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RPCH Modulation of a Multi-Oscillator Network: Effects on the Pyloric Network of the Spiny Lobster

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Dickinson, Patsy S., Jane Hauptman, John Hetling, and Anand Mahadevan. RPCH modulation of a multi-oscillator network: effects on the pyloric network of the spiny lobster. *J Neurophysiol* 85: 1424–1435, 2001. The neuropeptide red pigment concentrating hormone (RPCH), which we have previously shown to activate the cardiac sac motor pattern and lead to a conjoint gastric mill-cardiac sac pattern in the spiny lobster *Panulirus*, also activates and modulates the pyloric pattern. Like the activity of gastric mill neurons in RPCH, the pattern of activity in the pyloric neurons is considerably more complex than that seen in control saline. This reflects the influence of the cardiac sac motor pattern, and particularly the upstream inferior ventricular (IV) neurons, on many of the pyloric neurons. RPCH intensifies this interaction by increasing the strength of the synaptic connections between the IV neurons and their targets in the stomatogastric ganglion. At the same time, RPCH enhances postinhibitory rebound in the lateral pyloric (LP) neuron. Taken together, these factors largely explain the complex pyloric pattern recorded in RPCH in *Panulirus*.

INTRODUCTION

The often extensive role that neuromodulators play in altering the output of rhythmic pattern generators has been demonstrated in a number of systems (e.g., *Tritonia* swimming, Katz and Frost 1995, 1997; Katz et al. 1994; Willows et al. 1987; leech swimming, Angstadt and Friesen 1993; Mangan et al. 1994a,b; Willard 1981; leech heartbeat, Thompson and Calabrese 1992; crustacean stomatogastric system, Blitz et al. 1995; Cazalets et al. 1990; Dickinson and Marder 1989; Dickinson et al. 1990; Flamm and Harris-Warrick 1986; Harris-Warrick et al. 1997; Hooper and Marder 1987; Katz and Harris-Warrick 1990; Nagy and Dickinson 1983; Nusbaum and Marder 1989; Weimann et al. 1993). By altering such parameters as the cycle frequency, the number of neurons active in the pattern, and spike frequency in neurons of a target pattern generator, modulators can substantially change the motor output of the pattern generator. One such modulator, the peptide red pigment concentrating hormone (RPCH), has been shown to activate the cardiac sac motor pattern in the spiny lobster, *Panulirus interruptus* (Dickinson and Marder 1989; Dickinson et al. 1993), and to activate the pyloric rhythm in the crab, *Cancer borealis* (Nusbaum and Marder 1988). Immunocytochemical studies have shown that an RPCH-like peptide is present in all the ganglia of the stomatogastric system of *Panulirus* (Dickinson and Marder 1989). However, the effects

of RPCH on the pyloric rhythm in *Panulirus* have not yet been described.

In recent years, it has become clear that the pattern generators that underlie rhythmic behaviors in a variety of both vertebrate and invertebrate systems are not independent but instead interact in a variety of ways (Chrachri and Neil 1993; Chrachri et al. 1994; McFarland and Lund 1993; Meyrand et al. 1994; Weimann and Marder 1994). These interactions have been most extensively examined in the four motor pattern generators of the crustacean stomatogastric nervous system (for reviews, see Dickinson 1995; Dickinson and Moulins 1992). The functional boundaries of these pattern generators are fluid, and neurons can be shared between two networks, providing a pool from which appropriate combinations of neurons are selected to form the pattern generators needed at any given time, as is seen in the pyloric and gastric networks of the crab, *C. borealis* (Weimann and Marder 1994; Weimann et al. 1990, 1991). In other cases, single neurons can switch from one pattern generator to another, as occurs in *Palinurus vulgaris* (Hooper and Moulins 1989, 1990) and *P. interruptus* (Dickinson and Marder 1989), where neurons that commonly fire in pyloric or gastric time switch to fire in cardiac sac time. In more extreme cases, the gastric mill and cardiac sac patterns can fuse to form a single, conjoint pattern (Dickinson et al. 1990), or all of the stomatogastric pattern generators can be broken down and the neurons used to construct a novel pattern generator that is thought to control swallowing movements (Meyrand et al. 1991, 1994). Similar, though much less studied, interactions have been shown to occur in many other systems as well, including, for example, the respiratory and locomotor patterns in vertebrates (Viala 1986; for review, see Dickinson 1995).

Given that such interactions can occur, and in many cases are essential, to generate appropriate behavioral output, it seems likely that the different pattern generators might in some instances be modulated in concert rather than individually. Indeed, several neuromodulators or modulatory neurons have been shown to affect more than one network in the stomatogastric system. For example, the peptide proctolin co-activates the pyloric (Hooper and Marder 1987; Marder et al. 1986), gastric (Heinzel 1988; Heinzel and Selverston 1988), and cardiac sac (Dickinson and Marder 1989) pattern generators in the spiny lobster. The anterior pyloric modulator neuron modulates

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both the pyloric (Dickinson and Nagy 1983; Nagy and Dickinson 1983) and gastric (Dickinson et al. 1988; Nagy et al. 1988) pattern generators in the spiny lobster. Likewise, several of the modulatory commissural neurons influence both the pyloric and gastric mill patterns in the crab (Bartos and Nusbaum 1997; Coleman and Nusbaum 1994; Coleman et al. 1995; Norris et al. 1994, 1996; Nusbaum et al. 1992). RPH, like proctolin, can modulate more than one pattern, as seen by the fact that, in addition to activating the cardiac sac pattern, it can promote either the switching of neurons from gastric to cardiac sac time (Dickinson and Marder 1989) or the complete fusion of the cardiac sac and gastric mill patterns (Dickinson et al. 1990). Interestingly, the modulator dopamine, which activates the pyloric network, also alters the interactions between the cardiac sac and pyloric networks, essentially canceling the effects the cardiac sac normally exerts on the pyloric pattern and restoring a normal pyloric pattern even during intense cardiac sac bursting (Ayali and Harris-Warrick 1998).

The system we have chosen to study here presents an interesting opportunity to examine the actions of modulators on related interacting neural networks. In this system, there are several known interactions between two patterns, the cardiac sac pattern and the pyloric pattern, in spiny lobsters (Hooper and Moulins 1989, 1990; Moulins and Vedel 1977; Sigvardt and Mulloney 1982b). Additionally, both of these patterns are modulated by RPH in at least some species. We therefore examined the effects of RPH on the pyloric pattern in spiny lobsters to complement previous work documenting the peptide's influence on the cardiac and gastric networks of this species, and thus to give a more global view of the peptide's effects on this group of interacting neural networks.

METHODS

Experiments were conducted on a total of 82 male and female California spiny lobsters, *P. interruptus*, weighing 150–600 g. Animals were purchased from Marinus (Longbeach, CA) or Don Tomlinson Commercial Fishing (San Diego, CA) and were kept in recirculating seawater at 12–15°C for ≤ 6 wk before use.

Stomachs were removed from lobsters, and the complete stomatogastric system was dissected from the stomach wall (Selverston et al. 1976) and placed into cold *Panulirus* saline [composition (in mM/l): 479 NaCl, 12.8 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10 MgSO₄, 11 Trizma base, and 4.8 maleic acid; pH 7.5–7.6] in a silicone elastomer (Sylgard)-lined petri dish. Preparations included the four ganglia of the system (stomatogastric, STG; 2 commissural, CG; esophageal, OG), the connecting nerves, and the motor nerves, as shown in Fig. 1. The preparation was superfused with saline at 10–12 ml/min throughout the experiment. Temperature was maintained at 16–18°C using a Peltier cooling device.

The STG was desheathed to allow access to the cell bodies for intracellular recordings. Additionally, in some experiments, the stomatogastric nerve (*stm*) or the superior and inferior esophageal nerves (*sons*, *ions*) were desheathed so that conduction could be blocked with isotonic (750 mM) sucrose; in other experiments, these nerves were cut to block conduction irreversibly. A petroleum jelly wall across the dish allowed the STG to be superfused separately from the other ganglia.

