance determined with both measures (Fig. 5*B2*). No consistent differences were observed between the effects at the two time points, suggesting that DA does not produce substantial modulation of the current(s) responsible for the sag itself (see Harris-Warrick et al. 1995). This observation is consistent with attributing the principal action of exogenous DA to its activation of ligand-gated synaptic receptors.

If the depolarizations of B16 and B8 produced by dopamine reflect its activation of the postsynaptic receptors that respond to the excitatory neurotransmitter of B20, then these depolarizations could be expected to have an excitatory influence on these cells. This was tested by adjusting depo-

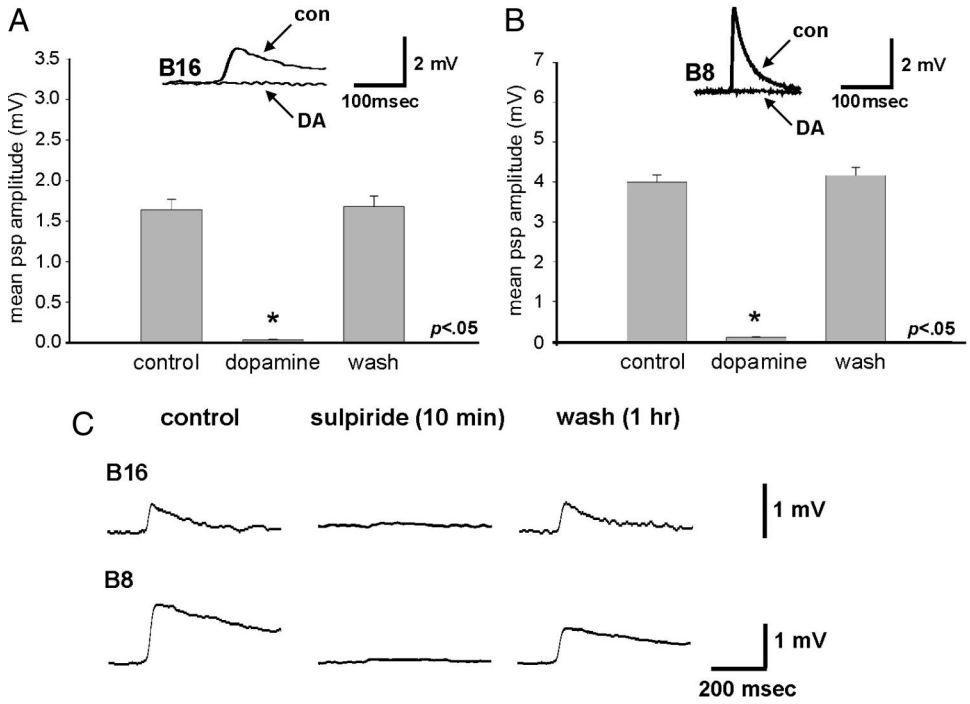

FIG. 6. Dopaminergic pharmacology of B20 fast synaptic signaling. *A*: exogenous application of dopamine occludes EPSPs from B20 to B16. *Inset*: under control conditions firing B20 produced an EPSP of ~1.5 mV (con) in the ipsilateral B16. When dopamine (1 mM) was introduced to the bathing solution, no PSP was detected in B16 when B20 was fired (DA). Records are aligned to facilitate comparison and do not reflect a 4-mV membrane depolarization of B16 that was produced by DA. Graph summarizes data from 3 experiments such as shown in the *inset.* Exogenous DA produced a significant ($t = 12.13$; $P < 0.05$) reduction (98.9 ± 3.2% decrease) of the EPSP produced by B20 in B16. *B*: exogenous application of dopamine occludes EPSPs from B20 to B8. *Inset*: under control conditions firing B20 produced an EPSP of ~3.5 mV (con) in the ipsilateral B8. When dopamine (1 mM) was introduced to the bathing solution, no PSP was detected in B8 when B20 was fired (DA). Records are aligned to facilitate comparison and do not reflect a 5-mV depolarization of B8 that was produced by DA. Graph summarizes data from 3 experiments such as shown in the *inset.* Exogenous DA produced a significant ($t = 39.2$; $P < 0.05$) reduction (97.6 ± 2.8% decrease) of the EPSP produced by B20 in B8. *C*: sulpiride blockade of synaptic signaling from B20 to its 2 direct postsynaptic followers. Simultaneous recordings from B16 (resting $V_m = -52$ mV) and B8 ($V_m = -47$ mV; control) show EPSPs resulting from firing of B20 (not shown). In the presence of the D2 antagonist sulpiride (1 mM, 10 min), the EPSPs in both B8 and B16 were nearly eliminated. The sulpiride blockade was reversed after 1-h wash with normal ASW (wash).

larizing current pulses to evoke a moderate number of impulses in each motor neuron (Fig. 5, *A3* and *B3,* top traces) prior to DA application. In both cases, identical current pulses produced a greater number of impulses in the presence of dopamine, indicative of an excitatory dopaminergic influence on B16 and B8 (Fig. 5, *A3* and *B3, bottom traces*). In B16, the number of evoked impulses was increased from 12.2 ± 2.4 to 31.6 ± 6.3 ($n = 7$) by DA ($t = 14.60$; $P < 0.05$; Fig. 5*A3*). In B8, an increase from 3.6 ± 1.5 to 6.8 ± 1.2 ($n = 6$) impulses was observed ($t = 14.26$, $P < 0.05$; Fig. 5*B3*). As these tests were made using the bath mode of DA application, they were subject to the same assumptions and limitations that applied to tests on input resistance (preceding text). Comparable increases in excitatory responsiveness were observed, however, when brief puffed applications of DA were applied to the somata of B16 and B8 and followed immediately by test current pulses (not shown).

If the effects of DA on B16 and B8 reflect its activation of the synaptic receptors that mediate fast signaling from B20, then application of a high concentration of exogenous DA could be expected to attenuate or occlude these signals. When B20 was fired in the presence of DA (1 mM), its EPSP was essentially eliminated in B16 (98.9 ± 3.2% reduction; $n = 3$) and B8 (97.6 ± 2.8% reduction; $n = 3$; Fig. 6, *A* and *B*).

