Dopaminergic Transmission Between Identified Neurons From the Mollusk, *Lymnaea stagnalis*

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**SUMMARY AND CONCLUSIONS**

1. Dopaminergic transmission was investigated in the central nervous system (CNS) of the freshwater snail, *Lymnaea stagnalis*.

2. The giant pedal neuron, designated as right pedal dorsal one (RPeD1), makes chemical, monosynaptic connections with a number of identified follower cells in the CNS. Previous work has shown that RPeD1 is an interneuron and an important component of the *Lymnaea* respiratory central pattern generator. In this study, the hypothesis that RPeD1 uses dopamine as its neurotransmitter was tested by chromatographic, pharmacological, and electrophysiological methods. Characterization of RPeD1's transmitter pharmacology is essential to clearly understand its role in *Lymnaea*.

3. Earlier studies demonstrated that the soma of RPeD1 contains dopamine. This was quantitated in the present study by high-performance liquid chromatography (with electrochemical detection) of isolated RPeD1 somata and growth cones, which yielded 0.8 ± 0.3 and 0.10 ± 0.08 pmol of dopamine per soma and growth cone, respectively.

4. Bath or pressure application of dopamine to follower cells of RPeD1, in situ, mimicked the effects of RPeD1 stimulation. Dose-response curves were constructed for the excitatory effect of dopamine on follower cells, visceral dorsal two and three (VD2/3) (ED50 = 39 µM, Hill coefficient = 1.03), and the inhibitory effect of dopamine on follower cell, visceral dorsal four (ED50 = 33 µM; Hill coefficient = 0.92).

5. The following dopamine agonists (100 µM) were tested by bath application: 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN), apomorphine, 2-bromo α-ergocryptine, deoxyepinephrine (DE), mesulergine, (-)quipazine, SKF 38393, and tyramine. Only the general dopamine agonists, ADTN and DE, mimicked RPeD1's effects on its follower cells.

6. When VD2/3 was isolated and plated in vitro, it maintained a depolarizing response to dopamine. This response was reduced by intracellular injection of the G-protein blocker, GDP-β-S (2 mM in electrode). Similarly, incubation of VD2/3, in vitro for ~18 h, with pertussis toxin (PTX, 5 µg/ml), the G-protein inactivating exotoxin, also reduced the dopamine response. Injecting GDP or incubating in heat-inactivated PTX did not effect the response.

7. Dopamine agonists were used in an attempt to block RPeD1's synapses: chlorpromazine, ergonovine, fluphenazine, haloperidol, 6-hydroxydopamine, SCH 23390, (+) sulpiride, and tubocurarine. Only the D2 dopamine receptor antagonist, (+) sulpiride, reversibly blocked synaptic transmission from RPeD1 to its follower cells. Both the (+) and the (-) enantiomer of sulpiride also antagonized synaptic transmission. A dose-inhibition curve for (+) sulpiride was constructed (IC50 = 47 µM). (+) Sulpiride also blocked the effects of bath applied dopamine.

8. Collectively, these data suggest that RPeD1 uses dopamine as a neurotransmitter and that dopamine may elicit its effects via a G-protein coupled, D2-like dopamine receptor.

**INTRODUCTION**

Dopamine, a monoamine belonging to the catecholamine family, is found in the central nervous system (CNS) of both vertebrates (Björklund and Lindvall 1984; Carlson 1987) and invertebrates (Barker et al. 1979; S. Rózsa 1984; Sweeney 1963; Walker and Holde-Dye 1989; Wendt and Homborg 1992). For invertebrates, in particular gastropod mollusks, dopamine has been detected and localized within many regions of the CNS (Carpenter et al. 1971; McCaman et al. 1979; Sweeney 1963). In some gastropods, dopamine has been associated with particular behaviors, for example, egg laying in *Lymnaea* (Werkman et al. 1990, 1991); feeding in *Helisoma* (Trimble and Barker 1984), *Limax* (Weiland and Gelperin 1983), and *Lymnaea* (Kyriakides and McCrohan 1989); and respiration in *Lymnaea* (Morozy and Winlow 1992, Syed et al. 1990). Furthermore, electrophysiological effects of exogenously applied dopamine have been observed in *Aplysia* (Ascher 1972; Gospe and Wilson 1980; Pellmar 1981; Safranova and Chemeris 1991), *Helix* (Nesic and Pasic 1992; Walker et al. 1968), *Lymnaea* (Audesirk 1989; Barnes et al. 1994; De Vlieger et al. 1986; Safranova and Chemeris 1991; Stoof et al. 1985), *Planorbius* (Bolshakov et al. 1993), and *Planorbus* (Berry and Cotterell 1975, 1979).

A gastropod mollusk that has been used extensively in neurobiological research is the freshwater snail, *Lymnaea stagnalis*. The focus of this article is dopaminergic synaptic transmission in the CNS of *Lymnaea*. Several studies, using high-performance liquid chromatography (HPLC), immunocytochemistry, histochemistry, or radiochemical assay, demonstrated the presence of dopamine in the *Lymnaea* CNS (Auditsirk 1985; Cotterell et al. 1979; Elekes et al. 1991; McCaman et al. 1979; Werkman et al. 1991). Furthermore, studies have shown that dopamine is metabolized by the CNS, indicating a neurotransmitter role for dopamine in *Lymnaea* (Werkman 1989). Quantitative measurements of dopamine in *Lymnaea* indicated that it is present at concentrations up to ~400 pmol per CNS (Cotterell et al. 1979; Elekes et al. 1991; Werkman et al. 1991).

