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Neuromodulation of the Crab Pyloric Central Pattern Generator by Serotonergic/Cholinergic Proprioceptive Afferents

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In the stomatogastric nervous system of the crab, *Cancer borealis*, a set of 4 serotonergic/cholinergic proprioceptive neurons, called gastropyloric receptor (GPR) cells, have effects on the pyloric motor pattern. In a semi-intact foregut preparation, the GPR cells are not activated by movements of the pyloric filter; instead they respond to the slower movements of the gastric mill (Katz et al., 1989). Thus, their activity is not synchronized to the pyloric motor pattern. However, when the GPR cells are stimulated in an *in vitro* preparation in a manner that resembles their normal firing pattern, they produce dramatic effects on the pyloric motor pattern. These effects include: (1) a prolonged increase in the pyloric cycle frequency, (2) a momentary pause in the motor pattern, (3) transient inhibition of some motor neurons, (4) strong excitation of other motor neurons,and (5) altered phase relationships of the different components of the motor pattern. These changes in the motor pattern are due to direct effects of the GPR cells on neurons in the pyloric central pattern generator (CPG). All of the cells in the pyloric circuit appear to receive GPR input. However, only 2 neurons receive detectable rapid nicotinic synaptic potentials. The other neurons receive only slower neuromodulatory input from GPR stimulation. The neuromodulatory effects include burst enhancement, plateau potential enhancement, excitation, and inhibition. These modulatory effects are largely mimicked by bath-applied serotonin (5-HT). Thus, primary sensory neurons can alter the production of motor patterns by a CPG through a phase-independent mechanism; these proprioceptors do not need to fire at a precise time in the cycle to be effective because their effects are mediated through the slower actions of the neuromodulator 5-HT.

Rhythmic motor patterns produced by central neural circuits must be constantly modified to fit changing conditions. Until recently, this modification was thought to occur through 3 general pathways. First, it could be directed by phasic synaptic input from other CNS neurons, such as command neurons (Kupfermann and Weiss, 1978), higher-order neural centers (Grillner, 1981; Armstrong, 1986), and coupled oscillators (Grillner, 1981; Robertson and Moulins, 1981a, b, 1984; Cohen, 1987; Nagy and Moulins, 1987). Second, it could be orchestrated by neuromodulatory input from the CNS, either directly through neural pathways or indirectly through the action of circulating neurohormones (Grillner, 1981; Livingstone et al., 1981; Weiss et al., 1981; Harris-Warrick, 1988). Third, it could result from phasic sensory feedback from proprioceptors acting to correct the motor output on a cycle-by-cycle basis (Andersson et al., 1981; Pearson et al., 1983). We have recently demonstrated the existence of a fourth alternative, representing a combination of the latter 2 schemes: primary sensory afferents, called gastropyloric receptor (GPR) cells, can have direct prolonged neuromodulatory effects on neurons in the central pattern generator (CPG) circuits of the crab stomatogastric ganglion (Katz and Harris-Warrick, 1989a).

The GPR cells are 2 bilaterally symmetrical pairs of proprioceptive cells that sense the movements of certain muscles in the crab foregut (Katz et al., 1989). Biochemical, immunohistochemical, and pharmacological studies demonstrate that these cells use both 5-HT and ACh as cotransmitters (Beltz et al., 1984; Katz et al., 1989; Katz and Harris-Warrick, 1989a). We have previously shown that the GPR cells have direct synaptic effects on some neurons in the stomatogastric ganglion that can be ascribed to one or the other of these transmitters: (1) transient synaptic potentials with nicotinic cholinergic pharmacology, and (2) prolonged modulatory effects, including burst enhancement, which are not blocked by cholinergic antagonists, but are mimicked by 5-HT (Katz and Harris-Warrick, 1989a).

The stomatogastric ganglion (STG) is a small ganglion, containing only 30 neurons that comprise the components for 2 CPG circuits, the pyloric and the gastric mill circuits. These CPGs produce repetitive motor patterns that control the striated musculature of the pyloric filter and the gastric mill apparatus in the decapod crustacean foregut (see Selverston and Moulins, 1987). In semi-intact foreguts, the GPR cells respond to the relatively slow movements of the gastric mill, but not to those of the faster pyloric filter (Katz et al., 1989). However, they are able to affect the activity of neurons in *both* the gastric mill and pyloric circuits (Katz and Harris-Warrick, 1989a). In this paper, we examine the effects of the GPR cells on the rhythmically active pyloric CPG.