Standard electrophysiological techniques were used in all experiments. Nerves were recorded extracellularly using A-M Systems AC amplifiers and stainless steel pin electrodes isolated from the bath with petroleum jelly. The same electrodes were used for nerve stimulation via a switch box. For intracellular recordings, we used glass microelectrodes filled with 2.5 M K Acetate or 3 M KCl (tip resistances:

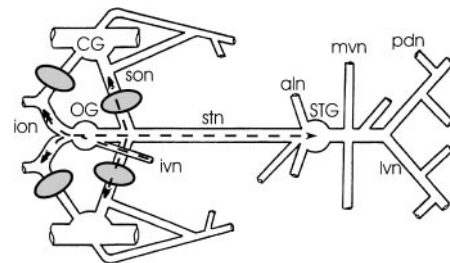


FIG. 1. The stomatogastric system of the spiny lobster. Both the superior and inferior esophageal nerves (*sons* and *ions*) were cut or blocked with isotonic sucrose (shaded ovals) to simplify the system and decrease modulatory inputs in all experiments. Axon trajectories of the inferior ventricular (IV) neurons (cell bodies located in supraesophageal ganglion, not shown and not present in experimental preparations) shown as dotted lines. CG, commissural ganglion; *ivn*, inferior ventricular nerve; *lvn*, lateral ventricular nerve; *mvn*, medial ventricular nerve; *pdn*, pyloric dilator nerve; OG, esophageal ganglion; STG, stomatogastric ganglion; *stm*, stomatogastric nerve.

10–30 M Ω), and WPI M707 microprobe systems, A-M Systems DC amplifiers, or an Axoclamp 2B. Data were recorded on a Gould TA4000 recorder and on videotape with a Vetter VCR adapter. In some experiments, data were taken directly onto a PC or Macintosh computer using PowerLab (AD Instruments) software for analysis. Current for controlling intracellular membrane potentials as well as for extracellular stimulation was from a Grass S88 stimulator via stimulus isolation units.

RPH, purchased from Peninsula, was dissolved in 5% dimethyl sulfoxide (DMSO). Deionized water was added to make a 10^{-3} M solution, which was kept frozen as a stock solution. It was diluted to the final concentration of 10^{-6} M in *Panulirus* saline just before use. At this RPH concentration, DMSO was at a final concentration of 0.005%, which had no effects in control experiments.

Data were analyzed using two-tailed paired Student's *t*-tests with GBStat (Dynamic Microsystems, Silver Spring, MD) or a binomial test, as indicated in the text. All *n* values reported refer to the number of preparations analyzed.

RESULTS

Since neurons originating in the commissural ganglia (CGs) release a number of neuromodulators into the STG that act in concert on the pyloric network, most of our experiments were conducted with input from the CGs removed, as shown in Fig. 1. This was accomplished either by cutting the *ions* and *sons* or by blocking conduction in those nerves. Under these conditions, the cardiac sac pattern is never spontaneously active, and the pyloric pattern is less active than in the unblocked condition; thus excitatory effects, particularly those that are state dependent (Dickinson and Nagy 1983; Nusbaum and Marder 1989), are clearer and more easily studied.

The pyloric pattern in RPH (10^{-6} M) displayed different patterns of activity in two time domains after the start of superfusion. Initially, the pyloric pattern alone was enhanced (Fig. 2A), while later, once the cardiac sac pattern was activated (see Fig. 5), the pyloric pattern assumed a more complex form because of the influence of the cardiac sac network on the pyloric network. In a first step to assess the influence of RPH on the pyloric network itself, we examined the changes that occurred in the pyloric pattern before the cardiac sac pattern was initiated.

While the effects of RPH may not have been maximal in the relatively short time (≤ 11 min after the onset of RPH application) before the cardiac sac pattern was activated, it was

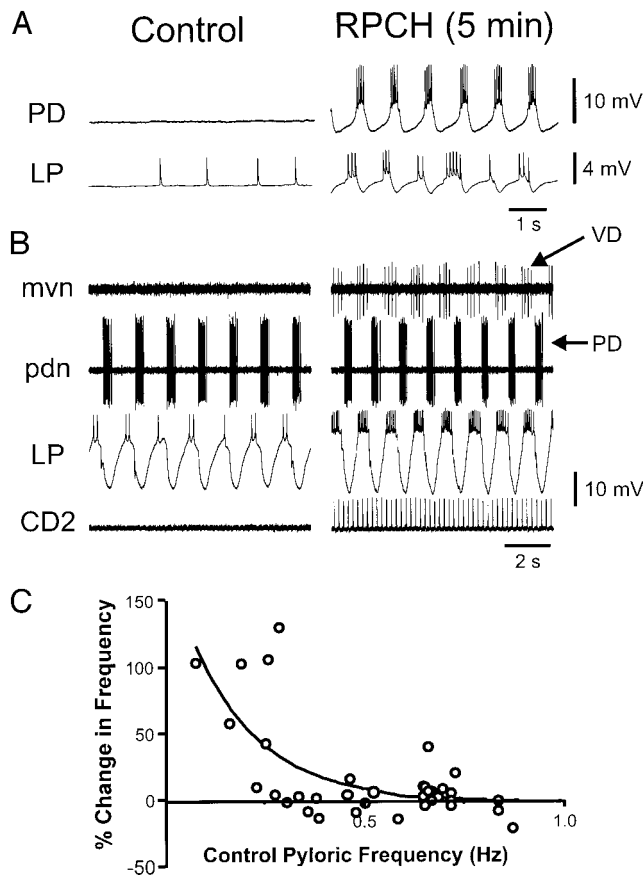


FIG. 2. Bath application of 10^{-6} M red pigment concentrating hormone (RPCH) causes state-dependent enhancement of the pyloric rhythm. *A*: in those preparations in which the pyloric pattern was inactive in the control, RPCH caused an activation of the pattern. *B*: in preparations already showing spontaneous pyloric activity, the pyloric pattern was intensified. *C*: cycle frequency increased in RPCH, in a frequency-dependent manner, such that the extent of the increase was dependent on the cycle frequency before the RPCH application, i.e., the control frequency. Cycle frequencies were measured 7–8 min after the onset of RPCH superfusion. $n = 38$ preparations. CD2, cardiac sac dilator neuron 2; LP, lateral pyloric neuron; PD, pyloric dilator neuron; VD, ventricular dilator neuron.

nonetheless clear that the peptide directly activates the pyloric network (Fig. 2). In the presence of RPCH, preparations that did not express spontaneous pyloric activity began to display a robust pyloric pattern (Fig. 2*A*), and pyloric activity increased in those preparations that were already cycling (Fig. 2*B*). This was seen, for example, as a significant increase in cycle frequency [from 0.53 ± 0.05 to 0.58 ± 0.04 (SE) Hz, $n = 38$, paired *t*-test; $P = 0.02$]. However, this increase was state dependent and varied as a function of control frequency (i.e., frequency before RPCH was applied; Fig. 2*C*). Thus, in preparations that were cycling slowly, cycle frequency increased substantially (by 46%, from 0.24 ± 0.02 to 0.35 ± 0.05 Hz in the slowest 25% of preparations, $P = 0.017$, paired *t*-test, $n = 10$), while it increased little or not at all in those preparations that were already cycling at a relatively high frequency.