Along with general evidence for dopamine as a neurotransmitter in *Lymnaea*, there are several maps of presumed dopaminergic neurons in the CNS (Auditsirk 1985; Elekes et al. 1991; Werkman et al. 1991). A finding common to these studies was the detection of a large neuron on the dorsal surface of the right pedal ganglion; this cell was both strongly immunoreactive to a dopamine antibody and in-
tensely histofluorescent for dopamine after glyoxylic acid staining (Audesirk 1985; Cottrell et al. 1979; Elekes et al. 1991; Werkmann et al. 1991). This large, dopamine-containing neuron has been designated as right pedal dorsal one (RPedD1) (Benjamin and Winlow 1981). McCaman et al. (1979), using a radiochemical assay, measured the level of dopamine within the excised soma of RPedD1 and found it to be ~0.5 pmol. Subsequent investigations into the physiology of RPedD1 found it to be an interneuron that makes a large number of synaptic connections with many identified follower cells in the visceral and right parietal ganglia (Benjamin and Winlow 1981; Winlow and Benjamin 1977; Winlow et al. 1981). Several of these follower cells have been shown to be motor neurons involved in cardiorespiratory behaviors (Buckett et al. 1990; Syed et al. 1991). Moreover, recent work indicated that RPedD1 is an important component of the central pattern generator responsible for abdominal respiration (Moroz and Winlow 1992; Syed et al. 1990; Syed and Winlow 1991). A key function of RPedD1 is to initiate the respiratory cycle (Park and Winlow 1994; Syed and Winlow 1991).

In the present study, synaptic transmission from interneuron RPedD1 to a selection of its follower cells was examined. Using chromatographic, pharmacological, and electrophysiological methods, the hypothesis that RPedD1 uses dopamine as its neurotransmitter was tested. RPedD1 is a readily identifiable cell and is part of a circuit responsible for respiratory function. There is ample morphological evidence to suggest that RPedD1 uses dopamine as its neurotransmitter. However, detailed pharmacological and electrophysiological evidence regarding the nature of RPedD1’s synaptic transmission has yet to be provided. In fact, truly rigorous pharmacological analysis of dopaminergic transmission, within a group of behaviorally defined neurons such as RPedD1 and its follower cells, has yet to be undertaken within the gastropoda. Furthermore, the increasing use of Lymnaea in molecular biological, electrophysiological, and developmental studies makes an investigation into dopaminergic transmission within this animal timely.

 METHODS

 Animals

 The experiments used a stock of the mollusk, L. stagnalis (Gastropoda, Pulmonata, Basommatophora, Lymnaeidae), raised and maintained in a large-scale aquaculture at the University of California. Animals used for electrophysiology and the production of conditioned medium had shell lengths of 20-25 mm (aged ~1-2 mo), whereas animals used in cell culture were 15-20 mm long (aged ~1-2 mo).

 Dissection and salines

 The CNS was removed and pinned-out dorsal surface up. For electrophysiology, the CNS was pinned to the silicone rubber base (General Electric RTV 616) of a small volume (~500 μl) recording chamber. For cell isolation, the CNS was pinned to the rubber base of a 55 × 15 mm petri dish. In both cases, the cerebral commissure was cut so that the brain lay flat (Fig. 1). Dissection, pinning-out of the brain, and electrophysiology on cultured neurons (in vitro) was performed in normal Lymnaea saline composed of (in mM) 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 5.0 N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), adjusted to pH 7.9 with 1 N NaOH. Electrophysiology on isolated brain preparations (in situ) was performed in 6×Ca²⁺/6×Mg²⁺ saline composed of (in mM) 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, 7.5 MgSO₄, and 5.0 HEPES, pH 7.9). This saline reduces the probability of polysynaptic effects (Austin et al. 1967; Berry and Pentreath 1976; Elliott and Benjamin 1989). Salts were obtained from Sigma. With the exception of the HPLC, all experiments were performed at room temperature (18-20°C).

 HPLC

 HPLC was performed on acutely isolated neuronal somata and on growth cones isolated from the ends of axons. HPLC methods were modified from Syed et al. (1993). The isolation of neuronal somata is described below. Growth cone isolation occurred after the cell had been plated and was actively extending processes. Large growth cones, 50-100 μm lamellopodial length, were severed from the ends of neurites by means of a broken microelectrode and gently aspirated into a siliconized (Sigma SL2), wide-bore, fire-polished pipette and then placed into 10 μl of 0.2 M pentafluoroproionic acid (PFPA, Pierce) and frozen (at −70°C) until analysis (within 3 h). Freshly isolated somata were treated identically. For a given aliquot of PFPA, there was one to three somata or several growth cones. Before analysis, the samples were taken though two freeze-thaw cycles and the contents diluted with PFPA to 100 μl in an autosampler microvial. The chromatographic system consisted of a Waters HPLC pump, an ESA Coulochem guard cell at +0.75 V, a Waters intelligent sample processor autosampler, a Beckman ultrasphere 5 μm octadecyl ion-pairing column (4.6 × 45 mm) thermostatted at 50°C, a graphite/cyanacrylate electrode, and a Bio analytical systems amperometric detector set for +0.65 V. The detector signal was digitized and recorded using the Waters/Dynamic Solutions Maxima data acquisition system. The autosampler introduced 90 μl of an individual sample, and the separation was performed at 50°C on a Beckman UltraspHERE 5 μm C-18 column (4.6 × 45 mm) using a mobile phase consisting of (in mM) 30 trisodium citrate, 10 citric acid, 1 EDTA, 100 sodium perchlorate, and 10 sodium dodecyl sulphate, at a flow rate of 2 ml/min. Standardization was achieved using a cocktail of freshly prepared external standards at a concentration of 1 pmol in 100 μl of 0.2 M PFPA. The standards used were norepinephrine, epinephrine, dihydroxybenzamine (an internal standard), dopamine, and serotonin. The salts for the mobile phase and the standards were all obtained from Sigma.

