

Dopamine Activates Two Different Receptors to Produce Variability in Sign at an Identified Synapse

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Magoski, Neil S. and Andrew G. M. Bulloch. Dopamine activates two different receptors to produce variability in sign at an identified synapse. *J. Neurophysiol.* 81: 1330–1340, 1999. Chemical synaptic transmission was investigated at a central synapse between identified neurons in the freshwater snail, *Lymnaea stagnalis*. The presynaptic neuron was the dopaminergic cell, Right Pedal Dorsal one (RPeD1). The postsynaptic neuron was Visceral Dorsal four (VD4). These neurons are components of the respiratory central pattern generator. The synapse from RPeD1 to VD4 showed variability of sign, i.e., it was either inhibitory (monophasic and hyperpolarizing), biphasic (depolarizing followed by hyperpolarizing phases), or undetectable. Both the inhibitory and biphasic synapse were eliminated by low Ca^{2+} /high Mg^{2+} saline and maintained in high Ca^{2+} /high Mg^{2+} saline, indicating that these two types of connections were chemical and monosynaptic. The latency of the inhibitory postsynaptic potential (IPSP) in high Ca^{2+} /high Mg^{2+} saline was ~ 43 ms, whereas the biphasic postsynaptic potential (BPSP) had ~ 12 -ms latency in either normal or high Ca^{2+} /high Mg^{2+} saline. For a given preparation, when dopamine was pressure applied to the soma of VD4, it always elicited the same response as the synaptic input from RPeD1. Thus, for a VD4 neuron receiving an IPSP from RPeD1, pressure application of dopamine to the soma of VD4 produced an inhibitory response similar to the IPSP. The reversal potentials of the IPSP and the inhibitory dopamine response were both approximately -90 mV. For a VD4 neuron with a biphasic input from RPeD1, pressure-applied dopamine produced a biphasic response similar to the BPSP. The reversal potentials of the depolarizing phase of the BPSP and the biphasic dopamine response were both approximately -44 mV, whereas the reversal potentials for the hyperpolarizing phases were both approximately -90 mV. The hyperpolarizing but not the depolarizing phase of the BPSP and the biphasic dopamine response was blocked by the D-2 dopaminergic antagonist (\pm) sulpiride. Previously, our laboratory demonstrated that both IPSP and the inhibitory dopamine response are blocked by (\pm) sulpiride. Conversely, the depolarizing phase of both the BPSP and the biphasic dopamine response was blocked by the Cl^- channel antagonist picrotoxin. Finally, both phases of the BPSP and the biphasic dopamine response were desensitized by continuous bath application of dopamine. These results indicate that the biphasic RPeD1 \rightarrow VD4 synapse is dopaminergic. Collectively, these data suggest that the variability in sign (inhibitory vs. biphasic) at the RPeD1 \rightarrow VD4 synapse is due to activation of two different dopamine receptors on the postsynaptic neuron VD4. This demonstrates that two populations of receptors can produce two different forms of transmission, i.e., the inhibitory and biphasic forms of the single RPeD1 \rightarrow VD4 synapse.

INTRODUCTION

The sign of transmission at a chemical synapse is usually considered invariant, although there are reports that the sign of a connection can vary between preparations (Park and Winlow 1993; Spencer and Winlow 1994). However, preparation-to-preparation variability in the sign of transmission at a single, identified synapse was not documented. Differences in the types of connections between the same neurons can be considered an extension of the “polymorphic network” concept. First proposed by Getting (1989), the polymorphic network theory suggests that physically defined circuits of neurons can produce more than one type of output. By changing the sign of a specific synapse, the functional output of the circuit that contains the neurons in question can be modified. There are instances where it may be advantageous for a presynaptic neuron to alter the excitability of a postsynaptic neuron in a specific manner, for example, Cl^- -dependent inhibition versus K^+ -dependent inhibition. The levels of second messengers may also be affected, depending on the types of synaptic connection, providing access to different forms of neuromodulation and neuroplasticity. We examine the physiological and pharmacological basis of different synaptic potentials at a single, identified molluscan synapse. The connection in question displays the unusual property of variability in the type of synaptic potential between preparations.

The focus here is a well-characterized, identified dopaminergic neuron known as Right Pedal Dorsal one (RPeD1) from the CNS of the freshwater snail, *Lymnaea stagnalis* (Audesirk 1985; Benjamin 1984; Cottrell et al. 1979; Elekes et al. 1991; Magoski and Bulloch 1997; Magoski et al. 1995; McCaman et al. 1979; Werkman et al. 1991; Winlow and Benjamin 1977; Winlow et al. 1981). One of RPeD1's many postsynaptic cells is the cardiorespiratory interneuron Visceral Dorsal four (VD4) (Benjamin 1984; Buckett et al. 1990; Skingsley et al. 1993; Syed and Winlow 1991). Neurons RPeD1 and VD4 are components of the central pattern generator responsible for aerial respiration (Moroz and Winlow 1992; Syed and Winlow 1991; Syed et al. 1990, 1992).

The synapse from RPeD1 to VD4 was observed in one of two forms. It is either inhibitory (monophasic and hyperpolarizing) (Syed and Winlow 1991; Syed et al. 1990) or biphasic (depolarizing followed by hyperpolarizing phases) (Benjamin 1984). Beyond these reports documenting the synapse, there is no information regarding physiological parameters such as latency or reversal potential; as well, tests for a chemical or monosynaptic connection were not carried out on the RPeD1 \rightarrow VD4 synapse. Furthermore, although Magoski et al.

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(1995) provided detail regarding the pharmacology of the inhibitory form of the synapse (as expected it is dopaminergic), there is no information on the pharmacology of the biphasic form. Interestingly, all previous reports on the RPeD1 → VD4 synapse implied that the connection is either inhibitory or biphasic. By thoroughly surveying the sign of transmission at the RPeD1 → VD4 synapse in many preparations this study will show that this connection can be either inhibitory or biphasic, i.e., the sign of transmission is variable between animals. The physiology and pharmacology of both forms of the RPeD1 → VD4 synapse will then be examined. We then provide evidence that the differences in sign at this synapse are due to the selective activation of two different postsynaptic dopamine receptors.

METHODS

Animals, dissection, and salines

A stock of the mollusk, *Lymnaea stagnalis* (Gastropoda, Pulmonata, Basommatophora, Lymnaeidae), raised and maintained in a large scale aquaculture at the University of Calgary was used. Animals had shell lengths of 15–25 mm (age ~1–4 mo). The CNS was removed and pinned dorsal surface up to the silicone rubber base (General Electric RTV 616) of a ~500- μ l recording chamber. The cerebral commissure was cut so that the CNS lay flat. Dissection, pinning of the CNS, and some electrophysiology were performed in normal *Lymnaea* saline (composition in mM was 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 5.0 HEPES, adjusted to pH 7.9 with 1 N NaOH). To reduce the probability of polysynaptic effects, most electrophysiology was performed in high Ca²⁺/high Mg²⁺ saline (composition in mM was 51.3 NaCl, 1.7 KCl, 24.6 CaCl₂, 1.5 MgCl₂, 7.5 MgSO₄, and 5.0 HEPES, pH 7.9). To test for chemical synapses, a low Ca²⁺/high Mg²⁺ saline was used (composition in mM was 51.3 NaCl, 1.7 KCl, 0.01 CaCl₂, 1.5 MgCl₂, 7.5 MgSO₄, and 5.0 HEPES, pH 7.9). Salts were obtained from Sigma. Experiments were performed at room temperature (18–20°C).