Several receptor antagonists were tested to further clarify the contribution of DA to synaptic signaling by B20. Two ergot alkaloids that act as antagonists of DA receptors in mollusks, ergonovine (Ascher 1972; Wieland and Gelperin 1983) and methylergonovine (Nargeot et al. 1999), were ineffective in blocking B20-evoked EPSPs (see also Teyke et al. 1993). Also without effect were the D1 receptor antagonist, butaclamol, the D2 antagonist, raclopride, and the typical and atypical neuroleptics fluphenazine, chlorpromazine, haloperidol, and clozapine (all tested ≤1 mM concentrations). Effects were observed with the D2 receptor antagonist sulpiride, which has been shown to block dopaminergic synapses and responses to dopamine in mollusks, including *Aplysia* (Due et al. 2004; Magoski et al. 1995; Quinlan et al. 1997). Application of sulpiride (1 mM, 10 min) produced a blockade of the EPSPs recorded in both B16 (reduction of 98.1 ± 2.2%, $n = 3$) and B8 (reduction of 98.8 ± 1.8%, $n = 3$; Fig. 6*C*). Block of the EPSPs in the two cells followed identical time courses. They were fully accomplished within 10 min of exposure and both required prolonged wash with normal ASW to achieve reversal (Fig. 6*C, right panels*).

Together, the observed dopaminergic actions on the membrane potential, input resistance, and synaptic responses in B16 and B8, coupled with the block of these PSPs by sulpiride, support the role of DA as the principal neurotransmitter me-

diating rapid EPSP signaling from B20 to these two buccal motor neurons.

Rapid excitatory signaling from B65 to B8 and B4/5

B65 is a moderately sized buccal interneuron that is located between the origins of the esophageal nerve and buccal nerve 1 near the caudal surface of each hemiganglion (Fig. 7*A*) (Díaz-Ríos et al. 2002; Kabotyanski et al. 1998). As B65 shares the GABA-immunoreactive/catecholamine phenotype with B20 (Díaz-Ríos et al. 2002; Due et al. 2004; Jing et al. 2003), its direct EPSPs to identified follower neurons provided an opportunity to explore the generality of the preceding observations implicating dopamine as the mediator of B20's fast synaptic signaling.

Unlike B20, the axonal projections of B65 are confined to the buccal ganglion, where its strongest synaptic connections occur in the contralateral hemiganglion (Kabotyanski et al. 1998). The somata of two direct synaptic followers, B8 (see also Fig. 1) and B4/5, were readily accessible from the caudal surface of the contralateral hemiganglion, due to their large size and marginal positions (Fig. 7*A*). B4/5 (actually 1 of a pair of cells that are not distinguishable by currently known physiological criteria) is a multifunctional neuron that is located in the most ventromedial edge of each hemiganglion (Fig. 7*A*). It projects an axon to the ipsilateral I3 muscle and is thought to have sensory, motor, and interneuronal functions (Church and Lloyd 1994; Gardner 1971; Jahan-Parwar et al. 1983; Rosen et al. 1982; Sossin et al. 1987). Simultaneous recording from B8 and B4/5 while firing B65 in high-divalent ASW (Fig. 7*B*) confirmed that each follower neuron received a fast PSP with a brief latency (4–5 ms) and rapid time to peak (~35 ms). In agreement with previous observations (Kabotyanski et al. 1998), the amplitude of the PSP recorded in the contralateral B4/5 cell body (5.25 ± 0.28 mV, $n = 5$) was larger than that