In the presence of RPCH, but with no cardiac sac pattern, activity in both the pyloric dilator (PD) and lateral pyloric (LP) motor neurons increased significantly as measured by both spike frequency within pyloric bursts and by the number of spikes per burst (Fig. 3*A*; LP spike frequency, $P = 0.003$; PD spike frequency, $P = 0.032$; Fig. 3*B*, LP spikes/burst, $P =$

0.039 ; PD spikes/burst, $P = 0.002$; paired *t*-test, $n = 17$). Additionally, burst duration increased in both the LP and PD neurons (Fig. 3*C*; LP, $P = 0.027$; PD, $P = 0.012$; paired *t*-test, $n = 17$). It should be noted that in nearly half of the preparations (30 of 74), the LP neuron was silent in the control. In 17% of these 30 preparations, the LP was activated by RPCH. Neither burst duration nor spike frequency within bursts increased significantly in the ventricular dilator (VD) neuron, although the number of spikes per burst increased significantly, as it did in the other neuronal types (Fig. 3; $P = 0.020$; paired *t*-test, $n = 14$). Activity in the inferior cardiac (IC) neuron also increased in some preparations ($n = 2/11$), though both IC and PY were usually silent in both control (IC, $n = 10/11$; PY, $n = 30/34$) and RPCH (IC, $n = 9/11$; PY, $n = 28/34$).

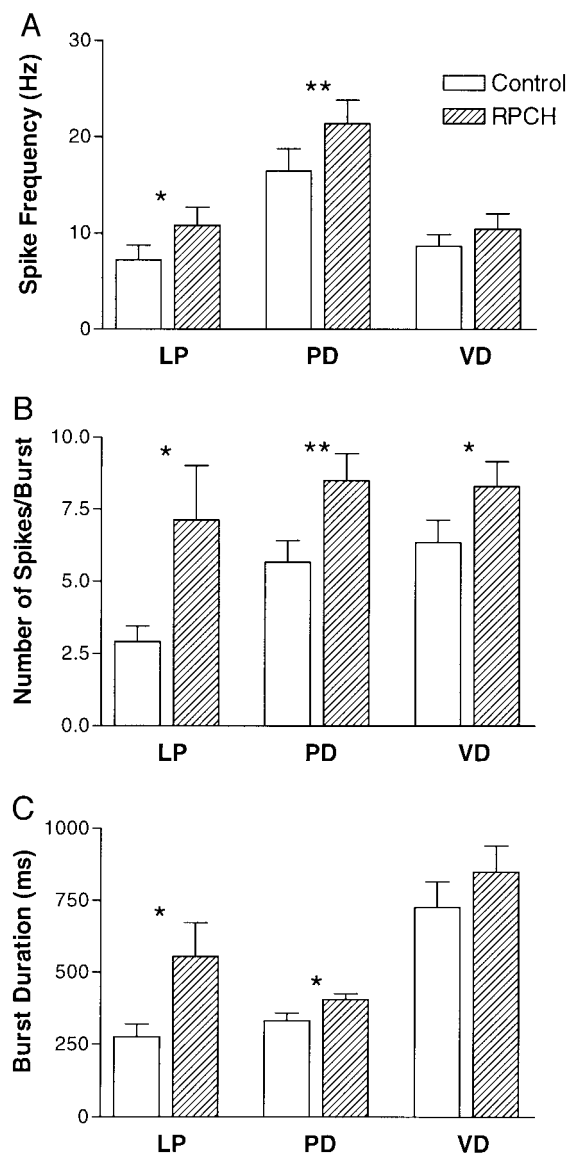


FIG. 3. Changes in burst parameters of the PD, LP, and VD neurons in RPCH, but in the absence of any cardiac sac pattern. *A*: spike frequency within bursts increased significantly in RPCH in the PD and LP, but not the VD, neurons. *B*: number of spikes per burst increased significantly in RPCH in the PD, LP, and VD neurons. *C*: burst duration increased significantly in RPCH in the PD and LP, but not the VD, neurons. $n = 17$ for PD and LP, 14 for VD. *, difference between control and RPCH significant at $P < 0.05$; **, difference between control and RPCH significant at $P < 0.01$.

An additional change seen in RPCH without cardiac sac activity was in the phase relationships of the PD, LP, and VD neurons (Fig. 4, $n = 7$; measured only in preparations in which all 3 neurons were active in the control). While the phases in which the PD and VD neurons were active did not change significantly, the end of the LP neuron burst was relatively later in the cycle in RPCH than in the control (paired t -test, $P = 0.01$). Consequently, the LP burst was relatively longer in RPCH than in control saline (paired t -test, $P = 0.01$). Additionally, the onset of firing in the VD neuron was in phase with that of the LP neuron in RPCH. As a result, the LP and VD bursts were closer to being in phase with one another in RPCH than in control, in which the VD burst began before that of the LP neuron (paired t -test, $P = 0.008$).

Effects of RPCH when the cardiac sac pattern is active

Later during RPCH superfusion, the pyloric pattern became complex because the peptide eventually also activates the cardiac sac pattern (Dickinson and Marder 1989) and because the cardiac sac pattern itself, via synapses from the inferior ventricular (IV) neurons onto a number of pyloric neurons (Russell and Hartline 1981; Sigvardt and Mulloney 1982a), can then influence the pyloric pattern (Moulins and Vedel 1977). Instead of the regular alternation of bursts in the pyloric neurons seen in the control (Fig. 5A), the pyloric pattern recorded after longer exposure to RPCH included regular interruptions in cycling, coincident with dilator bursts recorded in cardiac sac neurons (CD2 neuron, Fig. 5B). Although the details of the complex pattern differed considerably between preparations, RPCH generally enhanced the pyloric pattern, particularly in preparations that showed weak pyloric activity under control conditions (i.e., normal saline, with anterior inputs blocked). One reflection of this enhancement was a highly state-dependent increase in average pyloric cycle frequency, measured between cardiac sac bursts (Fig. 6). Thus in those preparations in which the initial cycle frequency was low, cycle frequency increased significantly [from a mean of 0.24 ± 0.02 to 0.42 ± 0.03 Hz ($P < 0.0001$, paired t -test, $n = 11$) in preparations in which initial cycle frequency was <0.35 Hz, and from a mean

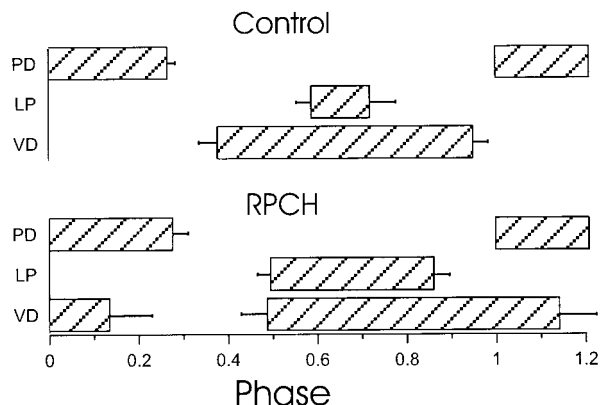


FIG. 4. Phase changes in the pyloric pattern in the absence of cardiac sac activity. The phase of the PD neuron was unchanged, while the LP neuron fired for longer. Additionally, the LP neuron started firing earlier in the cycle and the VD neuron started firing later in the cycle in RPCH, in contrast to the much earlier onset of VD firing in the control. Each bar represents the mean and SE of measurements from 7 experiments (10 cycles each).