 Identified cell culture

 Identified cell culture was performed to analyze somata and growth cones with HPLC and to study dopamine responses in vitro. The methods were modified from Ridgway et al. (1991). Before dissection, the deshelled animals were sterilized by soaking in a 10% (vol/vol) solution of Listerine in normal saline for 10 min. Animals were then placed in antibiotic saline (ABS; normal saline with 150 μg/ml gentamicin, Sigma G3632) and the brains removed in the aseptic conditions of a laminar flow hood. After a 15-min wash in ABS, the brains were placed in an enzyme cocktail of 1.33 mg/ml of collagenase/dispease (Boehringer Mannheim 269638) and 0.67 mg/ml trypsin (Sigma type III) in defined medium (DM) for 30-40 min. The DM used was serum-free, 50% (vol/vol) Liebowitz L-15 medium (GIBCO, special order) with added inorganic salts (concentration in mM: 40.0 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 5.0 HEPES, pH 7.9) and 20 μg/ml of gentamicin. After enzyme treatment, the brains were placed for 10 min in a 0.67 mg/ml solution of soybean trypsin inhibitor (Sigma type I S) dissolved in DM. The brains were then pinned to the rubber base of a 55 × 15 mm petri dish containing high
osmolarity DM (DM with 30 mM glucose, Sigma G7021). The inner sheath was removed from the ganglion of interest and identified neurons were isolated by using a siliconized, wide-bore, fire-polished pipette attached to a micrometer syringe (Gilmont) for vacuum or pressure. Isolated neurons were either placed in 10 μl PFPA for subsequent HPLC analysis or were plated on poly-L-lysine coated 35-mm petri dishes (Falcon 3001). Neurons used for electrophysiology were plated in dishes containing 2 ml of DM, under these conditions the cells were spherical. Neurons used for growth cone isolation were plated in a mix of 1 ml DM and 1 ml brain-conditioned medium (CM), at a density of 5 neurons per dish. In the presence of CM, these neurons exhibited neurite outgrowth concomitant with the presence of growth cones. CM is required to induce neurite outgrowth from cultured cells (Ridgway et al. 1991; Wong et al. 1981). CM was made by incubating DM with the CNS from other Lymnaea at 1 brain/ml for 72 h; subsequently, the CM was harvested, frozen in 1-ml aliquots in cryovials (Nalgene M5904) to reduce both noise and evaporative loss of electrolyte.

**Electrophysiology**

Current clamp recordings were made using single-barrel borosilicate micropipettes. When filled with 0.75 M KCl, these electrodes had a final resistance of 20–30 MΩ. Normally 0.75 M KCl was the electrolyte, although in a small number of cases saturated K₂SO₄ was used (electrode resistance 15–20 MΩ). The back end of the electrodes were filled with a small amount of mineral oil (Sigma M5904) to reduce both noise and evaporative loss of electrolyte. Data were collected with a Neurodata dual channel intracellular amplifier equipped with a bridge balance. Microelectrodes were connected to the amplifier headstage via a silver wire coated with AgCl. A second silver wire coated with AgCl served as ground. The voltage was displayed on a Tektronix dual beam storage oscilloscope and recorded on a Gould 2 channel chart recorder. Electrodes were balanced with 20-ms, 1-nA-square, hyperpolarizing current pulses delivered by a Grass stimulator and isolation unit. Current was injected into the neurons via the direct current injection function on the amplifier. To facilitate microelectrode penetration of neurons in the isolated brain, the sheath surrounding the CNS was exposed to a small pronase crystal (Sigma type XIV), held in place with normal saline. The final concentration of ethanol in these cases was <0.4% (vol/vol). When this amount of ethanol was applied as a control, no discernable effect on membrane potential, action potential wave form, firing pattern, or synaptic transmission was observed. Similar levels of ethanol have been used when applying drugs to the *Hermissenda crassicornis* and *Aplysia californica* nervous systems without deleterious effects (Fossier et al. 1994; Rogers et al. 1994). For pressure application onto neurons in the isolated brain, dopamine was dissolved in 6× Ca²⁺/6× Mg²⁺ saline, containing 0.01% fast green and 0.1% sodium metabisulphite. This solution was loaded into a wide-bore, fire-polished pipette that was connected to a Medical Systems pneumatic pressure unit. The pressure pipette was positioned directly over the soma of the neuron of interest. Only one synaptic connection or one identified follower cell was examined in each preparation.

**Application of GDP-β-S and PTX**

To test for the involvement of G proteins in the dopamine response, dopamine was applied to cultured visceral dorsal two and three (VD2/3) somata, before and after the injection of either GDP-β-S (2 mM in electrode, Sigma G7637), or as a control, GDP (2 mM, Sigma G7127). Nucleotides were dissolved in intracellular saline composed of (in mM) 50 KCl, 2.56 CaCl₂, 5 ethylene glycol-bis-(β-aminomethyl ether)-N,N,N',N'-tetracetic acid, and 10 HEPES, adjusted to pH 7.9 with KOH (Haydou et al. 1991), along with 0.01% fast green. Salts were from Sigma. This solution was loaded into an electrode that was connected to a Medical systems pneumatic pressure unit, allowing simultaneous membrane potential monitoring and nucleotide injection. Nucleotides were injected (3 s pulses at 1.5 kg/cm²) until the soma was the same color as the electrode solution (~1–5 min).