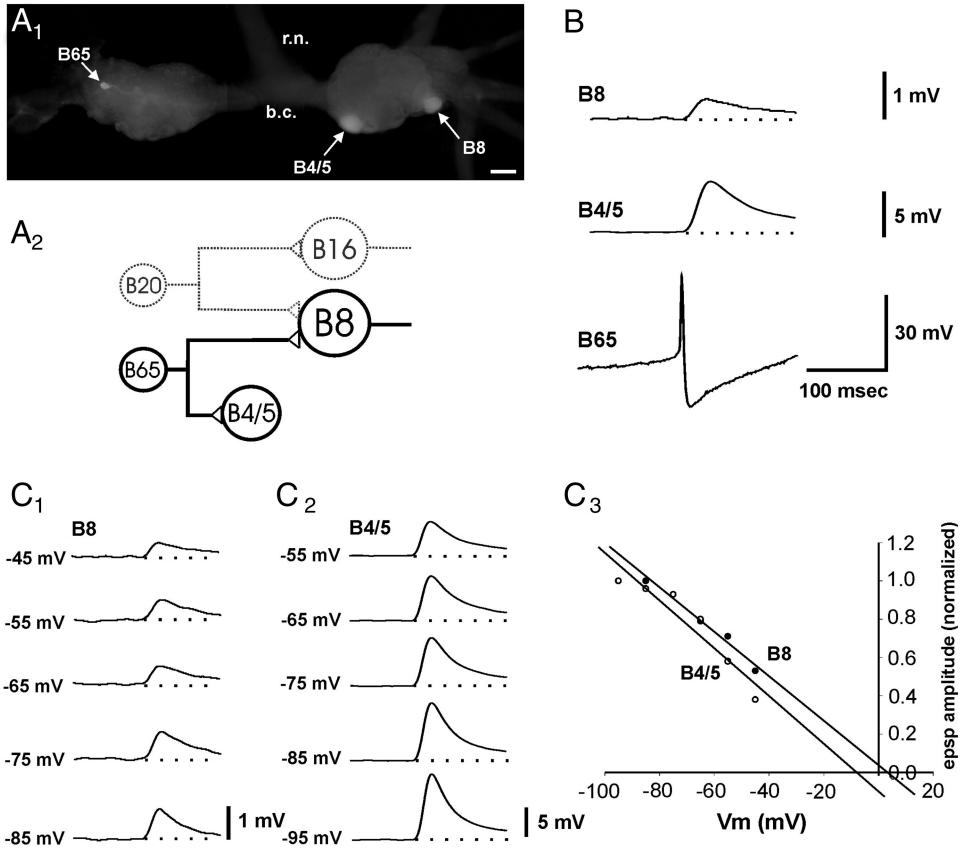

FIG. 7. Divergent signaling from B65 to B8 and B4/5. *A1*: neurobiotin fills of the GABA-immunoreactive and catecholamine-containing interneuron B65 and its 2 follower motor neurons B8 and B4/5. Caudal surface of the paired buccal ganglia. B65 is a moderately sized buccal interneuron located in the lateral region of the ganglion close at the confluence of the esophageal n. and buccal nerve 1. It projects a fiber to the contralateral hemiganglion via the buccal commissure (b.c.). B65 produces EPSPs in motor neurons B8 and B4/5 in both hemiganglia. As indicated by this image, postsynaptic responses were typically studied in contralateral follower neurons, as they tended to exhibit larger PSPs than their counterparts in the ipsilateral hemiganglion. Calibration bar = 100 μm. *A2*: B65 produces EPSPs in the radula closer motor neuron B8, where it converges with signaling from B20 (see Fig. 1). B4/5 is a multifunction neuron that produces diverse synaptic actions including a prominent inhibition of several buccal motor neurons. Diagram depicts direct divergent excitatory synaptic signaling from B65 to B8 and B4/5. Signaling is unidirectional in both cases, i.e., B65 does not receive direct synaptic input from either B8 or B4/5. The schematic from Fig. 1 is reproduced in gray to emphasize convergent signaling from B20 and B65 to B8. *B*: EPSPs evoked in B8 (*top;* $V_m = -44$ mV) and B4/5 (*middle;* $V_m = -56$ mV) by an impulse produced in B65 (resting $V_m = -46$ mV). Experiment was conducted in a raised divalent ASW solution that attenuates polysynaptic signaling. *C*: potential dependence of fast synaptic signaling from B65 to B8 and B4/5. *C, 1* and *2*: follower neurons were hyperpolarized to indicated levels using sustained current from a 2nd microelectrode. B65 was fired by passing depolarizing current through the recording electrode. In both B8 and B4/5, the fast EPSP increased in amplitude when the membrane potential of the postsynaptic cell was held at hyperpolarized levels. *C3*: plot of the fast EPSP amplitude as a function of postsynaptic membrane potential in B4/5 (○) and B8 (●). When amplitudes were normalized by setting the maximal EPSP = 1.0, the extrapolated *x* intercept was +5 mV for B8 and −7 mV for B4/5.

recorded in the contralateral B8 (0.55 ± 0.31 mV, $n = 5$). Both EPSPs were increased in amplitude when the membrane potentials of the postsynaptic cells were adjusted to more hyperpolarized levels (Fig. 7*C, 1* and *2*). Reversal of the B4/5 EPSP was in the range of −10 to –5 mV ($n = 3$), while that in B8 was estimated to occur at +5 to +10 mV ($n = 3$; Fig. 7*C3*).

RAPID SYNAPTIC SIGNALING OF B65: TESTING THE ROLE OF DOPAMINE. In view of the observations indicating the role of DA in mediation of the B20-evoked EPSPs (preceding text), the dopaminergic pharmacology of the EPSPs originating from B65 was examined initially. The EPSP produced in B8 was virtually occluded in the presence of 1 mM DA (98.7 ± 1.3% reduction, $n = 3$; Fig. 8*A*) and blocked by sulpiride (95.4 ± 3.5% reduction, $n = 3$; Fig. 8*C*) (see also Due et al. 2004). Surprisingly, however, the amplitude of the EPSP produced by B65 in the contralateral B4/5 was only partially reduced by 1 mM DA (37.2 ± 7.3% decrease, $n = 3$; Fig. 8*B*). Moreover, the antagonism of the B65-to-B4/5 synapse by sulpiride differed from that in B8 (Fig. 8*C*). After 10 min of sulpiride (1 mM) exposure, the B65-to-B8 EPSP was completely blocked (see also Due et al. 2004), but the EPSP in B4/5 was only partially reduced (48.4 ± 8.5% decrease, $n = 3$; Fig. 8*C,* compare *top* and *bottom*).

The observed differences in the synaptic pharmacology in the divergent signaling of B65 prompted us to examine the effects of dopamine on B4/5. Unlike the excitatory effects of DA on B16 and B8 (Fig. 4), focal or bath application of DA to B4/5 produced a small, but sustained, hyperpolarization of B4/5 (Fig. 9*A*) (see also Teyke et al. 1993) that was accompanied by a small decrease in input resistance (Fig. 9*B; n* = 4). When assessed with depolarizing intrasomatic current pulses, the DA-induced hyperpolarization was found to have an inhibitory influence on B4/5, reducing the number of evoked impulses from a control level of 14.7 ± 3.2 to 1.6 ± 1.2, $n = 5$; $t = 29.49$, $P < 0.05$, Fig. 9*C*) (see also Kabotyanski et al. 2000). With each of these tests, therefore the responses of B4/5 to application of DA differed qualitatively from those observed on B16 and B8 (see Figs. 4 and 5).