Electrophysiology

Current-clamp recordings were made with single-barrel borosilicate micropipettes filled with 2 M potassium acetate and having a resistance of 20–30 M Ω . Data were collected with a dual-channel intracellular amplifier equipped with a bridge balance. The voltage was displayed on a storage oscilloscope and recorded on a chart recorder as well as a digital storage oscilloscope. Current was injected into the neurons via the DC injection function on the amplifier. In some instances, to facilitate microelectrode penetration of neurons, the sheath surrounding the CNS was exposed to a small pronase crystal (Sigma type XIV), held by forceps. The CNS was then rinsed in cold (~4°C) normal saline to remove excess enzyme.

Identification of neurons

Neurons RPeD1 and VD4 are identifiable from preparation to preparation with a high degree of reliability. Neuron RPeD1 is the only large (>100 μ m) neuron in the right pedal ganglion and easily recognized on the basis of size, location, color, and relatively infrequent firing pattern (Benjamin and Winlow 1981). Neuron VD4 is a small (20–30 μ m), very white cell whose location in the visceral ganglion can differ between preparations. To ensure that the cell in question was VD4, one or more of three independent criteria were used: 1) VD4 always displayed a characteristic discharge of steadily broadening action potentials immediately after impalement; 2) VD4 very often displayed regenerative firing properties; and 3) VD4 always made an excitatory synapse with neurons Right Pedal Dorsal

TABLE 1. *Distribution of RPeD1 → VD4 synapses*

RPeD1 → VD4	%	<i>n</i>
Inhibition	39	104
Biphasic	48	129
Undetectable	13	34

RPeD1, Right Pedal Dorsal one; VD4, Visceral Dorsal four.

two or three (Nesic et al. 1996; RPeD2/3; Syed and Winlow 1989). We are confident that the variability of synapses described in this study were not due to discrepancies in neuronal identification.

Application of pharmacological agents

The chamber was perfused thoroughly at ~3 ml/min with a peristaltic pump. For bath application, a compound was dissolved in high Ca²⁺/high Mg²⁺ saline containing 0.01% (wt/vol) Fast Green (Sigma F7258), and the solution was introduced into the bath via a three-way valve system. For dopamine (Sigma H8502), which is prone to oxidation, 0.1% (wt/vol) sodium metabisulfite (Sigma S1516) was also included. When Fast Green and sodium metabisulfite were applied as a control, no discernible effect on membrane potential, action potential wave form, firing pattern, or synaptic transmission was observed. Sulpiride (Research Biochemicals International S116), a dopaminergic antagonist, was first dissolved in a small volume of 80% ethanol and then added to the saline. The final concentration of ethanol was 0.4% (vol/vol). When 0.4% ethanol was applied as a control, no discernible effect on neuronal physiology was observed. The only other drug bath applied was the Cl⁻ channel blocker picrotoxin (Sigma P167).

Dopamine was also pressure applied. Dopamine was dissolved in high Ca²⁺/high Mg²⁺ saline, containing 0.01% Fast Green and 0.1% sodium metabisulfite. This solution was loaded into a wide-bore, fire-polished pipette connected to a pneumatic pressure unit. Dopamine was applied directly to the soma or was pressure ejected slightly adjacent to the soma and allowed to rapidly pass over the cell body; this did not affect the type of response. The Fast Green that was coejected with dopamine allowed us to consistently observe where dopamine was being applied. In almost all cases, the bolus of dopamine spread over an area that was two to three times the diameter of the soma.

Data analysis

The mean and SE of the mean are given either in the text or graphically. The program Inplot 4.01 (ISI Software) was used to plot data and fit regression lines (least-squares method).

RESULTS

As outlined in the INTRODUCTION, the sign of the RPeD1 → VD4 synapse was reported as either inhibitory or biphasic. We examined whether the sign of this synapse in fact varied among a large number of CNS preparations. Neuron RPeD1 inhibited VD4 in 39% of the preparations and in 48% of the cases made a biphasic synapse onto VD4, and in the remaining 13% of preparations the synapse was undetectable (Table 1). The possibility that seasonal or environmental changes were related to the differences seen in the sign of transmission was examined. No obvious correlation between the month, day of the week, time of day, or feeding schedule and the sign of the RPeD1 → VD4 synapse was observed (data not shown). For the two detectable forms of the synapse, i.e., inhibitory and biphasic, we first sought to determine if the connections were indeed

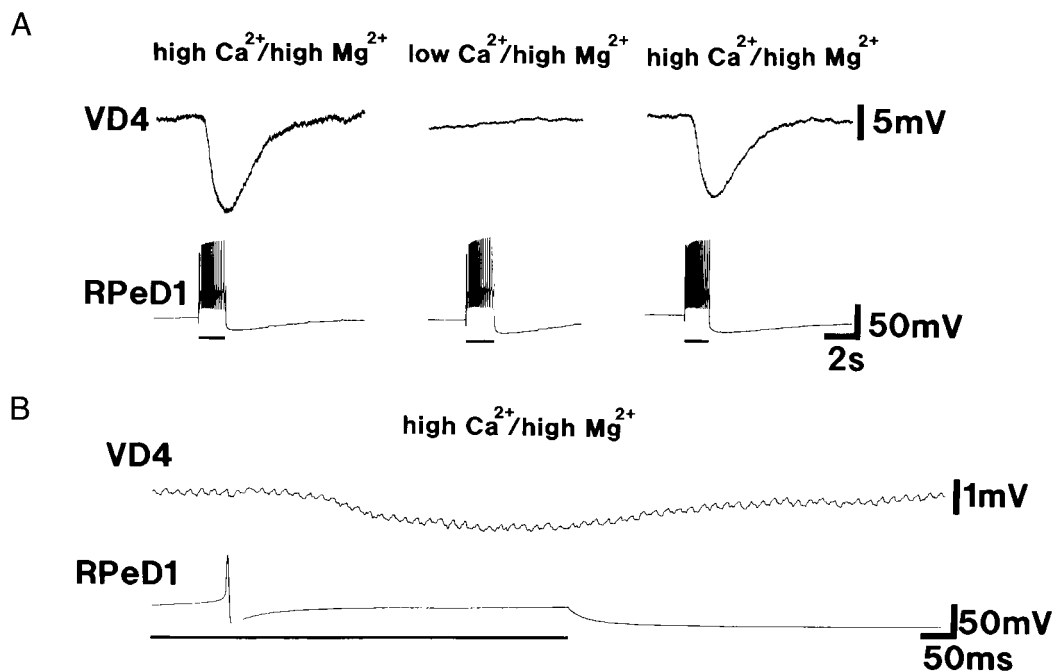


FIG. 1. Chemical and monosynaptic nature of the inhibitory Right Pedal Dorsal one (RPeD1) \rightarrow Visceral Dorsal four (VD4) synapse. *A*: synapse was maintained in high Ca^{2+} /high Mg^{2+} saline. This connection disappeared when low Ca^{2+} /high Mg^{2+} saline was applied but returned on wash with high Ca^{2+} /high Mg^{2+} saline. Membrane potentials: RPeD1 = -60 mV; VD4 = -55 mV. *B*: when recorded at high speed in high Ca^{2+} /high Mg^{2+} saline a 40-ms action potential-to-inhibitory postsynaptic potential (IPSP) inflection latency was observed. Membrane potentials: RPeD1 = -50 mV; VD4 = -50 mV. Bars indicate the duration of depolarizing current injection into RPeD1.

chemical and monosynaptic. We then compared the physiological properties of the synaptic response to the response evoked by applied dopamine (RPeD1's transmitter). Importantly, the response of VD4 to applied dopamine always mimicked the endogenous synaptic input from RPeD1. Finally, we examined the pharmacology of the biphasic synapse to test whether it is mediated by the activation of two different dopamine receptors.