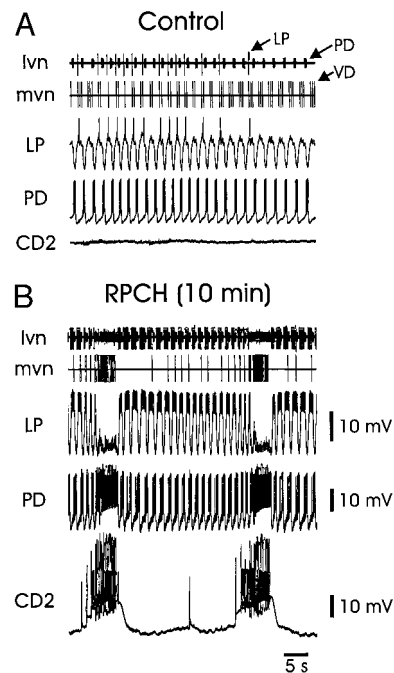


FIG. 5. Long-term exposure of the STG to RPCH activates the cardiac sac network and results in a complex pyloric pattern. *A*: in control conditions, pyloric cycling was regular, with short bursts in the PD neuron in each cycle. The LP neuron showed rhythmic membrane potential oscillations, largely due to synaptic inhibition from the PD neuron, but fired a single spike in only about half the cycles. The VD neuron, recorded on the *mvn*, fired 2–3 spikes per cycle. The cardiac sac dilator neuron 2, CD2, was silent. *B*: after 10 min in 10^{-6} M RPCH, bursts were more intense in the LP and PD neurons, and activity in the VD neuron (seen here on the *mvn*) differed considerably from that recorded in control. During each cardiac sac burst, indicated by the bursts in the cardiac sac motor neuron CD2, pyloric cycling was interrupted. In this preparation, the PD and VD neurons fired synchronously with CD2, while the LP neuron was inhibited and remained hyperpolarized for the duration of the cardiac sac burst. After each cardiac sac burst, pyloric activity was very strong. The VD neuron lost nearly all of its pyloric timing and fired primarily in cardiac sac time. The apparent changes in spike amplitude in the PD and CD2 neurons during cardiac sac bursts are due to the enhancement of the excitatory postsynaptic potentials (EPSPs, from the IV neurons) that drive these bursts. Thus the EPSPs become extremely large and can even surpass in amplitude the spikes, which are passively propagated into the somata, and therefore relatively small, in these neurons. Abbreviations as in Figs. 1 and 2.

of 0.33 ± 0.03 to 0.43 ± 0.02 Hz ($P = 0.027$, $n = 21$) in preparations in which cycle frequency was less than the mean of all initial cycle frequencies in control preparations with anterior inputs blocked, i.e., <0.53 Hz]. There was, however, no significant increase in cycle frequency in those preparations

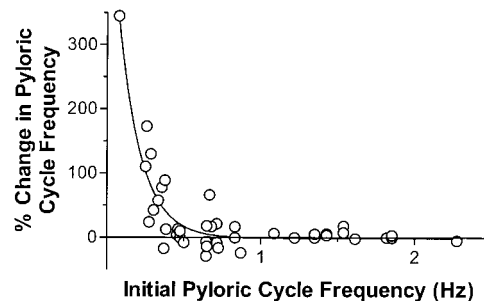


FIG. 6. Increase in pyloric cycle frequency in RPCH was state dependent with large increases in preparations that were cycling slowly in the control, but little or no increase in those preparations that were already cycling at >0.3 Hz. $n = 64$ preparations.

that were already cycling at higher frequencies (>0.53 Hz). It should be noted that because blocking the input from the commissural ganglia decreases the level of activity in the pyloric network, 80% of unblocked preparations (i.e., 16 of 20) fell into the latter category, whereas only 27% (i.e., 12 of 44) of the blocked preparations burst at these higher frequencies in control saline.

During cardiac sac bursts, seen in Fig. 5 as bursts of action potentials in the cardiac dilator 2 motor neuron (CD2), both the PD and VD neurons fired tonically, while the LP neuron was silent. Moreover, as was the case in RPCH before the onset of cardiac sac bursting, the PY and IC neurons (not shown) were completely silent in both control saline and RPCH in most preparations (PY, $n = 28/34$; IC, $n = 9/11$). At the end of a cardiac sac burst, the first pyloric neuron to fire after a cardiac sac burst was in most cases ($n = 33/38$) the LP. This was then followed by alternating bursts in the PD and LP neurons.

Between cardiac sac bursts, pyloric activity was not constant but instead gradually changed with time. Thus, although average pyloric cycle frequency, measured between cardiac sac bursts, was enhanced as described in the preceding text, these average frequencies are in some respects misleading. In fact, cycle frequency remained constant between cardiac sac bursts in only about half of the preparations, while it gradually increased in the others (see Fig. 7). Likewise, levels of activity within most of the pyloric neurons changed gradually between cardiac sac bursts. In most cases, pyloric activity, as measured by spike frequency and burst duration in the LP and PD neurons, was maximal either immediately after each cardiac sac burst or within the first two pyloric cycles after the cardiac sac burst ($n = 24/39$). Pyloric activity then gradually diminished until it either ceased or was interrupted by the next cardiac sac burst (Fig. 8, A–D), though these changes in burst characteristics over time were much less pronounced in the PD than in the LP neuron. In some cases ($n = 15/39$), however, as in the example shown in Fig. 5B, peak pyloric activity (i.e., maximum burst duration and amplitude in the LP neuron) was reached only after several pyloric cycles (3 cycles in 8 preparations; 4 cycles in 7 preparations).

In contrast to the PD and LP neurons, in which bursting activity was strongest within a few cycles after a cardiac sac burst, the VD neuron fired only weakly, if at all, immediately after a cardiac sac burst ($n = 44$). Instead, the VD neuron was often silent for a prolonged period after a cardiac sac burst, then gradually resumed its pyloric activity ($n = 21/44$; Fig. 5B; *mvn*). Thus, in most cases ($n = 27/44$), both spike frequency and burst duration increased with time during the cardiac sac interburst interval in the VD neuron (Fig. 8, E and F). In 31% of experiments ($n = 12/39$), however, there was little change in spike frequency with time after a cardiac sac burst. In the remaining five (of 44) preparations, VD remained entirely silent between cardiac sac bursts. It should be noted that, in many cases ($n = 21/44$), as in the one shown in Fig. 5B, the activity of the VD neuron was only marginally time-locked to the pyloric rhythm between cardiac sac bursts.

Role of the IV neurons

One major question raised by these results is the extent to which the effects of RPCH on the pyloric pattern are due to the peptide itself and the extent to which they are indirect, due to

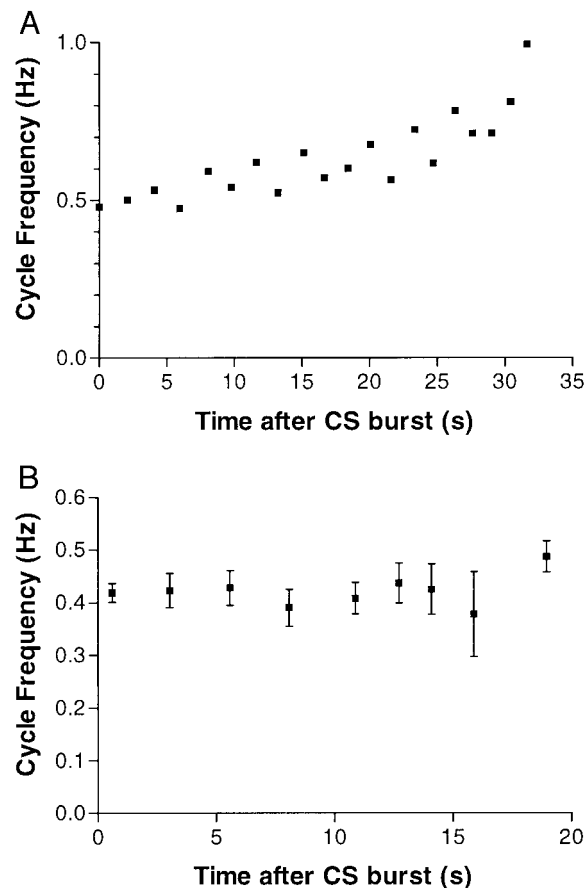


FIG. 7. Changes in pyloric cycle frequency as a function of time after a cardiac sac burst. The data shown represent the range of patterns seen in 27 preparations, but because of the variation between preparations, each represents data from a single preparation. A: data from 1 experiment representative of the $\sim 50\%$ of preparations in which cycle frequency gradually increased, with a concurrent decrease in burst duration. B: data from 1 experiment representative of the $\sim 50\%$ of preparations in which cycle frequency and burst duration did not change extensively after a cardiac sac burst. In B, error bars represent the SE of values from 10 cardiac sac cycles.

the activation of the cardiac sac pattern by RPCH. Because the IV neurons, which begin bursting in the presence of RPCH (Dickinson et al. 1993), synapse directly onto a number of pyloric neurons (Sigvardt and Mulloney 1982a), a number of the effects of RPCH on the pyloric network are certainly indirect and due to these synaptic potentials.