In sum, these experiments indicate that the rapid excitatory signaling from B65 to B8 is mediated by dopamine receptors that are similar or identical to those that transduce signaling from B20 (see also Due et al. 2004). However, the lower efficacy of DA and sulpiride in reducing the B65-to-B4/5

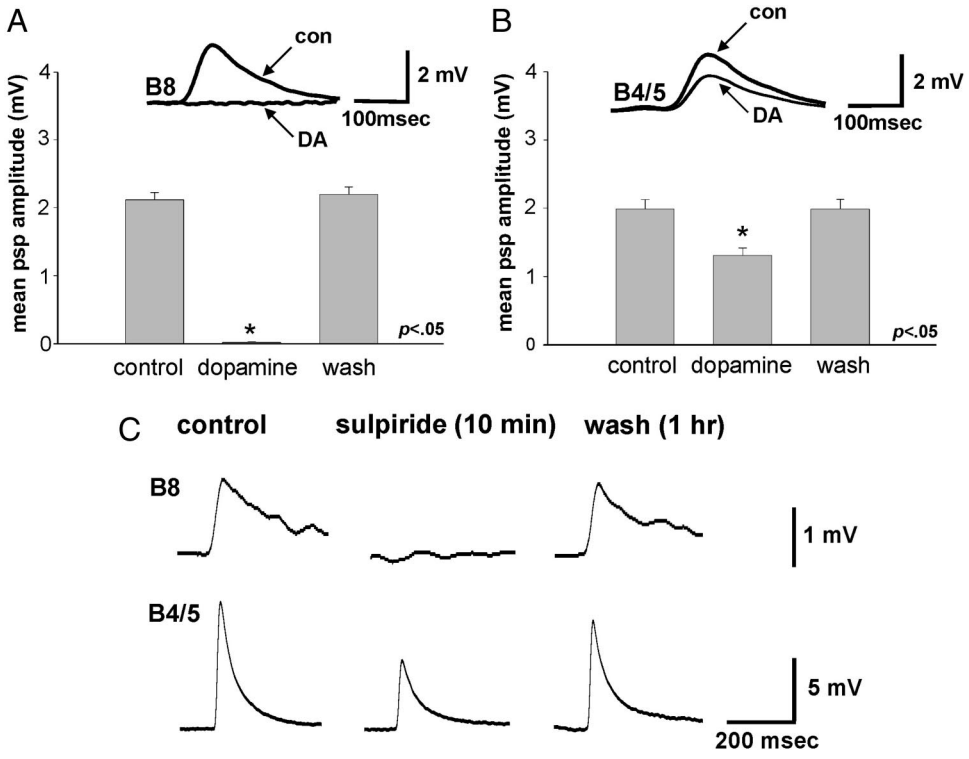

FIG. 8. Differential dopaminergic actions on synaptic signaling from B65 to 2 distinct follower cells. *A*: exogenous application of dopamine occludes synaptic transmission from B65 to B8. A single impulse was evoked by passing intracellular current into B65 (not shown). Under control conditions, the EPSP produced by B65 in B8 was ~2 mV in amplitude (record labeled con in *inset*). In the presence of dopamine (1 mM), the EPSP evoked by B20 was not detectable (record labeled DA; 5-mV DA-induced depolarization of B8 offset to facilitate comparison of traces). Graph summarizes data from 3 experiments such as shown in the inset. Exogenous dopamine produced significant ($t = 22.61$; $P < 0.05$) occlusion of the EPSP produced by B65 in B8 (98.7 ± 1.3% reduction; $n = 3$). *B*: application of DA reduces, but does not occlude, synaptic transmission from B65 to B4/5. Under control conditions, the EPSP produced by B20 in B16 was ~2 mV in amplitude (record labeled con in *inset*). In the presence of DA (1 mM), the EPSP evoked by B65 was reduced by ~35% (record labeled *DA*) Note: DA-induced hyperpolarization (2 mV) of B4/5 was offset to facilitate comparison of EPSPs. Graph summarizes data from 3 experiments such as shown in the *inset.* Exogenous DA produced a significant decrease (34.5 ± 5.8% reduction; $t = 12.27$, $P < 0.05$) in the amplitude of the EPSP produced by B65 in B4/5. *C*: differential actions of sulpiride on synaptic signaling from B65 to 2 distinct follower cells. Recordings were obtained simultaneously from B8 and B4/5 while impulses were evoked in B65. Under control conditions (control), B65 produced an EPSP >1 mV in B8 (resting $V_m = -48$ mV) and >9 mV in B4/5 (resting $V_m = -55$ mV). Application of sulpiride for 10 min produced complete block of synaptic transmission from B65 to B8 but only about a 50% reduction of the EPSP produced in B4/5 (sulpiride). These effects were largely reversed after 1 h wash with normal ASW (wash).