Physiology of the inhibitory RPeD1 \rightarrow VD4 synapse

The RPeD1 \rightarrow VD4 inhibitory synapse was first documented by Syed et al. (1990) and Syed and Winlow (1991). These studies demonstrated a one-to-one, action potential-to-inhibitory postsynaptic potential (IPSP) ratio in normal saline. For this study, the chemical and monosynaptic nature of this synapse was more rigorously examined. This included the standard criteria outlined by Austin et al. (1967) and Berry and Pentreath (1976): testing the Ca^{2+} dependence of transmission, observing the effects of elevated divalent ion concentrations (which raises the action potential threshold of any intervening interneurons) on transmission, and quantifying the consistency and magnitude of synaptic latency. The RPeD1 \rightarrow VD4 inhibitory connection was eliminated in a low Ca^{2+} /high Mg^{2+} saline and maintained in a high Ca^{2+} /high Mg^{2+} saline ($n = 5$ and 5 , respectively; Fig. 1A). In high Ca^{2+} /high Mg^{2+} saline, the inhibitory synapse had a consistent one-to-one action potential to IPSP ratio and displayed an action potential peak-to-IPSP inflection latency of 42.9 ± 1.0 ms ($n = 32$ IPSPs from 11 synapses; Fig. 1B).

To further characterize the physiology of this synapse, we tested the influence of VD4 membrane potential on IPSP

magnitude. As VD4 was hyperpolarized from -40 to -90 mV, the IPSP decreased in a linear fashion (Fig. 2A). Previous pharmacological work by Magoski et al. (1995) indicated that the RPeD1 \rightarrow VD4 inhibitory synapse was dopaminergic. To determine if the dopamine response in VD4 was similar to the IPSP, the effect of VD4 membrane potential on the response to exogenously applied dopamine was examined. When VD4 was held at -40 mV, pressure-applied dopamine (0.1 M in the pipette) produced a large hyperpolarization; holding the cell at more negative membrane potentials resulted in a steadily smaller hyperpolarization, which reversed at approximately -90 mV (Fig. 2B). The relationship between the membrane potential of VD4 and both the IPSP and the dopamine response are plotted together in Fig. 3. Linear regression of both relationships provided similar extrapolated reversal potentials of -90.5 mV for the IPSP ($n = 13$) and -90.4 mV for the dopamine response ($n = 7$). Note that the monophasic, inhibitory pressure-applied dopamine responses were always observed on VD4 neurons that received an inhibitory synaptic input from RPeD1.

Physiology of the biphasic RPeD1 \rightarrow VD4 synapse

Benjamin (1984) provided the first documentation of the RPeD1 \rightarrow VD4 biphasic synapse. For the current work, the biphasic synapse was subjected to the same tests for chemical and monosynaptic connections as the inhibitory form. The RPeD1 \rightarrow VD4 biphasic connection was eliminated in low Ca^{2+} /high Mg^{2+} saline and maintained in high Ca^{2+} /high Mg^{2+} saline ($n = 4$ and 4 , respectively; Fig. 4A). The biphasic synapse consistently displayed a one-to-one action potential-to-biphasic postsynaptic potential (BPSP) ratio. Because there

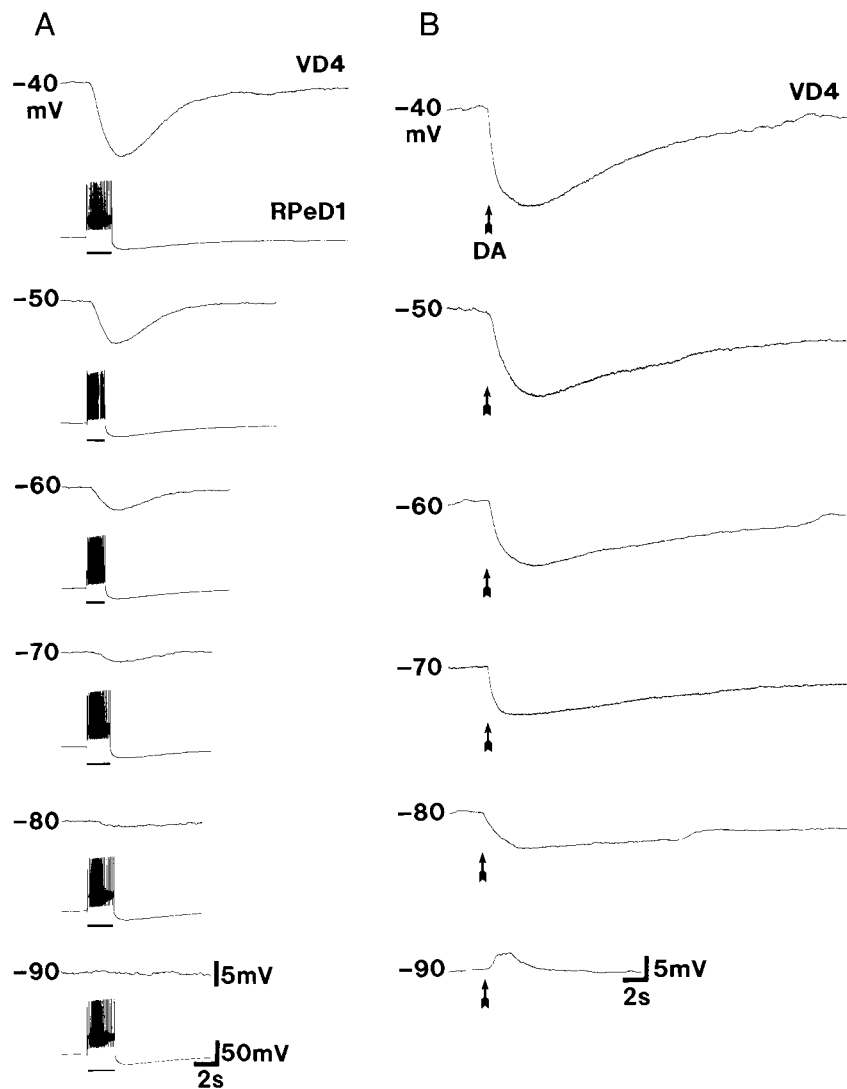


FIG. 2. Effect of postsynaptic membrane potential on the RPeD1 \rightarrow VD4 IPSP and the VD4 inhibitory pressure-applied dopamine response. *A*: neuron VD4 was held at the designated membrane potentials, and an IPSP was elicited. As the postsynaptic membrane potential was increased, the IPSP decreased. RPeD1 membrane potential = -50 mV. Bars indicate the duration of depolarizing current injection into RPeD1. *B*: neuron VD4 was held at different membrane potentials, and dopamine (0.1 M in the pipette) was pressure applied (at the arrow). As the membrane potential was increased, the dopamine response decreased and actually reversed at -90 mV. In this particular case, the ability to reverse the dopamine response may be explained by the voltage of the soma (where the dopamine response is produced) likely being under somewhat better control than the voltage of the axons (where the IPSP is produced). DA, dopamine.

is evidence that high cation concentrations can sometimes produce multiphased postsynaptic potentials (PSPs) in other molluscan neurons (Getting 1981), latency was demonstrated in both normal and high Ca^{2+} /high Mg^{2+} saline. The action potential peak-to-depolarizing phase inflection latency in normal saline was 12.0 ± 0.5 ms ($n = 36$ BPSPs from 8 synapses), and similarly in high Ca^{2+} /high Mg^{2+} saline it was 12.1 ± 0.4 ms ($n = 27$ BPSPs from 8 synapses; Fig. 4B).