In related experiments, VD2/3 somata were incubated, in vitro, in PTX (5 μg/ml, List Biological laboratories 180) for ~18 h and then tested for their ability to respond to dopamine (Spencer et al. 1994). As a control, cells were incubated in PTX that had been heat inactivated in boiling water for 15 min. The PTX was kept at 100 μg/ml in a storage solution supplied by the manufacturer composed of (in mM) 50 NaCl, 10 NaH₂PO₄, pH 7.0 with an added 20 mM of dithiothreitol (DTT, Sigma D 9779). It was then diluted down to 5 μg/ml for the incubation. DTT is required to activate the PTX (Kaslow et al. 1987).

**Statistical analysis**

When necessary, the mean and standard deviation are given either in the text or graphically. The graphing program, “Inplot 4” (ISI Software), was run on a personal computer and used to plot data, fit curves, and calculate half-maximal values and Hill coefficients.

**Chemical sources**

Sources of chemicals not yet mentioned in the methods are given below. 2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydroxaphenline (D002), R(−) apomorphine (D004), phfenazine (F100), mesulergine (M153), (+) quinpirole (Q102), SCH 23390 (D054), (+) SKF 38393 (D047), (+) sulpiride (S116), (+) sulpiride (S113), and (−) sulpiride (S112) were obtained from Research Biochemicals International. 2-Bromo-a-ergocryptine (B2134), cadmium chloride (C2544), chlorpromazine (C8138), deoxycyepine (D5886), dopamine (H8502), ergonovine (E7008), haloperidol (H1512), 6-hydroxydopamine (H6507), spiperone (S7395), tubocurarine (T2379), and tyramine (T2879) were obtained from Sigma.
Fig. 1. Schematic map of Lymnaea's central ring ganglia. Ganglia are numbered according to Syed and Winlow (1991): left and right cerebral (1 and 2); left and right pedal (3 and 4); left and right pleural (5 and 6); left and right parietal (7 and 8); visceral (9). Buccal ganglia are not shown. Cerebral commissure has been cut and cerebral ganglia placed off to the side, ventral surface up, so the brain is essentially flat, with the rest of the ganglia being dorsal surface up. Identified neurons: left pedal dorsal one (LPeD1); right pedal dorsal one (RPeD1); visceral dorsal two (VD2); visceral dorsal three (VD3); visceral dorsal four (VD4); visceral I cell (VI); visceral J cell (VJ). Neurons are shaded to denote their color in living CNS, i.e., a white circle indicates an orange cell, a black circle indicates a white cell, and a partially filled circle indicates a cell with a mixture of the 2 colors.

RESULTS

Identified neurons

This study used several identified neurons. Figure 1 is a schematic map of the dorsal surface of the Lymnaea CNS. Illustrated is the presynaptic neuron, RPeD1, which is an interneuron involved in respiratory central pattern generation (Syed et al. 1990; Syed and Winlow 1991). Also shown are a selection of RPeD1's follower cells, including the giant neurons, VD2/3 (Benjamin and Winlow 1981), the cardiorespiratory interneuron, visceral dorsal four (VD4) (Hickett et al. 1990; Skingsly et al. 1993; Syed et al. 1990; Syed and Winlow 1991), which is also a component of the respiratory pattern generator, and the cardiorespiratory motor neurons, visceral I and J cells (VI, VJ) (Hickett et al. 1990; Syed et al. 1991). Also illustrated is left pedal dorsal one (LPeD1), which was used for comparison in the HPLC analysis.

Soma and growth cones of RPeD1 contain dopamine

RPeD1 soma and growth cones were subjected to HPLC to detect and measure dopamine (Fig. 2). Dopamine was measured at 0.8 ± 0.3 pmol per soma (n = 10 somata in 5 pooled groups) and 0.10 ± 0.08 pmol per growth cone (n = 53 growth cones in 5 pooled groups). Assuming that the soma of RPeD1 is both 150 μm in diameter and a perfect sphere, the concentration in the cell body would be ~0.5 mM. For comparison, the serotoninergic neuron, LPeD1 (Audesirk 1985; Cottrell et al. 1979; Croll and Chiason 1989), was also analyzed; serotonin was measured at 0.9 ± 0.5 pmol per soma (n = 6 somata), whereas dopamine was not detected.

Dopamine agonists mimic the effect of RPeD1 stimulation

If RPeD1 uses dopamine as its neurotransmitter, the exogenous application of dopamine agonists to its follower cells should mimic the effects of RPeD1 stimulation. Four different follower cells were selected to assay the effects of dopamine application: VD2/3, which are excited by RPeD1; VD4 and VJ cells, which are inhibited by RPeD1; and VI cells, which receive a biphasic excitation followed by inhibition from RPeD1 (Fig. 3). Previous studies have found these connections to be chemical and monosynaptic in nature (Benjamin and Winlow 1981; Winlow and Benjamin 1977; Winlow et al. 1981). In the present experiments, the follower neurons were first visually identified and then physiologically confirmed to be the correct cell by their response to RPeD1 stimulation.