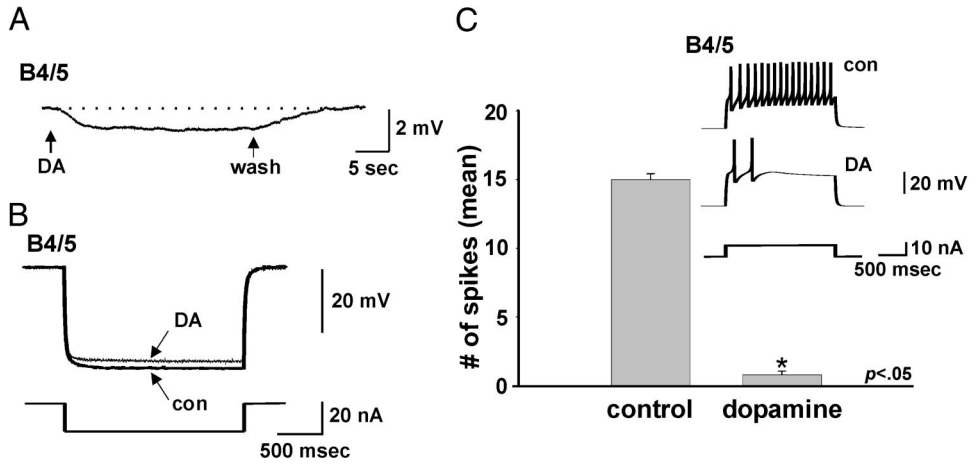

FIG. 9. Effects of exogenous application of DA on B4/5. *A*: effect of DA on the B4/5 membrane potential. DA (1 mM) was ejected from a pipette positioned near the soma/initial segment of B4/5 ($V_m = -56$ mV) while superfusion of the ganglion was briefly interrupted. It produced a persistent hyperpolarization (~1 mV) that reversed on resumption of the superfusion (wash). *B*: effects of dopamine on the B4/5 input resistance. Under control conditions a current pulse of 16 nA (*bottom*) produced a voltage deflection of ~35 mV (labeled con). In the presence of dopamine (1 mM), an identical current pulse (*bottom*, superimposed on control pulse) produced a hyperpolarizing deflection (labeled DA) that did not differ appreciably from the control pulse. Note that hyperpolarizing pulses did not reveal time-dependent responses in B4/5, either in the presence or absence of DA (compare with Fig. 6*B1*). *C*: dopamine decreases the excitability of B4/5. *Inset*: under control conditions, the amplitude of a 2-s depolarizing current pulse (*bottom*) was adjusted (8 nA in the example shown) to evoke 12–18 impulses in B4/5 (16 in the record shown, con; $V_m = -56$ mV). In the presence of dopamine (1 mM, bath application), an identical pulse (*bottom*, superimposed on control current pulse) produced 2 impulses (*middle*; $V_m = -57$ mV). Grouped data from four experiments in which the mean number of impulses evoked by ten pulses such as in *C1* was compared with the mean number evoked by 10 identical pulses in the presence of dopamine. DA produced a significant ($t = 29.49$, $P < 0.05$), denoted by *) decrease (from 14.7 ± 3.2 to 1.6 ± 1.2, $n = 5$) in the number of evoked impulses.

EPSPs (Fig. 8), coupled with the predominantly inhibitory actions of exogenous dopamine on B4/5 (Fig. 9), leave unresolved the identity of the mediator and receptor type responsible for transmission of these signals.

RAPID SIGNALING FROM B65 TO B4/5: TESTING THE ROLE OF GABA. The inconclusive results of experiments testing the role of DA in the mediation of fast EPSPs from B65 to B4/5 (preceding text) led us to examine the actions of GABA on these signals. Focal application of GABA to the cell body region of B4/5, as well as bath application to the entire cell, did not produce detectable changes in the somatic membrane potential (Fig. 10*A*). In agreement with this observation, no consistent effects were produced by bath applied GABA on the input resistance of B4/5 (Fig. 10*B*).

No effects were observed on the rapid B65-to-B4/5 EPSP after application of agents that block GABA-mediated IPSPs in *Aplysia* (picrotoxin and bicucculine) (Jing et al. 2003), a $GABA_B$ antagonist (saclofen), or compounds that block GABAergic EPSPs in the feeding system of *Clione limacina* (piperidine-4S and 5-aminovaleric acid) (Norekian 1999) (all tested ≤1 mM). Moreover, the GABA uptake inhibitor nipecotic acid did not influence B65-to-B4/5 signaling. Finally, application of GABA itself did not produce desensitization/occlusion of this EPSP. GABA did effect a partial (36.7 ± 5.4%, $n = 3$) decrease of the EPSP produced by B65 in B4/5 ($t = 8.66$, $P < 0.05$; Fig. 10*C1*). As observed with GABAergic actions on signaling originating from B20 (Fig. 4, preceding text), the effects of GABA on the B65-to-B4/5 EPSP were mimicked by the $GABA_B$ agonist baclofen (41.2 ± 6.8% reduction, $n = 4$; Fig. 10*C2*).

In sum, the absence of GABAergic actions on the B4/5 membrane potential and input resistance, coupled with the inefficacy of GABAergic pharmacological manipulations to influence synaptic signals originating from B65, do not support the participation of GABA in the mediation of the fast EPSP between these cells. As was observed with the signaling of B20 to its followers, GABA was found to be capable of modifying these signals via the activation of $GABA_B$-like receptors.

DISCUSSION

This study explored the contributions GABA and DA to the "conventional" or "fast" synaptic signaling of two influential interneurons in which they have been proposed to be colocalized (Díaz-Ríos et al. 2002). Our results indicate that dopamine acts as the primary neurotransmitter mediating the rapid EPSPs from B20 to two of its direct followers, the radula closer motor neurons B16 and B8 (Fig. 11). Recent findings have shown that fast excitatory signaling from B65 to neurons that participate in radula protraction (interneurons B31/32 and motor neuron B61) is also mediated by dopamine (Due et al. 2004). In agreement with observations reported in that study, we observed that convergent fast EPSPs produced in B8 by B65 are mediated by dopamine acting on receptors that are similar, or identical, to those activated by B20 (Fig. 11) (see Due et al. 2004).

These data do not support the participation of GABA in the mediation of rapid excitatory signaling from B20 or B65. Tests for GABAergic responses did, however, disclose its ability to modify these EPSPs and provided evidence that such modulation could be achieved by activation of $GABA_B$-like receptors. In the case of B8, such $GABA_B$-like receptors were found to be present on the motor neuron itself, but our experiments do not enable us to ascribe the actions of GABA to pre- versus postsynaptic loci (Fig. 11). Moreover, the source of GABAergic regulation, and in particular it's possible origin from the interneurons B20 and B65 themselves, was not addressed in this study.