We addressed this question in two ways. First, in preparations with CG inputs blocked to remove other modulatory inputs, we examined the effects of RPCH on the pyloric pattern before peptide-induced cardiac sac bursting started. As described in the preceding text, there were very clear effects of RPCH on the pyloric pattern as well as on characteristics of the firing of the LP neuron as is discussed later. Thus it is evident that RPCH exerts at least a part of its effects directly on the pyloric network.

The second way we addressed the contribution of the IV neurons to the RPCH activation of the pyloric pattern was by electrically stimulating the *ivn* in a burst pattern similar to that occurring during RPCH-evoked cardiac sac activity (average spike frequency, 21.4 Hz; average burst duration, 6.5 s). By stimulating the *ivn* in repeated bursts (5-s duration, 20 Hz, once every 30 s) in control saline, we produced a complex pyloric

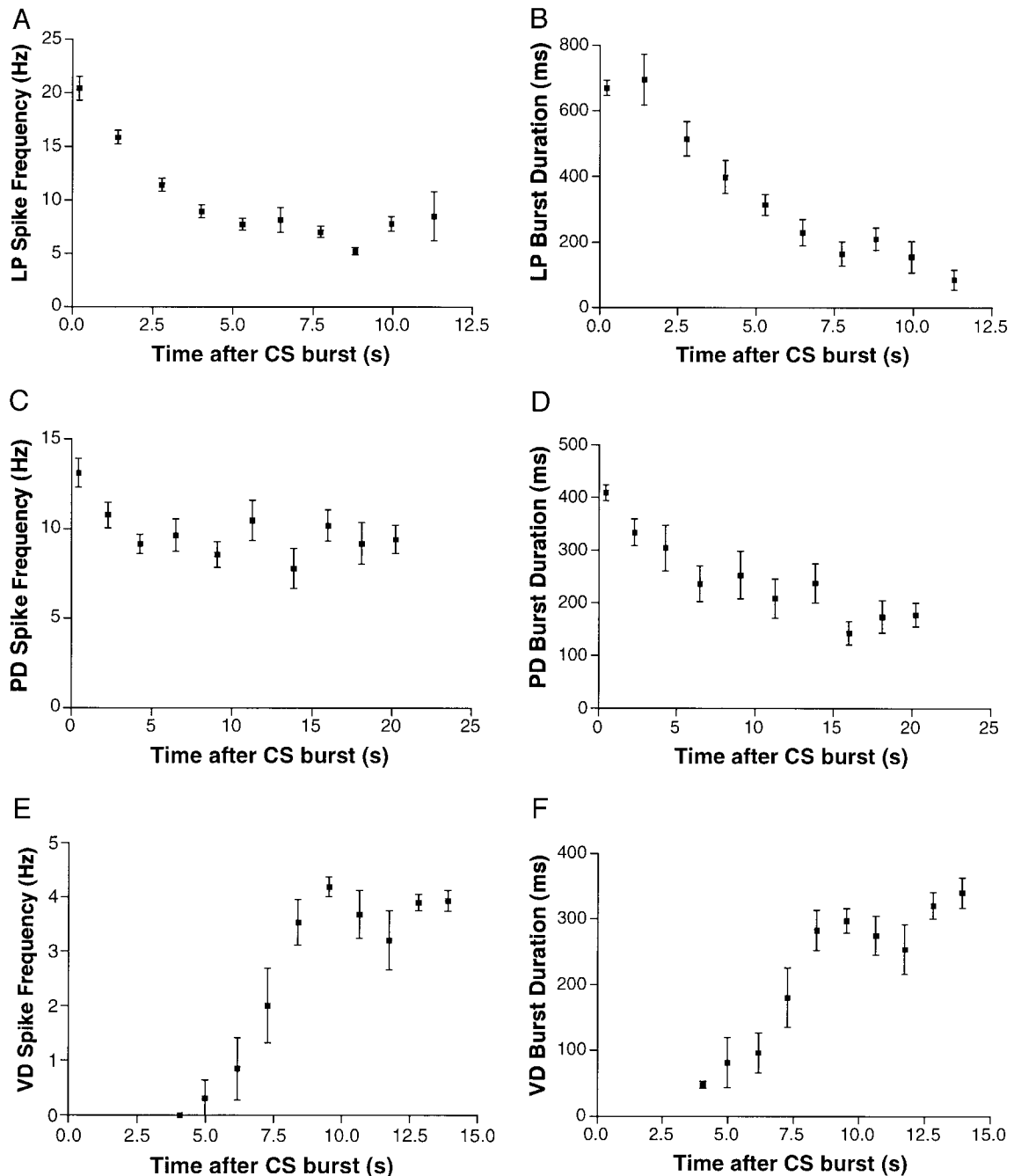


FIG. 8. Changes in spike frequency and burst duration between cardiac sac bursts in the 3 major pyloric neurons that are active under the conditions used in these investigations (i.e., *stm* blocked, with or without RPCH). In the LP neuron, both spike frequency (A) and burst duration (B) were greatest immediately after a cardiac sac burst, then gradually decreased with time to a new lower level. Similar trends were seen in the PD neuron (C and D), although the changes tended to be considerably smaller, and were less consistent from preparation to preparation. The VD neuron was silent for some time after the cardiac sac burst and only gradually resumed its pyloric activity, with both spike frequency (E) and burst duration (F) increasing with time after each cardiac sac burst. Each graph represents the averages of spike frequency or burst durations measured after 10 cardiac sac bursts in RPCH; *time 0* is the end of the cardiac sac burst. Data from the LP, PD, and VD neurons shown are from 3 different preparations.

pattern that was strikingly similar to that seen during superfusion with RPCH ($n = 12$; Fig. 9). Both the number of spikes per burst and spike frequency increased in the LP neuron immediately after each stimulated *ivn* burst ($P = 0.009$ for number of spikes/burst, $P = 0.015$ for spike frequency, paired *t*-tests, $n = 7$), then gradually decreased as they did after the cardiac sac bursts in RPCH. (See Table 1 for a comparison of burst parameters in the 2 bursts before and after each *ivn*

stimulation.) However, LP burst duration did not increase significantly. In the PD neurons, spike frequency, number of spikes per burst, and burst duration all increased after the stimulated *ivn* bursts, then gradually decreased ($P = 0.0004$ for spike frequency; $P = 0.0005$ for number of spikes/burst; $P = 0.043$ for burst duration, paired *t*-tests, $n = 8$; Table 1). In contrast, both burst duration and number of spikes per burst in the VD neurons decreased after stimulated *ivn* bursts ($P =$

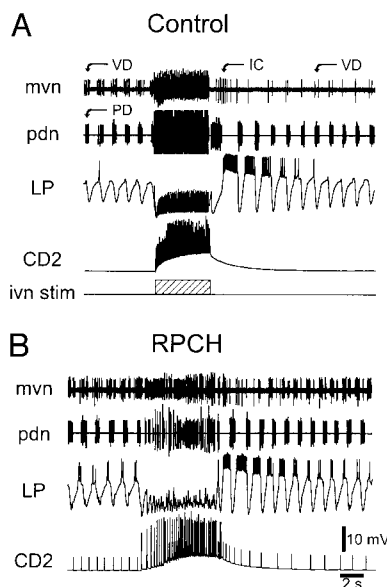


FIG. 9. The pyloric pattern in RPCH (B) is strikingly similar to that recorded in control (A) when the IV neurons, which normally drive the cardiac sac pattern, are stimulated in a burst (5 s, 20 Hz) that is similar to the cardiac sac pattern triggered by RPCH (average spike frequency, 21.4 Hz; average burst duration, 6.5 s). In both cases, the LP neuron was inhibited during the IV/cardiac sac burst but fired strongly in pyloric time, in alternation with the PD neuron, after the end of the burst. In this preparation, the IC neuron (larger spike on *mvn*) fired briefly after IV spiking, while the VD neuron remained silent for several seconds. One notable difference between the pattern observed in RPCH and that resulting from *ivn* stimulation, however, is that the PD neuron fired first after the stimulated *ivn* burst (A), whereas the LP neuron was first to fire strongly after the cardiac sac burst in RPCH (B).