Neuron RPeD1 is thought to exclusively use dopamine as a neurotransmitter (see INTRODUCTION). Thus we sought to determine if the BPSP and the VD4 biphasic dopamine response showed a similar dependence on VD4 membrane potential, the assumption being that if RPeD1 uses dopamine to produce the BPSP the BPSP should be mimicked by exogenously applied dopamine. For a biphasic connection, the effect of RPeD1 stimulation on VD4 at a range of postsynaptic membrane potentials can be seen in Fig. 5A. As VD4 was hyperpolarized, the depolarizing phase increased, and the hyperpolarizing phase decreased. Biphasic dopamine responses were always observed on VD4 neurons that received a biphasic synaptic input from RPeD1. Pressure-applied dopamine (0.1 M in the pipette) to the soma of VD4 produced a biphasic response ($n = 7$; Fig. 5B). Figure 6 shows a plot of both phases of the

RPeD1 \rightarrow VD4 BPSP and the VD4 biphasic pressure-applied dopamine response at various postsynaptic membrane potentials. The depolarizing phase of both the BPSP and the biphasic VD4 dopamine response had similar extrapolated reversal potentials of -44.4 and -43.6 mV, respectively. The hyperpolarizing phase of the BPSP and the biphasic VD4 dopamine response had also similar extrapolated reversal potentials of -91.9 and -88.5 mV, respectively.

In a few preparations, the depolarizing phase of the BPSP could be reversed, i.e., when VD4 was held at -40 mV the depolarizing phase of the BPSP was observed as a rapid hyperpolarization followed by a slow hyperpolarization. When the membrane potential of VD4 was held at -50 mV or greater, the initial phase was now the more typical, rapid depolarization (Fig. 7). This result shows that both phases of the biphasic synapse are functionally inhibitory.

Pharmacology of the biphasic RPeD1 \rightarrow VD4 synapse

To determine if two separate receptors mediate the RPeD1 \rightarrow VD4 BPSP, a pharmacological investigation of both BPSP and biphasic VD4 pressure-applied dopamine response was undertaken. Previously, we determined that of many do-

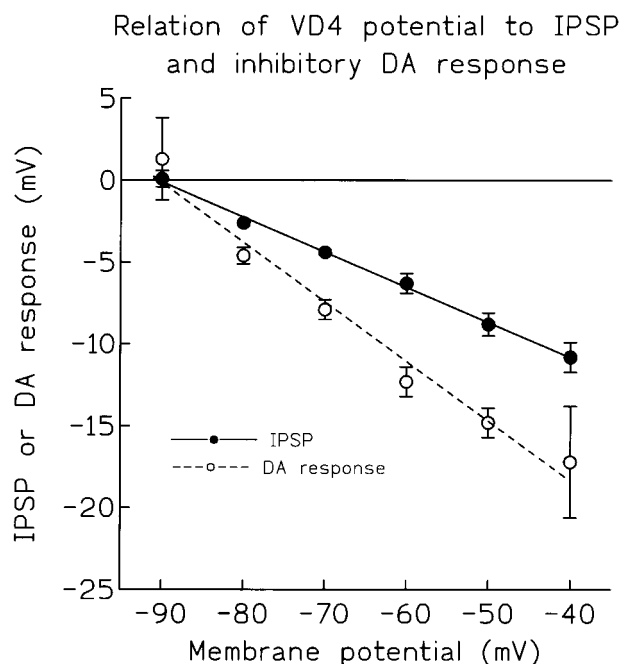


FIG. 3. Relationship between VD4 membrane potential and both the RPeD1 \rightarrow VD4 IPSP and the VD4 inhibitory pressure-applied dopamine response. The data and linear regression of both the VD4 IPSP and the inhibitory dopamine response are plotted. The reversal potentials were -90.5 mV for the IPSP and -90.4 mV for the dopamine response.

paminergic antagonists, the only effective drug at a number of RPeD1 synapses, including the monophasic inhibitory connection with VD4, was (\pm) sulpiride (Magoski et al. 1995). When (\pm) sulpiride ($100 \mu\text{M}$) was bath applied to a biphasic synapse, the hyperpolarizing phase but not the depolarizing phase of both the BPSP and the biphasic dopamine response was blocked ($n = 5$; Fig. 8).

Because an effective dopaminergic antagonist was not avail-

able for the depolarizing phase, a different approach was taken. The reversal potential of the depolarizing phase suggested the involvement of a Cl^- conductance, and therefore the Cl^- channel blocker picrotoxin was tested. The depolarizing phase but not the hyperpolarizing phase of both the BPSP and the biphasic response was reversibly blocked by picrotoxin ($100 \mu\text{M}$; $n = 4$; Fig. 9).

As a more conclusive test of whether the depolarizing phase was dopaminergic, the ability of exogenous dopamine to desensitize the receptor(s) on VD4 was examined. With continuous bath application of dopamine ($100 \mu\text{M}$), both phases of the BPSP and the biphasic pressure-applied VD4 dopamine response were effectively desensitized ($n = 5$; Fig. 10). The synaptic and pressure-applied dopamine responses were both desensitized ≤ 1 min after bath application of dopamine. To be certain that the membrane of VD4 possessed adequate resistance to carry synaptic input during bath-applied dopamine, a separate experiment was undertaken in which the input resistance of VD4 was measured during bath application of $100 \mu\text{M}$ dopamine. During dopamine exposure, the input resistance of VD4 decreased by only $32.5 \pm 9.7\%$ ($n = 6$). This would indicate that the response to RPeD1 stimulation and to pressure-applied dopamine was desensitized rather than shunted. Collectively, these data suggest that the RPeD1 \rightarrow VD4 biphasic synapse is mediated by dopamine acting on two different receptors.

DISCUSSION

By examining many preparations, we determined that the sign of synaptic transmission at the RPeD1 \rightarrow VD4 synapse varies in that it is either inhibitory or biphasic. In a minority of preparations, the connection was undetectable. We investigated the physiology and pharmacology of both the inhibitory and biphasic forms of the RPeD1 \rightarrow VD4 synapse. Both types of synapses appear to be chemical and monosynaptic, suggesting

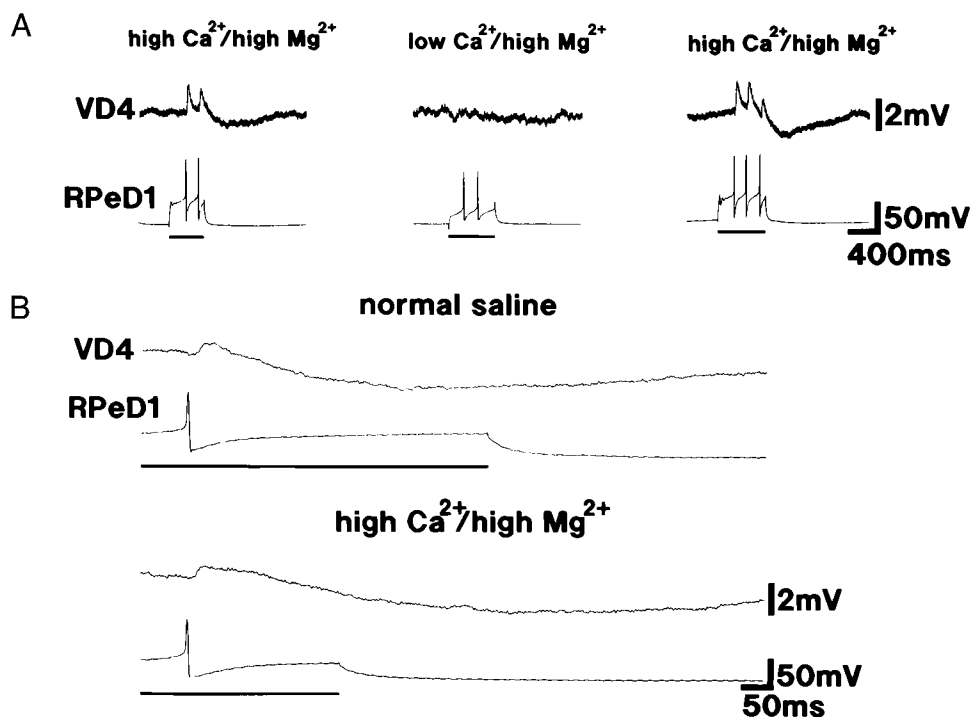


FIG. 4. Chemical and monosynaptic nature of the biphasic RPeD1 \rightarrow VD4 synapse. A: synapse was maintained in high Ca^{2+} /high Mg^{2+} saline, with a one-to-one action potential-to-postsynaptic potential (PSP) relationship. The synapse disappeared when a low Ca^{2+} /high Mg^{2+} saline was applied but returned on wash with high Ca^{2+} /high Mg^{2+} saline. Membrane potentials: RPeD1 = -55 mV; VD4 = -60 mV. B: when recorded at a high speed, a 15-ms action potential-to-biphasic PSP (BPSP) inflection latency was observed. This one-to-one action potential-to-PSP relationship was observed in either normal saline (top trace) or high Ca^{2+} /high Mg^{2+} saline (bottom trace). Membrane potentials: RPeD1 = -65 mV; VD4 = -70 mV. Bars indicate the duration of depolarizing current injection into RPeD1.