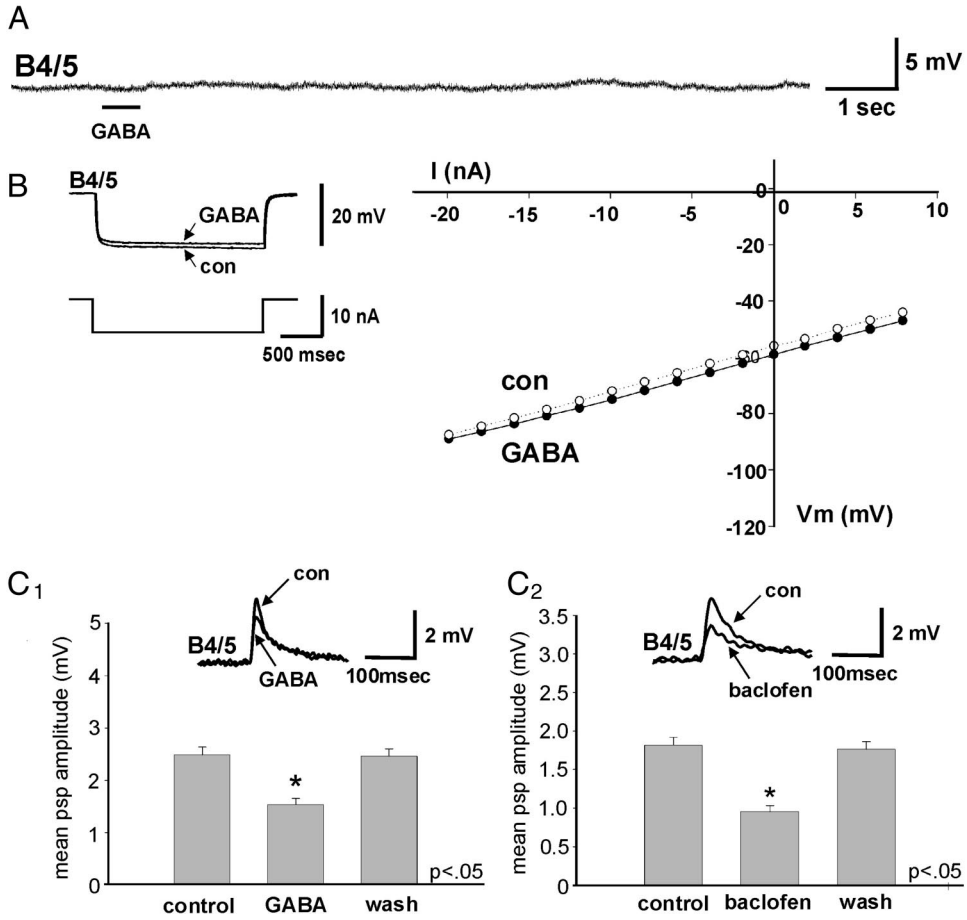

FIG. 10. Actions of exogenous GABA on B4/5. *A*: puffed application of GABA (1 mM) to the soma/initial segment region of B4/5 did not produce detectable effects on the resting membrane potential ($V_m = -56$ mV). *B*: bath application of GABA did not produce effects on the input resistance of B4/5 as assessed with hyperpolarizing (8 nA, 2 s in *inset*) and depolarizing current pulses. *C*: GABAergic reduction in amplitude of the B65-to-B4/5 EPSP. *C1*: application of GABA (1 mM) reduced synaptic transmission from B65 to B4/5. *Inset*: under control conditions, the EPSP produced by B65 in B4/5 was ~2.5 mV in amplitude (trace labeled con). In the presence of GABA (1 mM) the EPSP evoked by B65 was reduced by ~30% (trace labeled GABA). Graph summarizes data from 3 experiments such as shown in the *inset*. Application of GABA produced a partial (36.7 ± 5.4% reduction, $n = 3$), but statistically significant ($t = 8.66$, $P < 0.05$) decrease of the EPSP produced by B65 in B4/5. *C2*: application of baclofen (1 mM) reduced synaptic transmission from B65 to B4/5. Under control conditions, the EPSP produced by B65 in B4/5 was ~2.2 mV in amplitude (record labeled con in *inset*). In the presence of baclofen (1 mM) the EPSP evoked by B65 was reduced by ~45% (record labeled baclofen). Graph summarizes data from 3 experiments such as shown in the *inset*. Application of baclofen produced a partial (41.2 ± 6.8% reduction, $n = 4$), but statistically significant ($t = 14.76$, $P < 0.05$) decrease of the EPSP produced by B65 in B4/5.

Dopaminergic signals and receptors in molluscan feeding

The pharmacological observations reported in this study support the role of dopamine as the principal neurotransmitter mediating rapid EPSP signaling from two buccal interneurons to their direct follower motor neurons. While dopaminergic signaling in the vertebrate nervous system is commonly mediated by G-protein-coupled receptors that act via second messengers (Civelli et al. 1993; Greengard 2001), considerable evidence supports the presence of ligand-gated dopaminergic synaptic signaling in mollusks (Due et al. 2004; Green et al. 1996; Magoski et al. 1995; Quinlan et al. 1997).

The occlusion of synaptic signals in the presence of high concentrations of DA observed in this study could be produced by one, or a combination, of several mechanisms including: activation of nonsynaptic receptors that depolarize the postsynaptic neuron to the reversal potential of the B20-evoked PSPs, shunting of synaptic currents, desensitization of the synaptic receptors, or complete occupancy of synaptic receptors preventing their further activation by the synaptic transmitter. Nonspecific depolarization is unlikely to be responsible because elimination of B20-evoked PSPs by DA was also observed when the follower motor neurons were set at membrane potentials 40 mV more hyperpolarized than rest (not shown). Some shunting of synaptic current is likely to occur, but the moderate decreases in motor neuron input resistance produced by DA (Fig. 5, *A* and *B*) would not be expected to produce such complete elimination of PSPs. A contribution from desensitization is suggested by the observation that the depolarizations produced by sustained application of DA were modest in comparison to the apparent reversal potential of its predominant responses (Fig. 4) (see also Ascher 1972) and the reversal potential of the B20-evoked EPSPs (Fig. 1*C*). However, once the DA-induced depolarizations were achieved, no decay was observed in their amplitude during prolonged (≤30 min) bath application of the agonist. Thus although the relative contributions of desensitization and occlusion remain unresolved, these results support the proposal that exogenous DA attenuates B20- and B65-evoked PSPs in B8 and B16 by acting as an agonist at the receptors that mediate these signals.