When dopamine was pressure applied (0.1 M in pipette) to the soma of a follower neuron, the response of the cell was similar to that produced by RPeD1 stimulation (Fig. 4, also compare Figs. 3 and 4). Specifically, VD2/3 was excited (n = 6), VD4 and VJ cells were inhibited (n = 4 and 4), and VI cells were excited and then inhibited (n = 4) by pressure applied dopamine. When dopamine (100 μM) was bath applied to identified neurons, similar results were observed (Fig. 5); however, although the effects of dopamine on VD2/3, VD4, and VJ cells were appropriate (n = 13, 12, and 10, respectively), VI neurons consistently responded to bath applied dopamine with only inhibition and lacked an excitatory response (n = 8). Desensitization of the response produced by bath applied dopamine was not observed (5–10 applications of 100 μM dopamine over ~1.5 h; data not shown). The majority of the bath applied agonist studies was performed in 6 Ca2+/6 Mg2+ saline; however, a number

Fig. 2. HPLC analysis of RPeD1. Chromatograms show retention times of (A) 1 pmol standards, (1) norepinephine, (2) epinephine, (3) dihydroxybenzamidine (an internal standard), (4) dopamine, and (5) serotonin; (B) monoamine content of 1 RPeD1 soma; (C) monoamine content of 1 LPeD1 soma; and (D) monoamine content of 4 RPeD1 growth cones. Amount of monoamine detected is proportional to area under the peak. Artifact at time zero is due to passage of the solvent front.
of experiments were performed in 6× Ca^{2+}/6× Mg^{2+} saline that had been supplemented with 100 μM CdCl₂. This concentration of CdCl₂ blocks chemical transmission (Magoski and Bulloch, unpublished observation). In these experiments, the results were identical to those seen in the absence of CdCl₂ (n = 4, 5, 4, and 3 for VD2/3, VD4, VJ, and VI, respectively; data not shown), indicating that dopamine acts directly on the neurons in question.

Dose–response curves to bath applied dopamine for VD2/3 (n = 10) and VD4 (n = 9) were constructed (Fig. 6). These neurons were chosen because they displayed a representative excitatory (VD2/3) and inhibitory (VD4) response, and their voltage responses were readily quantified. The dose–response curves for both VD2/3 and VD4 were similar, with half-maximal doses of stimulation (ED₅₀) of 39 μM and 33 μM for VD2/3 and VD4, respectively. The Hill coefficients for the curves were 1.03 for VD2/3 and 0.92 for VD4.

To further characterize the dopamine response of RPeD1’s follower cells, a number of dopamine agonists, at 100 μM, were tested (Table 1). During screening, an individual agonist was compared with dopamine for its ability to elicit a response (n = 5–7 for each agonist on various follower cells). Of the seven agonists tested, only the general agonists, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN) and deoxyepinephine (DE), were capable of eliciting a response in the follower cells. The responses of VD2/3 (n = 11), VD4 (n = 10), a VJ cell (n = 10), and a VI cell (n = 7) to dopamine, ADTN, and DE are given in Fig. 7. For all four follower cells, the agonists mimicked the effects of bath applied dopamine.

G-protein blocking agents reduce the dopamine response of VD2/3

To determine whether dopamine exerts its effects via a G-protein coupled receptor, experiments involving G-protein
FIG. 5. Response of RPeD1 follower cells to bath applied dopamine. At the bar, dopamine (100 μM) was bath applied for 10 sec via a 3-way valve system. Membrane potentials: VD2/3 = -75 mV; VD4 = -54 mV; VJ = -48 mV; VI = -45 mV.

Blocking agents were undertaken. This involved using isolated VD2/3 somata, cultured in DM for 18-30 h. Cultured neurons were used because preliminary experiments with the whole brain suggested that applied dopamine activated receptors on both the soma and axon collaterals, with the latter being outside the influence of intracellularly injected G-protein blockers, such as GDP-β-s. Furthermore, the G-protein inactivating exotoxin, PTX, was loaded into the cells by incubating in a PTX-containing solution, and this was achieved most readily using cultured VD2/3 somata (Spencer et al. 1994).

Typically, VD2/3 in vitro had a more depolarized resting potential (about -50 vs. -75 mV) and a higher input resistance (about 300 vs. 50 MΩ) than in situ. As a result, bath applied dopamine (100 μM) produced long depolarizations with high spiking activity. When GDP-β-S (2 mM in electrode) was intracellularly injected into VD2/3, the dopamine response was considerably reduced, without obvious changes in membrane potential or excitability (n = 5; Fig. 8). As a control, injection of GDP (2 nM) did not reduce the dopamine response (n = 3; data not shown). Incubation of VD2/3 in DM containing 5 pg/ml of PTX virtually eliminated the dopamine response, although the neurons maintained their resting potentials and remained excitable (n = 5; Fig. 9A). When VD2/3 was incubated in heat-inactivated PTX, the dopamine response was maintained (n = 4; Fig. 9B).

Different forms of sulpiride block synaptic transmission from RPeD1 to its follower cells

Pharmacological blockade of a synaptic response is a common way to provide evidence that a particular neurotransmitter is being used by a presynaptic cell. Several dopaminergic antagonists were used in an attempt to block synaptic transmission from RPeD1 to its follower cells (Table 2). Antagonists were tested on single preparations, at a concentration of 100 μM (n = 4–7) on various connections. Of the nine antagonists tested, only (+) sulpiride, a D-2 dopamine receptor antagonist, was able to block transmission from RPeD1 to its follower cells. At a concentration of 100 μM, (+) sulpiride either completely blocked or clearly reduced synaptic transmission in a reversible manner (Figs. 10 and 11): RPeD1 → VD2/3 (n = 11), RPeD1 → VI (n = 4), RPeD1 → VJ (n = 5), and RPeD1 → VD4 (n = 4). Table 3 provides further information regarding the extent of the (+) sulpiride block. As a control for the nonspecific effects of (+) sulpiride, Fig. 11C also shows that the reciprocal synaptic connection from VD4 to RPeD1 (transmitter unknown) is not affected by the presence of the drug. The connection from VD4 to RPeD1 was present in control and wash situations as well (data not shown).