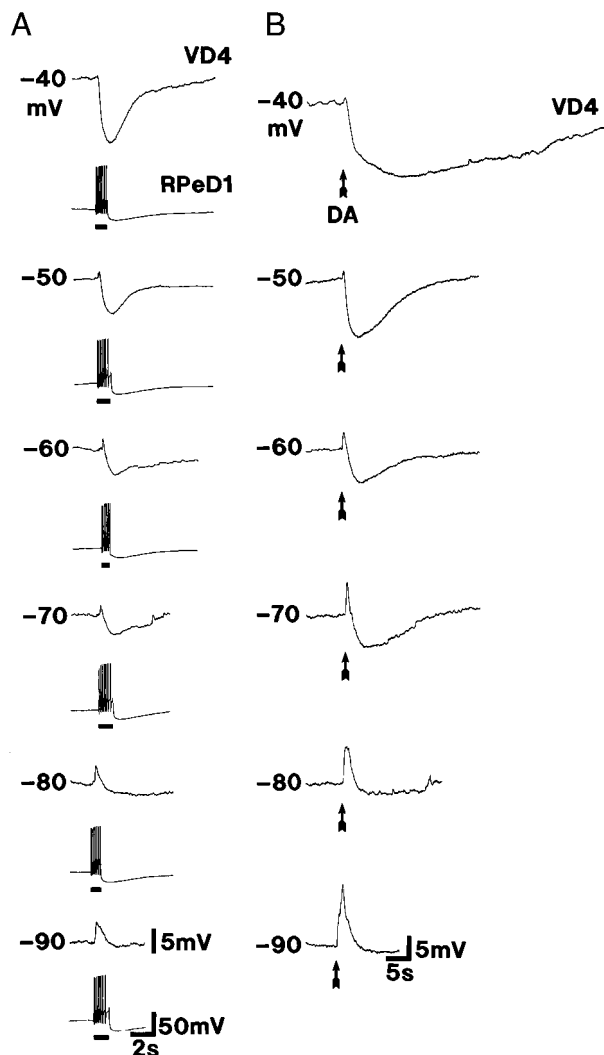


FIG. 5. Effect of postsynaptic membrane potential on the RPeD1 \rightarrow VD4 BPSP and the VD4 biphasic pressure-applied dopamine response. *A*: neuron VD4 was held at the designated membrane potentials, and a compound BPSP was elicited. As the postsynaptic membrane potential was increased, the depolarizing phase of the BPSP increased, and the hyperpolarizing phase decreased. RPeD1 membrane potential = -67 mV. Bars indicate the duration of depolarizing current injection into RPeD1. *B*: neuron VD4 was held at the designated membrane potentials, and dopamine (0.1 M in the pipette) was pressure applied (at the arrow). As the membrane potential was increased, the depolarizing phase of the dopamine response increased, and the hyperpolarizing phase of the dopamine response decreased. In this case, VD4 also received biphasic synaptic input from RPeD1. DA, dopamine.

that different synaptic responses are not due to polysynaptic effects. Importantly, dopamine (RPeD1's transmitter) mediates both the monophasic as well as the biphasic synapse. These data indicate that the different synaptic responses at this connection are due to the activation of two different postsynaptic dopamine receptors. We have shown that *variability* in synaptic sign at a *single* identified synapse is achieved by a transmitter activating two different receptors. The uniqueness of our study is that the same synapse between the same identified neurons (RPeD1 \rightarrow VD4) can manifest itself differently in different preparations, i.e., inhibitory versus biphasic.

Both forms of the RPeD1 \rightarrow VD4 synapse are likely chemical and monosynaptic. Elimination of transmission by a low Ca^{2+} /high Mg^{2+} saline indicates a chemical rather than an

electrical connection for the two types of synapse (Figs. 1 and 4) (Berry and Pentreath 1976). Furthermore, the ability of RPeD1 to evoke single IPSPs or BPSPs in high Ca^{2+} /high Mg^{2+} suggests that transmission is monosynaptic (Figs. 1 and 4). Elevated Ca^{2+} increases action potential threshold and reduces the likelihood of spiking by putative interneurons

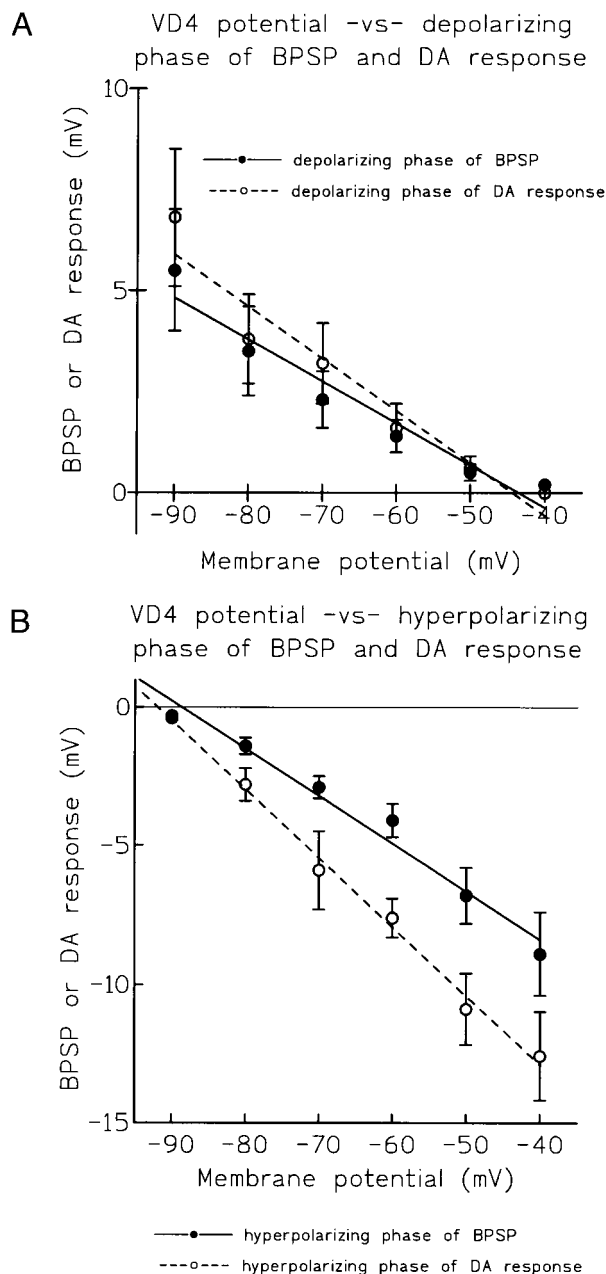


FIG. 6. Comparison of the RPeD1 \rightarrow VD4 BPSP and the VD4 biphasic pressure-applied dopamine response. *A*: relationship between VD4 membrane potential and the depolarizing phase of both the RPeD1 \rightarrow VD4 BPSP and the biphasic VD4 pressure-applied dopamine response. The data and linear regression of the depolarizing phase of both the BPSP ($n = 11$) and the biphasic dopamine response ($n = 7$) are plotted. The predicted reversal potentials were -43.6 mV for the PSP and -44.4 mV for the dopamine response. *B*: relationship between VD4 membrane potential and the hyperpolarizing phase both of the RPeD1 \rightarrow VD4 BPSP and the biphasic VD4 pressure-applied dopamine response. The data and linear regression of the hyperpolarizing phase of both the BPSP and the biphasic dopamine response are plotted. The predicted reversal potentials were -88.5 mV for the PSP and -91.9 mV for the dopamine response.