Exposure to comparatively low concentrations (1×10^{-6} M to 1×10^{-5} M) of dopamine is known to promote coordinated BMPs in several molluscan feeding systems (Kabotyanski et al. 2000; Kyriakides and McCrohan 1989; Quinlan et al. 1997; Trimble and Barker 1984; Wieland and Gelperin 1983). Thus in addition to the receptors mediating fast synaptic signaling originating from B20 and B65, additional and more sensitive dopaminergic receptor types are likely to be present in the buccal system. The presence of such receptors, and their probable role in configuring the buccal circuit, is supported by the demonstrated ability of the DA antagonist ergonovine (10^{-8}–10^{-7} M) to block BMPs produced by firing B20 without affecting the B20-to-B8 EPSP (Teyke et al. 1993; our observations). Also, low concentrations methylergonovine block the enhancement of motor patterns produced by stimulation of the esophageal nerve in an analogue of operant conditioning (Nargeot et al. 1999). Interestingly, there is evidence that additional

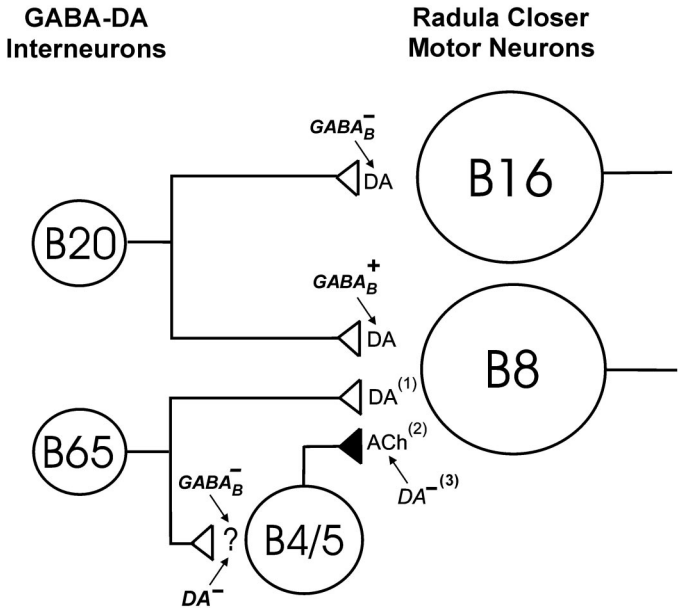

FIG. 11. Schematic summary of observations. Diagram depicts direct excitatory synaptic transmission (◁) from B20 and B65, 2 interneurons in which dopamine and GABA are colocalized. The configuration of this circuit includes divergent and convergent signaling to radula closer motor neurons B16 and B8. In addition, the multifunctional interneuron B4/5 receives divergent synaptic excitation from B65 and, in turn, inhibits B8 (◀). At each terminal, the neurotransmitter proposed to be mediating fast synaptic transmission appears between the synaptic terminal and the postsynaptic membrane. Neurotransmitters that can modify these EPSPs are shown in italics outside each terminal, with the direction of their modulatory actions indicated by superscripts. The depiction of the modulators is intended to underscore the present uncertainty regarding their cellular origin and their locus of action. 1, Due et al. (2004); 2, Gardner and Kandel (1977); 3, Kabotyanski et al. (2000).

DA receptor types are present on B8 and B4/5 themselves. In the case of B4/5, an EPSP that is thought to be produced by dopaminergic fibers in the esophageal nerve is blocked by ergonovine (Nargeot et al. 1999), an antagonist that did not affect EPSPs originating from B65. Also, the presence of presynaptic DA receptors is indicated by the observation that cholinergic inhibitory PSPs from B4/5 to B8 are down-regulated by low concentrations (5×10^{-6}) of dopamine (Kabotyanski et al. 2000) (see Fig. 11 of this article).

The apparent presence of multiple dopamine receptor types in direct follower neurons (preceding text) raises the possibility that DA originating from B20 and B65 could be acting both as the fast excitatory neurotransmitter and as a modulator at these synapses. In a previous study, Kabotyanski et al. (1998) demonstrated that, in addition to producing EPSPs in B8 and B4/5, repetitive trains of B65 impulses elicited slow and persistent actions in both follower neurons. It was proposed that such actions resulted from progressively increased levels of released dopamine. In this respect, B65 was likened to an intrinsic modulatory element (the dorsal swim interneuron: DSI) in the escape swimming CPG of *Tritonia diomedea* (Katz and Frost 1995, 1996; Katz et al. 1994). We have observed similar long-lasting effects in B8 with repeated stimulation of B20 (Díaz-Ríos and Miller 2002; unpublished data). If dopamine is responsible for both rapid and persistent signaling from B65 and B20, then these interneurons would share another property with the DSIs, i.e., rapid and prolonged signaling by a single biogenic amine neurotransmitter (Getting 1983; Katz and Frost 1995; McClellan et al. 1994). The ability to block the fast conventional EPSPs originating from B20 and B65 with sulpiride (see also Due et al. 2004) should facilitate characterization of additional synaptic actions and clarify the possible contribution of intrinsic dopaminergic modulation to regulation of the buccal CPG of *Aplysia*.

GABAergic signals and receptors in molluscan feeding

Although participation of GABA in the rapid synaptic signaling of B20 and B65 was not substantiated in this study, considerable evidence supports the involvement of GABA in the regulation of molluscan feeding (Arshavsky et al. 1991, 1993; Cooke et al. 1985; Norekian 1999; Norekian and Satterlie 1993; Richmond et al. 1991). Within the feeding-related circuits of *Aplysia*, two GABA-immunoreactive cerebral-buccal interneurons (CBIs; CBI-11 and -3) and two buccal-cerebral interneurons (BCIs: B34 and B40) have been shown to produce fast picrotoxin-sensitive IPSPs in identified follower neurons (Jing et al. 2003; Wu et al. 2003). These demonstrations of GABAergic signaling by GABA-immunoreactive neurons seem to support the validity of this method for the localization of authentic GABA. However, it remains plausible that the dopaminergic neurons examined in this study contain a specific epitope that is speciously detected with this antiserum. Although GABAli is colocalized with additional transmitters in some of the inhibitory interneurons [CBI-3: APGWamide (Jing and Weiss 2001); B34: ACh (Hurwitz et al. 2003)], in no case is it colocalized with dopamine (see also Díaz-Ríos et al. 2002).