0.036 for number of spikes/burst; $P = 0.048$ for burst duration, paired t -tests, $n = 6$) though average spike frequency did not change (Table 1). In 33% of preparations (4 of 12), the VD neuron completely stopped firing in the pyloric pattern between stimulated *ivn* bursts, instead firing only during the *ivn* stimulation.

These data suggest that the complex pyloric pattern recorded in RPCH was due largely to the effects of the synapses from the IV neurons. However, the pyloric pattern recorded when the *ivn* was stimulated in control saline was not identical to that recorded when the *ivn* was stimulated in RPCH (not shown) or when the IV neurons fired in spontaneous bursts in RPCH. In many preparations, one of the most striking differences was the order in which the LP and PD neurons resumed firing after a cardiac sac burst. After a stimulated *ivn* burst, the PD neuron fired strong bursts and was most often the first pyloric neuron to fire (75%; 9 of 12 preparations). During the PD burst, the LP neuron was inhibited and so only fired at the end of the PD burst (Fig. 9A). In contrast, after a cardiac sac burst in RPCH,

the LP neuron was, nearly always the first neuron to fire (87%; $n = 33$ of 38; Fig. 9B). It fired strongly after each burst, then gradually decreased its firing intensity.

RPCH enhances postinhibitory rebound in the LP neuron

Many of the effects of RPCH and *ivn* bursts on the pyloric pattern can be explained by the synaptic input the pyloric neurons receive from the IV neurons, but the LP neuron is not one of the neurons that has been shown to receive a direct postsynaptic potential (PSP) from the IV neurons (Sigvardt and Mulloney 1982a). In spite of this, it was strongly affected by the cardiac sac pattern in RPCH. The strong firing in the LP neuron after cardiac sac bursts might be explained by the enhanced inhibition it would receive from the PD neuron during such a burst, for the IV neurons powerfully and directly excite the PD neurons. However, this would not explain the fact that the LP neuron fired before the PD neuron in RPCH but after it in response to stimulated *ivn* bursts. Because postinhibitory rebound makes a major contribution to the firing of the LP neuron (Miller 1987), we postulated that RPCH might alter the extent or characteristics of postinhibitory rebound in the LP neuron. To determine whether this was the case, we held the LP neuron at a constant membrane potential using a second microelectrode, then injected pulses of hyperpolarizing current into the neuron in both control saline and RPCH. The resultant postinhibitory rebound was stronger in RPCH, as evidenced by a higher spike frequency and a larger depolarization after the hyperpolarizing pulses in RPCH compared with control (Fig. 10; spike frequency, $P = 0.006$; depolarization, $P = 0.003$; $n = 13$; binomial test).

RPCH potentiates synaptic potentials from the IV neurons to its pyloric targets

In addition to its direct effects on the LP neuron, RPCH enhanced the effects of the IV neurons on the pyloric pattern by potentiating the excitatory synaptic potentials from the IV neuron to the PD and VD neurons. To demonstrate this, we recorded the PSPs generated in response to *ivn* stimulation with an intracellular electrode in either the PD or VD neuron, while controlling the membrane potential of the neuron with a second electrode. The amplitude of the PSPs was substantially greater in RPCH than in control saline (Fig. 11), though this difference was significant (paired t -tests, $P < 0.05$ and $P < 0.01$) only at membrane potentials that were relatively hyperpolarized (more hyperpolarized than -60 mV for the PD neuron and -70 mV for the VD neuron) and thus further from the predicted reversal potential. Surprisingly, however, the amplitude of the PSPs in control saline appeared to remain constant in amplitude rather

TABLE 1. The effects of stimulating the *ivn* in control saline on burst parameters in the PD, LP, and VD neurons

	PD ($n = 8$ preps)			LP ($n = 7$ preps)			VD ($n = 6$ preps)		
	Before <i>ivn</i> stimulation*	After <i>ivn</i> stimulation*	P value (paired t -test)	Before <i>ivn</i> stimulation*	After <i>ivn</i> stimulation*	P value (paired t -test)	Before <i>ivn</i> stimulation*	After <i>ivn</i> stimulation*	P value (paired t -test)
Number of spikes/burst	6.8 \pm 1.1	18.1 \pm 1.5	0.0005	2.4 \pm 0.4	12.0 \pm 2.4	0.009	6.5 \pm 0.9	4.1 \pm 1.0	0.036
Spike frequency, Hz	17.5 \pm 2.9	40.2 \pm 3.7	0.0004	4.9 \pm 0.9	15.2 \pm 2.7	0.015	6.2 \pm 0.9	11.4 \pm 2.6	NS
Burst duration, ms	382.1 \pm 49.7	487.1 \pm 55.5	0.043	468.9 \pm 237.4	715.1 \pm 120.6	0.387	1059 \pm 243	364.0 \pm 74.0	0.048

* Averages of the 2 bursts just before and just after the *ivn* stimulation (5 s, 20 Hz bursts every 30 s) for 10 *ivn* stimulations in each neuron.