### Table 1. Effect of dopamine agonists on RPeD1 follower cells

<table>
<thead>
<tr>
<th>Agonist*</th>
<th>Active</th>
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<tr>
<td>ADTN</td>
<td>Yes</td>
</tr>
<tr>
<td>Apomorphine</td>
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</tr>
<tr>
<td>2-Bromo-α-ergocryptine</td>
<td>No</td>
</tr>
<tr>
<td>Deoxyepinephrine</td>
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</tr>
<tr>
<td>Mesulergine</td>
<td>No</td>
</tr>
<tr>
<td>(-) Quiniprole†</td>
<td>No</td>
</tr>
<tr>
<td>SKF 38393†</td>
<td>No</td>
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<tr>
<td>Tyramine</td>
<td>No</td>
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ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronephrine. * Agonists were bath applied individually at a concentration of 100 μM. † (-) Quiniprole is specific for D-2 dopamine receptors, whereas SKF 38393 is specific for D-1 dopamine receptors. All other compounds are general dopamine agonists.
DOPAMINERGIC TRANSMISSION IN LYMNAEA

FIG. 7. Response of RPeD1 follower cells to bath-applied dopamine agonists (100 μM). Neurons were exposed to dopamine for 10 sec. On return to baseline, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydroanaphthene (ADTN) was applied. After recovery, deoxycarpine (DE) was applied. A: VD2/3 was excited by dopamine and the agonists. Initial membrane potential = −76 mV. B: VD4 was inhibited by dopamine and the agonists. Initial membrane potential = −53 mV. C: VJ cell was inhibited by dopamine and the agonists. Initial membrane potential = −39 mV. D: VI cell was inhibited by dopamine and the agonists. Initial membrane potential = −46 mV.

(±) Sulpiride is a racemic mixture of two enantiomers, (+) sulpiride and (−) sulpiride. Previous work has indicated that the (−) enantiomer is more potent in blocking evoked dopamine responses than the (+) enantiomer (De Vlieger et al. 1986; Kebabian and Calne 1975; Stoof et al. 1985). The ability of the individual enantiomers to block synaptic transmission in Lymnaea was tested. Interestingly, at a concentration of 100 μM, both (+) and (−) sulpiride either completely or partially blocked synaptic transmission (Fig. 12): RPeD1 → VD2/3 (n = 5, 2 complete and 3 partial blocks) and RPeD1 → VD4 (n = 2, 1 complete and 1 partial block).

Using the RPeD1 to VD2/3 synapse as an assay, preliminary work with all three forms of sulpiride (±, +, and −) showed that little or no block could be achieved with any sulpiride at a concentration of 10 μM (n = 2; data not shown). Given that block could not be achieved at 10 μM but was achieved at 100 μM, it suggested that the range of concentrations required to block transmission was similar for all three forms of sulpiride. Because of this similarity in potency, a dose-inhibition curve was constructed for only (±) sulpiride. The synapse chosen for the inhibition assay was the RPeD1 → VD2/3 connection (Fig. 13), which often displayed individual postsynaptic potentials that could be readily quantified. The half-maximal concentration of inhibition (IC₅₀) required for (±) sulpiride to block transmission was 47 μM.

Sulpiride blocks the effects of exogenously applied dopamine

To test whether the synaptic and evoked responses involved the same receptor, the ability of (±) sulpiride to

FIG. 8. Intracellular injection of GDP-β-S reduces the dopamine response of VD2/3, in vitro. Top: control action potential. Dopamine (100 μM) was bath applied, and a long depolarization (~20 mV) was elicited. The full recovery of the response is not shown. GDP-β-S (2 μM) was then injected. Fifteen minutes after nucleotide injection, another action potential was elicited, using the same amount of current as for control. This spike had a very similar threshold and waveform when compared with control. Dopamine was applied for a second time, but produced only a small depolarization (~4 mV). Resting membrane potential throughout experiment = −50 mV. Bars indicate duration of current injection into VD2/3.
block bath applied dopamine was investigated. The presence of (+) sulpiride substantially reduced the dopamine response of VD2/3 (n = 6; average 70% reduction) and VD4 (n = 5; average 75% reduction) and blocked the dopamine response of VI (n = 5) and VJ (n = 6) cells in a reversible manner (Figs. 14 and 15).

**DISCUSSION**

**A chemically defined dopaminergic interneuron**

There is strong evidence to indicate that RPeD1 contains dopamine. Both the present work and previous studies provide either chemical (McCaman et al. 1979; this work) or histological and immunocytochemical (Audesirk 1985; Cotrell et al. 1979; Elekes et al. 1991; Werkman et al. 1991) data demonstrating the presence of dopamine in the soma of RPeD1. Using an enzyme-based, radiochemical assay, McCaman et al. (1979) found 0.52 ± 0.12 pmol of dopamine in the soma of RPeD1, whereas in the present study, using HPLC, 0.8 ± 0.3 pmol of dopamine was detected in the isolated soma of RPeD1 (Fig. 2). The similarity between these results is remarkable. Possible explanations for the small difference may include animal size, animal supply stock, seasonal variability, or the detection method. In the present study, the measurement of dopamine was taken one step further with the analysis of dopamine in growth cones isolated from the neurites of RPeD1, in vitro. Dopamine was detected at a level of 0.10 ± 0.08 pmol in isolated growth cones. The growth cone may be considered a developmental precursor of the synaptic terminal; this is supported by the fact that growth cones contain synthetic enzymes for neurotransmitters (Gotow and Sotelo 1987; Westenbroek et al. 1988), synaptic vesicles and vesicle associated proteins (Ivgy-May et al. 1994), and release neurotransmitters (Haydon and Man-Son-Hing 1988; Young and Poo 1983). If the growth cones of RPeD1 may be considered precursors of its synaptic terminals, the detection of dopamine in these growth cones suggests that RPeD1 uses dopamine at its synapses.