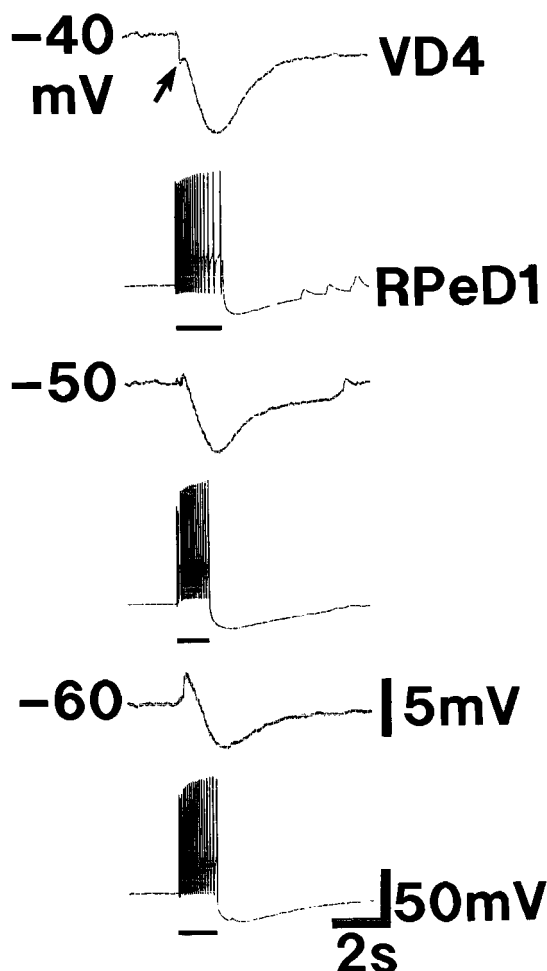


FIG. 7. Reversal of the depolarizing phase of the biphasic RPeD1 \rightarrow VD4 synapse. Neuron VD4 was held at the designated membrane potentials. In this case, when VD4 was held at -40 mV, the depolarizing phase of the BPSP was observed as a reversed, rapid hyperpolarization (marked by the arrow), followed by a more gradual hyperpolarization. When VD4 was hyperpolarized to -50 or -60 mV, the depolarizing phase took on its more conventional form of a rapid depolarization. RPeD1 membrane potential = -57 mV. Bars indicate the duration of depolarizing current injection into RPeD1.

(Austin et al. 1967; Berry and Pentreath 1976; Elliot and Benjamin 1989). The constant and rapid latency of both the inhibitory and biphasic synapses is consistent with a monosynaptic connection (Berry and Pentreath 1976). The difference in latencies between the inhibitory and the biphasic synapses (~ 43 vs. ~ 12 ms) presumably reflects different rates of postsynaptic transduction, or the depolarizing phase of the BPSP may be initiated electrotonically closer to the soma (the recording site) than the IPSP. There is good evidence to suggest that hyperpolarizing, K^+ -dependent transmitter responses in *Aplysia* neurons are mediated by the relatively slow process of G-protein-coupled receptors (Brezina 1988; Bolshakov et al. 1993; Sasaki et al. 1997). A similar mechanism may be involved in producing the long latency of the hyperpolarizing PSP at the RPeD1 \rightarrow VD4 synapse. Incidentally, previous reports, from both *Lymnaea* (Winlow et al. 1981) and the related pulmonate mollusk, *Planorbis corneus* (Berry and Cottrell 1975), show that the latency of inhibitory connections made by the giant dopamine cell (RPeD1 in *Lymnaea* or GDC in *Planorbis*) are usually 4–10 times slower than excitatory

and biphasic connections. Although we cannot completely rule out the possibility that the inhibitory synapse is the result of a polysynaptic pathway, the high concentration of divalent cations would make such a possibility very remote. Both ourselves (Magoski and Bulloch 1997) and other investigators (Winlow et al. 1981) never reported a single spike in RPeD1 eliciting a spike in a follower cell to which RPeD1 made an excitatory connection while in the presence of high divalent cations. This would have to be the case if the inhibitory RPeD1 \rightarrow VD4 synapse were polysynaptic. Parenthetically, both the inhibitory (Syed et al. 1990) and the biphasic (O. Nestic, personal communication) synapses form in vitro when the RPeD1 and VD4 are isolated and plated in culture.

Previous work shows that the soma of RPeD1 contains dopamine (Audesirk 1985; Cottrell et al. 1979; Elekes et al. 1991; Magoski et al. 1995; McCaman et al. 1979; Werkman et al. 1991). Also, Magoski et al. (1995) and Magoski and Bulloch (1997) provided pharmacological evidence that RPeD1 uses dopamine at a number of synapses, including its inhibitory synapse with VD4. This is supported by the observation that the inhibitory synapse and the inhibitory dopamine response of VD4 have similar reversal potentials (Figs. 2 and 3), indicating that RPeD1 and applied dopamine both activate a similar conductance. The approximately -90 mV reversal potential suggests that the conductance is K^+ selective. Similar inhibitory responses to dopamine were reported for identified neurons in *Achatina* (Emaduddin et al. 1995), *Aplysia* (Ascher 1972), *Helix* (Nestic and Pasic 1992), *Lymnaea* (Audesirk 1989; De Vlioger et al. 1986), *Planorbis* (Berry and Cottrell 1979), and *Planorbarius* (Bolshakov et al. 1993).

It was important to determine that dopamine was involved in both phases of the RPeD1 \rightarrow VD4 biphasic synapse. This would reinforce the conclusion that variability in sign at this

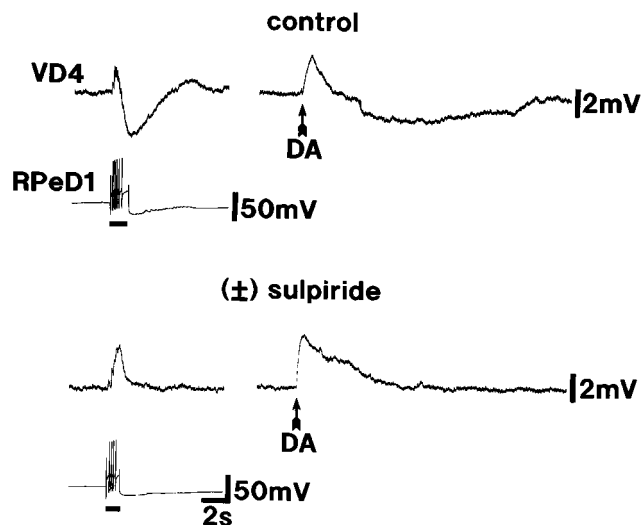


FIG. 8. Block of the hyperpolarizing phase of both the biphasic RPeD1 \rightarrow VD4 synapse and the biphasic VD4 pressure-applied dopamine response by (\pm) sulpiride. Control: burst of action potentials in RPeD1 elicited a BPSP in VD4. Pressure application of dopamine (0.1 M in the pipette, at the arrow) to VD4 produced a biphasic response. (\pm) Sulpiride: After 5-min exposure to (\pm) sulpiride (100 μ M), the hyperpolarizing phase of both the BPSP and the pressure-applied dopamine response was completely blocked. In addition, the magnitude of the depolarizing phase increased during the (\pm) sulpiride exposure. Membrane potentials: RPeD1 = -62 mV; VD4 = -70 mV. Bars indicate the duration of depolarizing current injection into RPeD1. DA, dopamine.