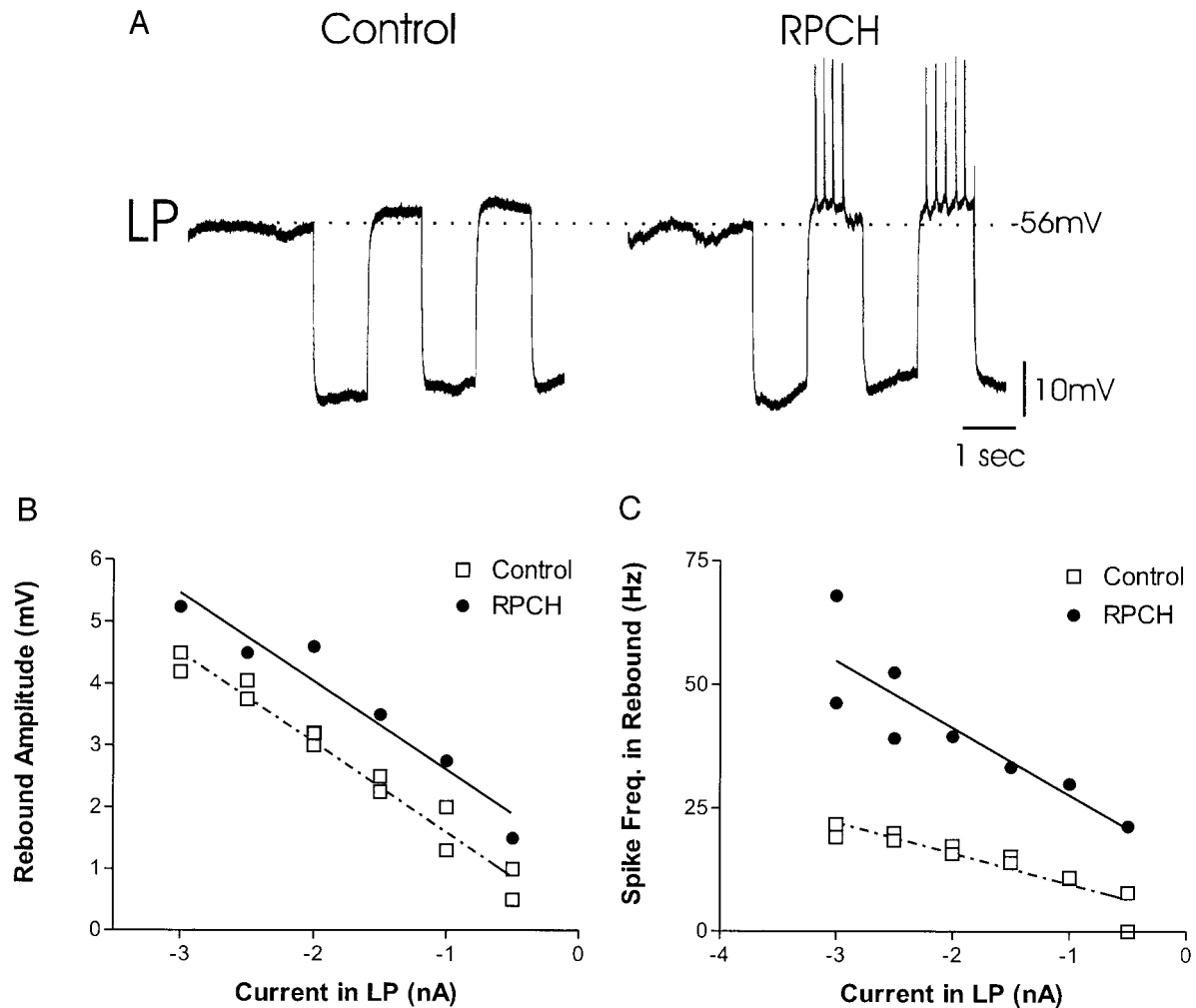


FIG. 10. RPCH enhanced postinhibitory rebound in the LP neuron. *A*: traces from an LP neuron in control saline and in RPCH. Repeated 1-s pulses of hyperpolarizing current (2 nA in traces shown) were injected into the LP via a 2nd electrode. The LP neuron depolarized further and fired a burst of action potentials in RPCH but not in control saline. *B*: when 5-s pulses of negative current (similar in duration to cardiac sac bursts) of different magnitudes were injected into an LP neuron, the depolarization beyond control membrane potential was greater in RPCH for all sizes of currents. *C*: on rebound from the same 5-s current pulses, the spike frequency of the action potentials recorded during rebound was greater for all amplitudes of currents injected.

than increasing with hyperpolarization as we would predict. One factor that may partially explain this phenomenon is the increased amplitude of the hyperpolarization-activated inward current, I_h , and the resulting decrease in membrane input resistance at hyperpolarized membrane potentials. However, this current was clearly present in RPCH as well as in control saline, so this cannot be the only explanation.

DISCUSSION

For a number of years, the stomatogastric system has served as a model in elucidating many fundamental principles regarding the functioning of rhythmic motor pattern-generating networks (Marder and Calabrese 1996; Pearson and Ramirez 1992). In the present study, we used this system to continue our investigations into the ways that neuromodulatory substances can alter, in concert, a number of different, but functionally related, motor patterns. The role of neuromodulators in determining behavioral output is extensive; they can specify a particular physiological configuration from an anatomically defined but physiologically flexible neuronal network (Getting

1989; Marder 1991), thus playing an important role in the selection of motor patterns that are appropriate for a given set of conditions. By simultaneously affecting more than one motor program, neuromodulators also have the potential to alter suites of related behaviors in concert. Moreover, motor pattern generators interact in a variety of systems (e.g., vertebrate respiration and swallowing or locomotion, McFarland and Lund 1995, 1996; Viala 1986; *Aplysia* feeding, Perrins and Weiss 1996; goldfish escape and swimming, Svoboda and Fetcho 1996; for review, see Dickinson 1995), and, as we have shown here, and has been seen previously (e.g., Ayali and Harris-Warrick 1998; Dickinson et al. 1990; Hooper and Moulins 1989, 1990), neuromodulators can alter the strength of such interactions. Consequently modulators may also play key roles in coordinating related behaviors or in preventing incompatible behaviors from occurring simultaneously.

Thus, in the present case, RPCH not only activates the cardiac sac motor pattern (Dickinson and Marder 1989) and causes the gastric mill and cardiac sac patterns to fuse, thus creating a novel conjoint pattern (Dickinson et al. 1990), but it

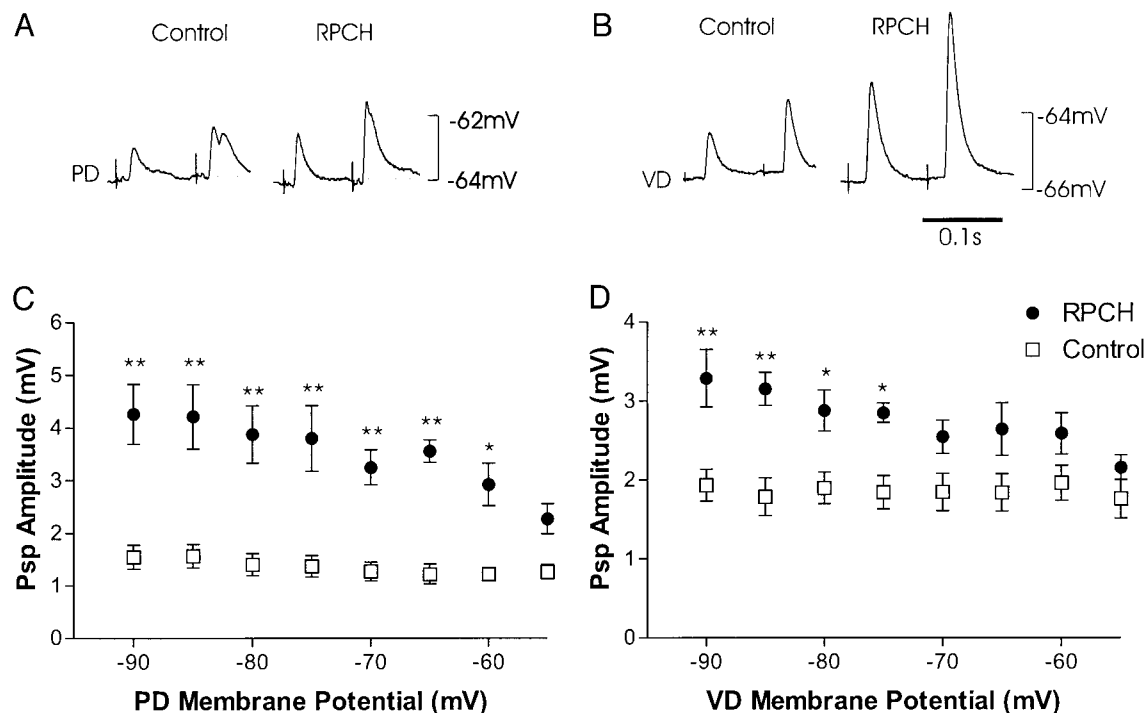


FIG. 11. RPCH causes potentiation of synaptic potentials from the IV neurons onto the PD and VD neurons. In single experiments, PSPs recorded in PD (A) and VD (B) in response to pairs of current pulses delivered to the *ivn* increased in amplitude. C and D: data pooled from 8 PD (C) and 7 VD (D) neurons with the membrane potential held at a variety of levels ranging from -55 to -90 mV show an increase in PSP amplitude. *, PSP amplitude differed significantly between control and RPCH, $P < 0.05$; **, a significant difference at $P < 0.01$.

also activates the pyloric pattern (as it does in crabs; Nusbaum and Marder 1988) and enhances the effects of the cardiac sac pattern on the pyloric pattern. Consequently, the motor behavior of an entire anatomical part (the foregut) of the animal is altered. The fact that similar interactions between motor patterns have been seen in other systems under a variety of conditions suggests that the existence of such interactions—and perhaps more importantly, the possibility of changes in such interactions, controlled by either hormonal or neuronally released modulators—is widespread among nervous systems.

Interestingly, we found that the time course of the modulation of the cardiac sac and pyloric patterns by RPCH differed. Early during a bath application of RPCH, the pyloric pattern alone was modulated. The cardiac sac pattern was activated later and so did not begin to influence the pyloric pattern until later in the perfusion. This difference in timing could be due to different thresholds, with that for the pyloric pattern being lower, so that it was enhanced before the concentration reached its final value of 10^{-6} M in these experiments. It might also be attributed to different mechanisms in the two systems. The cardiac sac pattern might, for example, involve a longer and/or slower second messenger pathway. Regardless of the mechanism, this difference in the time of onset of these different effects of RPCH has important functional consequences: it allows a single modulator to produce a series of sequential changes in the overall motor output.