Although the evidence for the presence of dopamine in the soma of RPeD1 is strong, there are no data demonstrating the presence of dopamine in the terminals of RPeD1, in vivo. However, insight on this matter may be gained from work on a related pulmonate mollusk, *Planorbis corneus*. Planorbis possesses a large, dopamine-containing neuron that is homologous to RPeD1 in its chemical content, morphology, and large number of follower cells (Berry and Cotrell 1973, 1975; Bulloch and Ridgway 1995). Using a combination of electron microscope autoradiography and 3H-dopamine injection, the terminals of the Planorbis neuron were examined, in vivo, and found to possess dense-cored vesicles containing labeled dopamine (Pentreath and Berry 1975).

**Pharmacology of dopaminergic transmission**

Several pieces of pharmacological evidence have been presented to suggest that RPeD1 uses dopamine as a neurotransmitter: 1) exogenously applied dopamine, or the general dopamine agonists ADTN and DE, mimicked the effects of RPeD1 stimulation on its follower cells (Figs. 4–7); 2) the D-2 dopaminergic antagonist, (+) sulpiride, reversibly blocked synaptic transmission from RPeD1 to its follower cells, in a dose-dependent manner (Figs. 10–13); and 3) (+) sulpiride also antagonized the effects of exogenously applied dopamine on RPeD1’s follower cells (Figs. 14 and 15). These facts, along with the chemically and morphological evidence discussed above, provide compelling evidence that RPeD1 uses dopamine as a neurotransmitter at its synapses.

**TABLE 2. Effect of dopamine antagonists on synaptic transmission from RPeD1 to its follower cells**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Receptor Specificity</th>
<th>Synaptic Blockade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpromazine</td>
<td>General</td>
<td>No</td>
</tr>
<tr>
<td>Ergonovine</td>
<td>General</td>
<td>No</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>D-1/D-2</td>
<td>No</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>D-2/D-1</td>
<td>No</td>
</tr>
<tr>
<td>6-Hydroxydopamine†</td>
<td>General</td>
<td>No</td>
</tr>
<tr>
<td>SCH 23390</td>
<td>D-1</td>
<td>No</td>
</tr>
<tr>
<td>(+) Sulpiride</td>
<td>D-2</td>
<td>Yes</td>
</tr>
<tr>
<td>Tubocurarine†</td>
<td>General</td>
<td>No</td>
</tr>
</tbody>
</table>

* Antagonists were bath applied at a concentration of 100 μM. † Although 6-hydroxydopamine and tubocurarine are not normally considered dopamine antagonists, they have been used to block dopamine evoked responses in *Aplysia* (Ascher 1972) and dopaminergic transmission in Planorbis (Berry et al. 1974; Berry and Cotrell 1975).
Furthermore, the fact that (±) sulpiride is a D-2 dopaminergic antagonist suggests that dopamine may exert its effects via a D-2-like dopamine receptor.

Electrophysiological responses of identified neurons to exogenously applied dopamine have been investigated in a number of gastropods (Ascher 1972; Audesirk 1989; Barnes et al. 1994; Berry and Cottrell 1975, 1979; Bolshakov et al. 1993; De Vlieger et al. 1986; Gospe and Wilson 1980; Nesic and Pasic 1992; Pellmar 1981; Safranova and Chemeris 1991; Stoof et al. 1985; Walker et al. 1968). The dose-
TABLE 3. Complete vs. partial block of transmission by 100 μM (±) sulpiride

<table>
<thead>
<tr>
<th>Connection</th>
<th>Complete Block</th>
<th>Partial Block*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPeD1 → VD2/3</td>
<td>6</td>
<td>5 (74)</td>
</tr>
<tr>
<td>RPeD1 → VD4</td>
<td>3</td>
<td>1 (86)</td>
</tr>
<tr>
<td>RPeD1 → V1</td>
<td>3</td>
<td>1 (92)</td>
</tr>
<tr>
<td>RPeD1 → VJ</td>
<td>4</td>
<td>1 (83)</td>
</tr>
</tbody>
</table>

RPed1, right pedal dorsal one; VD2/3, visceral dorsal two and three; VD4, visceral dorsal 4; VI, VJ, visceral I and J cells. * Values in parentheses are %, and the % partial block was calculated only from those preparations where partial block was observed. Specifically, the partially blocked PSP was divided by the control PSP to give a percentage; this percentage was then subtracted from 100% to arrive at the % partial block.

response curves for VD2/3 and VD4 (Fig. 6) exhibit a range of dopamine concentrations within that required to elicit responses in other molluscan neurons. Typically, although not exclusively, dopamine elicits a response at concentrations between 10 and 100 μM in neurons from several different mollusks (Audesirk 1989; Barnes et al. 1994; De Vlieger et al. 1986; Gospe and Wilson 1980; Matsumoto et al. 1988; Nesic and Pasic 1992).

Several agonists were also used to investigate the dopamine response in RPeD1’s follower cells (Table 1). The two effective compounds were the general dopamine receptor agonists, ADTN and DE (Fig. 7). In part, these agonists were chosen on the basis of their effectiveness in other molluscan preparations. For example, ADTN is an agonist for dopamine-induced fictive feeding in Limax (Weiland and Gelperin 1983), and ADTN, along with dopamine itself, induced stereotypical respiratory behavior when injected into Lymnaea (Moroz and Winlow 1992). Furthermore, DE is an agonist for dopamine-stimulated adenylyl cyclase in the gill of Aplysia (Weiss and Drummond 1981). Because ADTN and DE are both general dopamine agonists, it is difficult to make a conclusion from this data regarding dopamine receptor subtypes present on RPeD1’s follower cells.