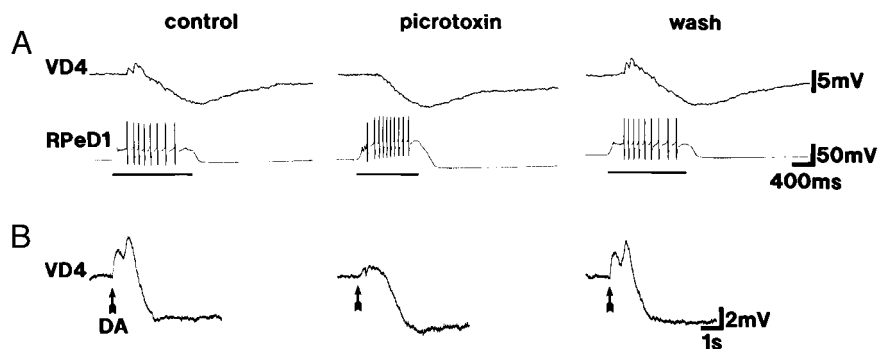


FIG. 9. Block of the depolarizing phase of both the RPeD1 \rightarrow VD4 BPSP and the biphasic VD4 pressure-applied dopamine response by picrotoxin. *A*: burst of action potentials in RPeD1 elicited a BPSP in VD4. After 7-min exposure to picrotoxin (100 μ M), the depolarizing phase of the BPSP was completely blocked. This was reversible on wash with high Ca^{2+} /high Mg^{2+} saline. Membrane potentials: RPeD1 = -55 mV; VD4 = -72 mV. Bars indicate the duration of depolarizing current injection into RPeD1. *B*: in the same preparation, pressure application of dopamine (0.1 M in the pipette, at the arrow) to VD4 produced a biphasic response. After 8-min exposure to picrotoxin (100 μ M), the depolarizing phase of the dopamine response was reduced by $\sim 75\%$. This was reversible on wash. The “dual peak” shape of the depolarizing phase was likely caused by a somewhat slow removal of dopamine by the perfusion system, allowing for a second dopamine exposure to occur. Because of the length of this particular biphasic response, only the depolarizing phase and the beginning of the hyperpolarizing phase are shown. VD4 membrane potential = -60 mV. DA, dopamine.

connection is due to activation of either one or both of two receptors. It would also rule out the unlikely possibility that the depolarizing phase was a polysynaptic effect. At a biphasic RPeD1 \rightarrow VD4 synapse, pressure-applied dopamine produced a biphasic response in VD4 (Fig. 5). The reversal potentials of the BPSP and the biphasic dopamine response were essentially the same (Fig. 6), indicating that RPeD1 input and dopamine activate a similar set of conductances. Both hyperpolarizing phases reversed at approximately -90 mV, implicating a K^+ conductance, and were blocked by 100 μ M (\pm) sulpiride (Fig. 8). This concentration of (\pm) sulpiride was previously found to be effective at blocking both the RPeD1 \rightarrow VD4 inhibitory synapse as well as other synapses of RPeD1 (Magoski et al. 1995). The reversal potentials of both depolarizing phases (approximately -44 mV) implicated a Cl^- conductance. Thomas (1977) showed that the Cl^- Nernst potential for certain *Helix* neurons is approximately -50 mV. Consistent with

this, both depolarizing phases were blocked by the Cl^- channel blocker picrotoxin (Fig. 9). Picrotoxin, at similar concentrations, blocks Cl^- -dependent responses to GABA in locust neurons (Jackel et al. 1994) as well as histamine (Hashemzadeh-Gargari and Freschi 1992) and glutamate responses (Cleland and Selverston 1995) in lobster neurons. Picrotoxin is thought to either directly block the pore of the Cl^- ionotropic receptor or bind to an associated, nonreceptor site on the protein (Barker et al. 1983).

Both the BPSP and the biphasic dopamine response desensitized when dopamine was bath applied (Fig. 10). This suggests that bath-applied dopamine competes with dopamine released at the synapse. It is unlikely that the input resistance of VD4 was reduced during the response to bath-applied dopamine to such an extent that the BPSP was shunted rather than desensitized, given that in separate experiments there was only a one-third reduction of input resistance during bath-applied

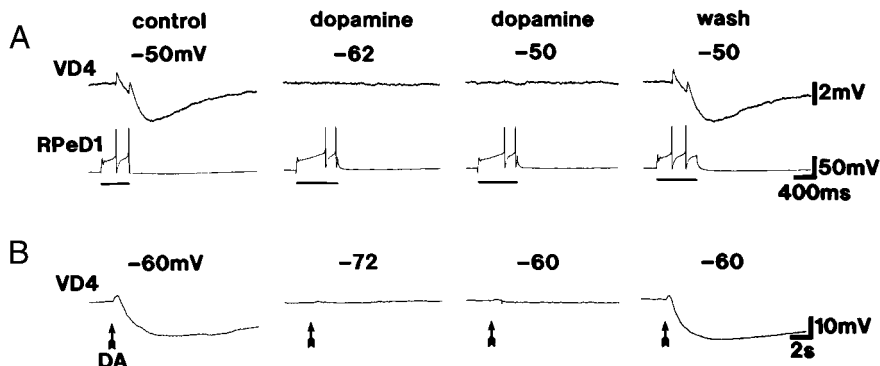


FIG. 10. Desensitization of the biphasic RPeD1 \rightarrow VD4 synapse and the biphasic VD4 pressure-applied dopamine response. *A*: neuron VD4 was held at -50 mV (membrane potential is given across the top), and two action potentials in RPeD1 elicited a BPSP. Dopamine was then continuously bath applied, causing VD4 to hyperpolarize by 12 mV to a membrane potential of -62 mV within 1 min. At this postsynaptic voltage, stimulation of RPeD1 could not elicit any form of response in VD4. Similarly, when constant current was used to depolarize VD4 to -50 mV (the membrane potential before dopamine application), a BPSP could still not be elicited. The desensitized BPSP was restored after wash to high Ca^{2+} /high Mg^{2+} saline. RPeD1 membrane potential = -45 mV. Bars indicate the duration of depolarizing current injection into RPeD1. *B*: in a different preparation, VD4 was held at -60 mV, and dopamine was pressure applied (0.1 M in the pipette, at the arrow) to elicit a biphasic response. Dopamine was then continuously bath applied, causing VD4 to hyperpolarize by 12 mV to a membrane potential of -72 mV. At this voltage, pressure-applied dopamine could not elicit a response in VD4. Furthermore, when VD4 was depolarized back to -60 mV, pressure-applied dopamine again elicited virtually no response. After wash, the desensitized biphasic dopamine response was restored. DA, dopamine.

dopamine responses. Furthermore, on a number of occasions we observed what is likely nondopaminergic synaptic input occurring in VD4 during the peak of a response to bath-applied dopamine (e.g., see Fig. 15 of Magoski et al. 1995). Finally, we also noted that both the RPeD1 \rightarrow VD4 inhibitory and biphasic synapses are maintained in the presence of 10 μ M GDPFLR-Famide, a *Lymnaea* peptide that hyperpolarizes VD4 (Magoski and Bulloch, unpublished observations). Thus it is likely that the lack of a response of VD4 to RPeD1 stimulation in the presence of 100 μ M dopamine is due to desensitization rather than shunting. Bath-applied dopamine desensitizes ionophoretic dopamine responses in *Aplysia* (Ascher 1972); furthermore, repeated or prolonged agonist application to *Aplysia* (Matsumoto et al. 1987) and mammalian dopamine receptors (Seeman and Van Tol 1994) causes desensitization.