Once the cardiac sac pattern was activated, the pyloric pattern became more complex, with regular interruptions of pyloric bursting followed by the return of strong pyloric bursting, which then gradually diminished. In some respects, the overall pattern resembled a pyloric rhythm with a cardiac sac

pattern superimposed on it. This contrasts with the conjoint pattern, in which two patterns have completely merged, that is seen when the gastric and cardiac sac patterns are recorded in RPCH (Dickinson et al. 1990). It should be noted that both the gastric mill and cardiac sac patterns were silent before RPCH application in the previous study, whereas in the experiments reported here, the pyloric pattern was generally ongoing while the cardiac sac pattern was silent. Moreover, even in those cases in which the pyloric network was initially silent, it was activated before the cardiac sac pattern, whereas gastric mill and cardiac sac activity began simultaneously in RPCH, with the two patterns fused from the onset of rhythmic activity.

As is the case with the gastric-cardiac sac interactions in RPCH, a major mechanism responsible for the increased influence of the cardiac sac pattern on the pyloric pattern was the increased amplitude of the PSPs from the IV neurons that drive the cardiac sac pattern onto several pyloric neurons. These synapses, previously described by Sigvardt and Mulloney (1982a,b), Russell and Hartline (1981) and Claiborne (1983, 1984), are shown diagrammatically in Fig. 12. In particular, the IV neurons synapse onto the PD and VD neurons. In both cases, the synapses are primarily excitatory and are potentiated by RPCH. In addition, however, the PD neurons are subject to a delayed inhibition during high-frequency firing (Sigvardt and Mulloney 1982a,b) and display enhanced bursting after the end of a train of IV PSPs (Russell and Hartline 1981). The pattern of activity in the PD neurons can be largely explained by this combination of effects as follows. During the cardiac sac burst, the PD neurons were usually driven one for one by the larger amplitude excitatory PSPs (EPSPs) they received from the IV neurons. After the cardiac sac burst, bursting in the PD neurons

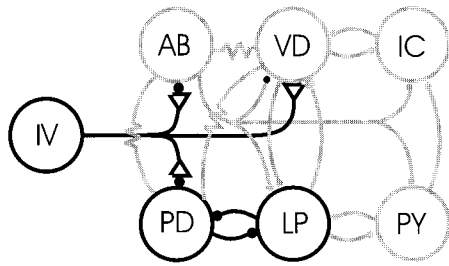


FIG. 12. The pyloric circuit and known synapses from the IV neurons onto pyloric neurons. Relevant synaptic connections shown in black, others in gray. The IV neurons synapse directly onto the anterior burster (AB), PD, and VD neurons, but not onto the LP neuron. Filled circles indicate inhibitory synapses, open triangles indicate excitatory synapses. Mixed synapses shown with both symbols. IV synapses in diagram based on data from Sigvardt and Mulloney (1982a).

was enhanced, as always occurs after a burst in the IV neurons. There is at present no clear evidence that the burst enhancement in RPCH differs from that in control saline. As this burst enhancement decreased with time, so did the bursting in the PD neurons and the entire pyloric pattern, until it was once again interrupted by the next cardiac sac burst.

The pattern of activity in the VD neurons, which fired vigorously during each cardiac sac burst, was likewise due at least in part to the enhanced PSPs from the IV neurons. Again, the large IV PSPs were able to drive the VD neurons to fire a higher frequency burst, generally one for one with the IV neurons. After the burst, the VD neurons were silent for some time before gradually resuming their activity. Additionally, it is possible that the plateau properties in the VD neurons of *Panulirus*, like those of *Palinurus*, were suppressed by synaptic input from the cardiac sac pattern generator (Hooper and Moulins 1989, 1990). In contrast to the case in both the LP and PD neurons, in which neuronal activity is enhanced by RPCH both in the presence and absence of a cardiac sac pattern, pyloric activity in the VD neuron decreased in RPCH during cardiac sac activity, but increased slightly (as evidenced by an increased number of spikes per burst) in RPCH when the cardiac sac pattern was not activated. This suggests that the cardiac sac pattern (presumably via the IV neurons) more strongly influences the activity of the VD neuron than does RPCH itself.

Both the PY and the IC neurons were generally silent both in the control, with the *stm* blocked, and during bath application of RPCH. Neither neuron receives a PSP from the IV neurons (Sigvardt and Mulloney 1982a), and these data suggest that RPCH does not directly affect either neuron.

The pyloric neuron whose activity in RPCH is perhaps most intriguing is the LP neuron. The LP neuron does not receive a direct PSP from the IV neurons, so its firing cannot be attributed to enhancement of that synaptic activity. In addition, we noted that the activity of the LP neuron was enhanced even before the onset of cardiac sac activity, as well as in preparations in which RPCH concentration was subthreshold for the cardiac sac pattern, but still enhanced pyloric activity. At least three separate factors contribute to the LP neuron's enhanced firing. First, postinhibitory rebound, which occurs when the anterior burster (AB) and PD neurons stop firing and thus release the LP neuron from inhibition, plays a major role in the onset of bursts in the LP neuron (Miller 1987). The strong bursts generated by the PD

neurons when they were driven by cardiac sac bursts in the IV neurons produced strong inhibition in LP, leading in turn to strong LP neuron rebound. Second, Manor et al. (1997) have shown that the synapses from the LP to the PD neurons are depressed during ongoing pyloric activity. During the long inhibition of the LP neuron that occurs during the IV and PD neuron bursts in RPCH, that depression would be removed. Thus, when the LP neuron was released from the PD inhibition at the end of the cardiac sac burst, it was able to inhibit the PD neuron more strongly than in previous cycles. This in turn would lead to a greater inhibition of the LP neuron and consequently a stronger postinhibitory rebound on the next cycle. As the synaptic depression set in once again and the enhanced rebound decreased, the intensity of both inhibition and of postinhibitory rebound would gradually decrease, resulting in the observed decrease in pyloric intensity. Third, we showed here that RPCH also enhanced postinhibitory rebound itself in the LP neuron. This would at least partially account for the enhanced LP neuron activity in RPCH before the cardiac sac pattern was initiated as well as the enhanced activity between cardiac sac bursts after that pattern had been activated. In combination with the enhanced inhibition due to recovery from depression, enhanced postinhibitory rebound would be expected to result in stronger and longer bursts in the LP neuron, as were observed. While we have not yet examined the mechanisms that might be responsible for the increased postinhibitory rebound in the LP neuron, the sag voltage clearly increased in 9 of the 13 preparations in which we measured postinhibitory rebound (unpublished data; see also Fig. 10A), suggesting the possibility that a hyperpolarization-activated inward current (I_h) might contribute to the enhanced rebound.

Thus, these results clearly illustrate the ways in which multiple mechanisms can contribute to the modulation and altered coordination of multiple motor networks. Using several mechanisms and acting on several parts of the stomatogastric networks, including both synaptic sites and membrane properties, a single neuropeptide (RPCH) is able to alter several neural circuits in concert. Consequently, the activity of three networks, namely the pyloric, gastric mill, and cardiac sac networks, as well as the interactions between them, are altered, resulting in a motor output that is quite different from that observed in the absence of this neuromodulator. Additionally, we have shown that because it can activate different patterns with different time courses, a single modulator, even if released hormonally and thus synchronously onto more than one neural network, can lead to progressive changes in both a single rhythmic motor pattern and a group of interacting motor patterns. Finally, these data demonstrate that not only can neurons (e.g., the LP neuron) within a network be influenced, via network interactions, by other neurons (e.g., the command-like IV neurons) with which they have no direct connections, but also that these indirect effects can be enhanced by changes in the membrane properties of the network neurons themselves as well as by changes in other parts of the network.

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