Regarding the biphasic response of the VI cells, pressure applied dopamine caused excitation followed by inhibition (like RPeD1’s effect), whereas bath applied dopamine or dopamine agonists caused only inhibition (compare Figs. 4, 5, and 7D). Similar results have been reported for Aplysia and Planorbin (Asher 1972; Berry and Cottrell 1975). Because the effect of pressure applied dopamine on VI neurons is appropriate, the results of bath applied dopamine do not necessarily jeopardize the conclusion regarding RPeD1’s transmitter. The excitatory response to dopamine in the VI neurons may desensitize very rapidly, and it is possible that the excitatory phase, seen with RPeD1 stimulation and pressure applied dopamine, may require a more rapid delivery of agonist then that produced by bath application.

Dopamine antagonists that block synaptic transmission have been studied in the mollusks Aplysia (Swann et al. 1978) and Planorbin (Berry et al. 1974; Berry and Cottrell 1975). In Aplysia, ergonovine, at a concentration of 900 μM, reversibly blocked transmission from putative dopaminergic motor neurons to gill musculature (Swann et al. 1978). In Planorbin, 6-hydroxydopamine (at concentrations ranging from 400 μM to 1 mM), reversibly blocked inhibitory transmission from a dopaminergic interneuron (homologous to RPeD1) to several of its follower cells (Berry and Cottrell 1975). Although both ergonovine and 6-hydroxydopamine were ineffective in Lymnaea (Table 2), the concentrations used for Aplysia or Planorbin are comparable with the concentrations of (±) sulpiride required to block dopaminergic synapses in Lymnaea (Figs. 10–13). Unfortunately, dose-inhibition curves for antagonists of dopamine-mediated synaptic transmission have not been published for other mollusks; however, there is a report of a dose-inhibition curve for the effects of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on excitatory amino acid neurotransmission in Aplysia (Trudeau and Castellucci 1993). The IC50 for CNQX in Aplysia was similar to the IC50 for (±) sulpiride in Lymnaea, that is, ~50 μM (Fig. 13B) (Trudeau and Castellucci 1993). Collectively, these data suggest that the concentrations of (±) sulpiride required in this study were not unreasonably high.

G proteins and the dopamine response

Dopamine receptors are considered to be G-protein coupled (Civelli et al. 1993). For VD2/3, a representative follower cell of RPeD1, dopamine appears to elicit its effects though a G-protein coupled receptor. When GDP-β-S, an inhibitor that competes with GTP for the binding site on the G protein (Eckstein et al. 1979), was injected into the soma of VD2/3, the dopamine response was reduced (Fig. 8). GDP-β-S has been shown to inhibit neuronal responses to serotonin and carbachol in Aplysia (Kehoe 1994; Kudo et al. 1991) and dopamine in Planorbin (Bolshakov et al. 1993). Additional evidence that G proteins mediate the effects of dopamine in Lymnaea is seen by the virtual elimination of VD2/3’s dopamine response after incubation of the soma in a PTX containing solution (Fig. 9A). Previous work...
has demonstrated that PTX can inhibit responses to dopamine in Aplysia and Lymnaea (Kudo et al. 1991; Spencer et al. 1994) and carbachol in Aplysia (Kehoe 1994). PTX causes ADP ribosylation of the α subunit on some G proteins; consequently, the G protein is unable to exchange GTP for GDP, and effector activation by the receptor is prevented (Dolphin 1987). An α-G-protein subunit from Lymnaea was recently cloned, and appropriately, the particular cys-
containing neurons in Helisoma and Planorbis are of unknown origin. Both phenotype and circuitry, the dopamine cells in these systems, consisting of RPeD1, a dopamine-containing presynaptic neuron, and a number of follower cells or follower cell clusters, such as VD2/3, VD4, VI cells, and VJ cells. Previous work indicates that many of the cells in this system are involved in respiratory behavior and that RPeD1 is part of the respiratory central pattern generator (Syed and Winlow 1991; Park and Winlow 1994; Syed et al. 1990, 1991). Incidentally, the injection of either dopamine or dopamine agonists into Lymnaea can induce stereotypical respiratory behavior (Moroz and Winlow 1992). Nevertheless, RPeD1 is presumably not the only Lymnaea neuron that uses dopamine; as the immunocytochemical data indicates, there are many candidate dopamine-immunoreactive neurons (Audesirk 1985; Cottrell et al. 1979; Elekes et al. 1991; Werkman et al. 1991). Furthermore, evidence suggests that dopamine plays a role in other Lymnaea behaviors, such as egg laying (Werkman et al. 1990, 1991) and feeding (Kyriakides and McCrohan 1989).

With respect to other pulmonate mollusks, there is a great deal of homology in dopaminergic systems. Both Helisoma trivolvis and P. corneus contain similar dopaminergic systems, consisting of a large, pedal, dopamine-containing presynaptic neuron and a number of follower cells (Berry and Cottrell 1975; Bulloch and Ridgway 1995; Syed et al. 1993). These neurons, from three different genera, contain picomolar levels of dopamine (McCaman et al. 1979; Syed et al. 1993), have a similar morphology, and make synapses with a large number of follower cells in similar regions of the CNS (Berry and Cottrell 1975; Syed et al. 1993; Winlow and Benjamin 1977; Winlow et al. 1981). The dopamine-containing neurons in Helisoma and Planorbis are of unknown function; however, given RPeD1's role in respiratory behavior (Syed and Winlow 1991) and the similarities in both phenotype and circuitry, the dopamine cells in these pulmonates may also be involved in respiration (Arshavsky et al. 1994). Interestingly, equivalent dopaminergic systems have not been identified in either of the commonly used opisthobranch mollusks, A. californica or Tritonia diomedia (Bulloch and Ridgway 1995; McCaman et al. 1979). This may reflect the need of the pulmonate mollusks to undergo aerial respiration.

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