Biphasic dopaminergic synapses and/or dopamine responses were observed in identified neurons from *Aplysia* (Ascher 1972), *Helisoma* (Syed et al. 1993), *Lymnaea* (i.e., other types of neurons in *Lymnaea*) (Magoski et al. 1995; Winlow and Benjamin 1977; Winlow et al. 1981), and *Planorbis* (Berry and Cottrell 1975, 1979) as well as neurons from the dorsal root ganglion of the rat (Molokanova and Tamarova 1995). Similarly, cholinergic (Gardner and Kandel 1972; Kehoe 1969, 1972a,b,c; Wachtel and Kandel 1967) and histaminergic (McCaman and Weinreich 1982, 1985) biphasic synapses were described in identified *Aplysia* neurons. The inferior ventricular nerve interneurons of the lobster stomatogastric ganglion also make biphasic synapses with some follower neurons (Sigvardt and Mulloney 1982). However, in none of these cases did the synapses or transmitter responses change, i.e., they consistently gave the same response. There are some examples in the literature (Kehoe 1972a,b,c; McCaman and Weinreich 1985) showing that the multicomponent nature of a small number of cholinergic and histaminergic responses is not always evident but requires pharmacological separation to demonstrate different components. This is not the situation here, where we are investigating physiological differences in the sign of transmission that do not require pharmacological manipulations to be revealed. Furthermore, the actual number of inhibitory and biphasic synapses we observed between RPeD1 and VD4 was substantial, suggesting that we are not studying some subset of synapses that on rare occasions shows differences in the sign of transmission.

We conclude that RPeD1 uses dopamine as a neurotransmitter at its synapse with VD4, whether that synapse is inhibitory or biphasic. The biphasic synapse is mediated by dopamine acting on two different receptors, one that is (\pm) sulpiride sensitive and likely activates a K^+ conductance and a second that is (\pm) sulpiride insensitive and probably activates a Cl^- conductance, whereas the inhibitory RPeD1 \rightarrow VD4 synapse is likely mediated exclusively by the (\pm) sulpiride-sensitive, K^+ conductance-coupled receptor.

Variability in the sign of transmission at the RPeD1 \rightarrow VD4 connection could be achieved by altering the functional expression of receptors. Colocalization of different types of dopamine receptors is not unusual. For example, D-1 and D-2 receptors are colocalized in approximately one-half of medium spiny projection neurons in the neostriatum (Surmeier et al. 1996). The receptors may be localized on different axon collaterals of VD4. Spike propagation blockade of a particular innervating RPeD1 axon might cause only certain receptors to

be activated, resulting in the loss of the depolarizing phase. Also, the depolarizing phase could elude detection if its portion of the PSP decayed before reaching VD4's soma (the recording site). For instance, there were preparations in which strong stimulation of RPeD1 could overwhelm the depolarizing phase of a biphasic synapse, resulting in only monophasic hyperpolarization (data not shown). However, we would contend that, whereas the two phases of the BPSP can vary in amplitude at the biphasic synapse, the difference between the inhibitory and biphasic synapse is likely not due to the depolarizing component being obscured. At the inhibitory synapse, a depolarizing component does not present itself at any time, even when VD4 was held at hyperpolarizing voltages, or when only one or two presynaptic spikes were elicited—conditions that assure detection of a depolarizing phase. Furthermore, we have shown previously (Magoski et al. 1995) that a depolarizing component does not appear when the inhibitory RPeD1 \rightarrow VD4 synapse is exposed to sulpiride nor does a depolarizing component appear when a VD4 neuron, responding to pressure-applied dopamine with only hyperpolarization, is also exposed to sulpiride (Magoski 1996).

It is not possible to be absolutely certain that a particular PSP or transmitter response does not possess additional, masked components. Pressure pipette position was known to affect the type of response elicited by exogenous transmitters (see Ascher and Kehoe 1975 for review). In this study, however, positioning the pipette directly over the soma or just adjacent to the soma (see METHODS) did not alter a given response nor did it affect the correlation between dopamine response and the type of PSP. Accordingly, we feel that the application technique is sufficient to distinguish between inhibitory and biphasic responses. Receptor localization combined with electrotonic distance from the recording site or the presence of barriers to transmitter access such as the ganglion's inner sheath could also confound the results of the pressure-applied dopamine experiments. The soma of VD4 in our preparations is quite small (~ 20 – 30μ m) and its major axonal arbor is located close by (see Benjamin 1984 or Syed and Winlow 1991 for morphological details). It is likely that a portion of the applied dopamine reached this arbor in the neuropile, which is the presumed location of receptors mediating transmission. Thus the dopamine responses we observed probably reflect the activation of receptors on both the soma and on the adjacent axons and axon collaterals. Although we cannot rule out the inner sheath as a barrier to transmitter access, there is no obvious indication from this or previous work involving pressure- and bath-applied transmitters (Hermann et al. 1997; Magoski et al. 1995; Netic et al. 1996) that the *Lymnaea* inner sheath is a significant obstacle to transmitters. Additional support for this conclusion comes from the observation that a number of *Lymnaea* neurons respond in the same manner to applied transmitter (dopamine or glutamate) whether they are in the brain and covered by the inner sheath or isolated in culture (Magoski et al. 1995; Netic et al. 1996). Given these arguments and the consistent correlation between dopamine response and synaptic input in VD4 we would suggest that, rather than experimental manipulation, differential localization or possibly differential expression of the two dopamine receptors more likely underlies variability. Cloning of *Lymnaea* dopamine receptors and the production of antibodies may offer tools to address the localization and expression issues.

To our knowledge, the RPeD1 \rightarrow VD4 synapse provides the first example of a connection between two identified neurons

where variability in synaptic sign is achieved by a transmitter activating two different receptors. Although an inhibitory or biphasic synapse mediated by a single transmitter is not new, we have shown that this single, identified synapse can vary between inhibitory and biphasic from animal to animal. As well, we provided an explanation for this variability, i.e., VD4 displays two distinct responses to dopamine (the presynaptic transmitter), and from preparation-to-preparation this correlates exactly with the form of endogenous synaptic response evoked by RPeD1 stimulation. Variability aside, the functional differences between the inhibitory and biphasic synapses remain to be determined. For both synapses, the overall effect of the input from RPeD1 is to inhibit VD4. The initial depolarization in the biphasic synapses could serve one or more purposes. The Cl^- -dependent phase is rapid in comparison with the K^+ -dependent phase. The biphasic synapse could offer short-duration inhibition at a low frequency of input and long-duration inhibition at a high frequency of input. Additionally, a voltage-dependent phenomenon may be triggered by the depolarizing phase of the biphasic synapse. For example, dendritic Ca^{2+} levels could be elevated during the depolarizing phase, which might modulate the response to subsequent hyperpolarization or some other input. As well, the activity of adenylate cyclase can be synergistically enhanced by depolarization (Reddy et al. 1995). Facilitation at the crayfish neuromuscular junction is believed to be the result of activating a presynaptic adenylate cyclase via the voltage change during tetanus (Wojtowicz and Atwood 1988). Finally, there is a recent report that intracellular Cl^- concentration can affect G-protein-mediated conductances (Lenz et al. 1997). The changes in intracellular Cl^- that would occur during the depolarizing phase could modulate G-protein-dependent responses in VD4. Collectively, mechanisms such as these could play a role in regulating the excitability of VD4.

The documentation of two synaptic responses at the RPeD1 \rightarrow VD4 synapse indicates a level of complexity not previously recognized at this connection and may represent a means by which the CNS synaptically configures neural networks in different ways. This complexity also does not support an organizational principle proposed by Segal (1983), i.e., that "... synapses with the same neurotransmitter will all produce the same synaptic action on any particular nerve cell." Rather individual synaptic connections may display different responses, depending on the specific receptors that are activated.

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