Although there is presently little evidence for the presence of rapid excitatory GABAergic signals in the feeding system of *Aplysia,* this possibility warrants further scrutiny in view of the their established role in the consummatory behaviors of other mollusks (Arshavsky et al. 1991, 1993; Norekian 1999; Norekian and Satterlie 1993). Within the *Aplysia* CNS, GABA is known to be capable of producing excitatory responses on specific neurons (Yarowsky and Carpenter 1977). Our observations do suggest the presence of GABA receptors that share pharmacological properties with the $GABA_B$ classification of vertebrates. The ability of these receptors to modify the excitatory signaling of key interneurons like B20 and B65 is consistent with the observations of Richmond et al. (1993), who showed that activation of $GABA_B$-like receptors could regulate the feeding CPG of the snail *Helisoma*. In that study, and in ours, baclofen mimicked several long-lasting GABAergic responses (see also Díaz-Ríos et al. 2003) but did not appear to activate $GABA_A$-like receptors. Interestingly, the GABAergic interneuron B40 produces a long-lasting depolarization of B8 that follows its inhibitory actions (Jing and Weiss 2002; Jing et al. 2003), but the possible participation of GABA in this long-lasting signal has not been explored. Baclofen should serve as a selective pharmacological tool for further disclosing the properties and functions of pre- and postsynaptic $GABA_B$-like receptors in the *Aplysia* CNS (see Philippe et al. 1981).

Functional considerations

Recent studies on the neural circuit controlling *Aplysia* consummatory behavior have led to an increased appreciation

for its multifunctionality and its diverse sources of activation (Horn and Kupfermann 2002; Hurwitz et al. 1996; Kupfermann and Weiss 2001; Morton and Chiel 1994; Proekt and Weiss 2003). Multifunctionality of the *Aplysia* CPG is achieved, in part, by a type of multiplexing of behavioral components. Although ingestive and egestive behaviors share common and fairly invariant features of radula protraction and retraction, it is the phasing of radula closure with respect to its protraction and retraction that determines whether food will be ingested or egested (Church and Lloyd 1994; Hurwitz et al. 1996; Jing and Weiss 2001, 2002; Morgan et al. 2002; Morton and Chiel 1993a,b). If the radula is closing during its retraction phase, it will push food toward the esophagus (ingestion), and if it is closing during the protraction phase, it will tend to push food out of the mouth (egestion). B20 and B65 both fire primarily during the protraction phase of biting, and both are thought to dictate egestive motor patterns, largely due to their direct activation of the radula closer motor neurons B8 and B16 (Jing and Weiss 2001; Kabotyanski et al. 1998; but see following text). In both instances, the fast excitatory signaling from B20 and B65 that achieves radula closure during protraction and thus promotes egestive behavior, appears to be mediated by dopamine (Fig. 11). In the case of B65, the significance of this signaling is underscored by observations of Due et al. (2004), who showed that sulpiride and bilateral hyperpolarization of B65 produced comparable decreases in the egestiveness of evoked buccal motor programs.

B4/5 fires during the retraction phase of feeding and has widespread interneuronal actions that influence the phasing of many neurons involved in radula protraction-retraction and closure (Church and Lloyd 1994; Gardner 1971; Gardner and Kandel 1977; Kabotyanski et al. 1998; Nagahama and Takata 1990; Sossin et al. 1987). Kabotyanski and coworkers (1998) showed that repeated bursts of B65 firing could exert a prolonged inhibition of B4/5. These investigators also found that application of dopamine (5×10^{-5} M) produced a downregulation of fast IPSP signaling from B4/5 to B8 during retraction (Kabotyanski et al. 2000) (see Fig. 11 of this article). They proposed that a build-up of DA with repetitive B65 bursts could achieve a transition from an egestive (radula closure during protraction) to an ingestive (radula closure during retraction) buccal motor pattern (Kabotyanski et al. 1998, 2000). The inhibitory actions of DA on B4/5 observed in this study are consistent with this proposal. By using the same neurotransmitter, dopamine, to both excite B8 directly and to downmodulate the interposed inhibitory neuron B4/5, B65 may achieve such a transition by imposing a graded downregulation of B4/5 that is proportional to its cumulative excitation of B8 over a period of time (Fig. 11) (see also Kabotyanski et al. 1998).

In common with most complex behaviors, *Aplysia* feeding can be activated by multiple stimuli (Horn and Kupfermann 2002; Kupfermann and Weiss 2001; Kupfermann et al. 1991). B20 and B65 both have the capacity to evoke coordinated motor programs in buccal ganglia that are isolated from the remainder of the CNS (Jing and Weiss 2001; Kabotyanski et al. 1998; Teyke et al. 1993). Recently, B20-induced BMPs were shown to be egestive (Jing and Weiss 2001). Moreover, B20 was shown to be preferentially active during egestive motor programs that were elicited by specific higher order cerebral-buccal interneurons (Jing and Weiss 2001) or stimulation of the esophageal nerve (Proekt et al., 2004). B65, which is not recruited into CBI-evoked motor programs (Jing et al. 2003), has been studied primarily in isolated buccal ganglia, where it appears to produce egestive patterns that gradually become ingestive during prolonged firing of the interneuron (Kabotyanski et al. 1998) (see preceding text). Interestingly, a catecholaminergic buccal neuron in the snail *Helisoma* (designated N1a) that is thought to correspond to B65 also promotes ingestive BMPs in isolated buccal ganglia. Although the natural mode of stimulation of B65 remains uncertain, N1a was shown to be activated by natural feeding stimulants (watermelon) within the oral cavity (Murphy et al. 2001). It appears, therefore that although B20 and B65 are likely to differ in their implementation and consequences, they share the ability to influence the polymorphic buccal CPG in a qualitative fashion. Their unusual GABA-immunoreactive/dopaminergic phenotype, which is only known to occur in one additional neuron in the *Aplysia* CNS (Díaz-Ríos et al. 2002), is likely to contribute to this common capability. The use of dopaminergic signaling by these cells is consistent with observations in mammals, where DA is thought to be critically involved in coordinating information flow and behavioral responses to changing environmental and internal conditions (Grace 2002).

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