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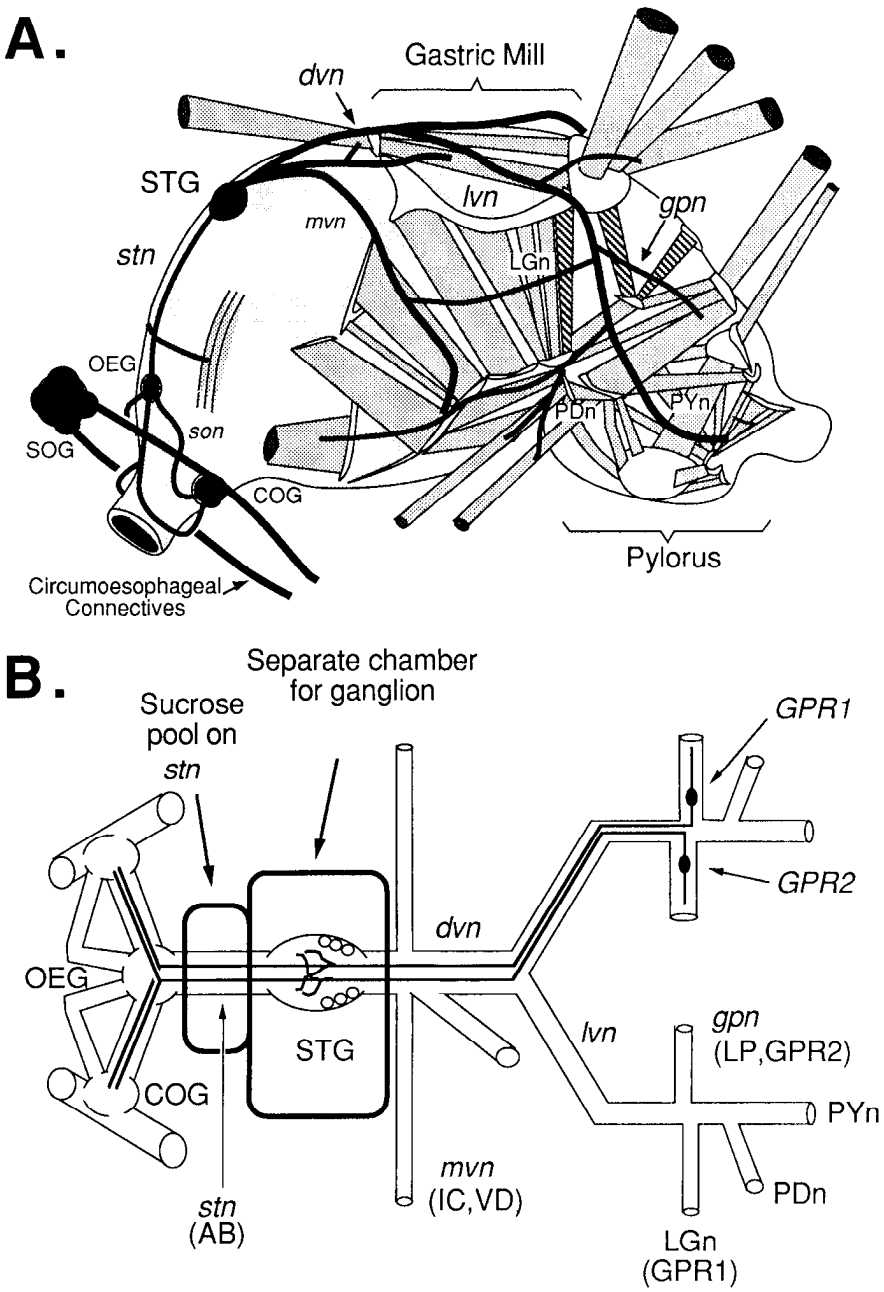

Figure 1. Schematic diagrams of the stomatogastric nervous system in the crab. *A*, The crab foregut, showing the locations of the major nerves with respect to gastric mill and pyloric musculature. The muscles that are innervated by the GPR sensory dendrites are highlighted. *B*, A schematic dorsal view showing the layout of the nerves as they appear in a Petri dish for physiological recording, and the projection pattern of the 2 GPR cell types. The STG neurons whose axons are in the various nerves are listed under each nerve. Abbreviations: The names of ganglia and neurons are capitalized. Nerves are indicated by lower-case, italicized letters, except in cases when a nerve contains the axon of a single motor neuron type; in those cases the nerve is named after that motor neuron [PD nerve (*PDn*) and PY nerve (*PYn*)]. *AB*, Anterior burster cell; *COG, commissural ganglion; dvn,* dorsal ventricular nerve; *gpn,* gastropyloric nerve; *GPR,* gastropyloric receptor; *IC,* inferior cardiac cell; *LGn,* lateral gastric nerve; *LP,* lateral pyloric cell; *lvn,* lateral ventricular nerve; *mvn,* medial ventricular nerve; *OEG,* esophageal ganglion; *PD,* pyloric dilator cell; *PY,* pyloric (constrictor) cell; *SOG,* superior esophageal ganglion; *son,* superior esophageal nerve; *STG,* stomatogastric ganglion; *stn,* stomatogastric nerve; *VD,* ventral dilator cell.

The pyloric circuit is one of the best-characterized CPG circuits. This ensemble of neurons is capable of generating a repetitive motor pattern in the absence of sensory input. However, when deprived of both sensory input and tonic modulatory inputs from other ganglia, rhythmic activity is dramatically reduced or silenced. A number of neuromodulatory substances, including 5-HT, restore rhythmic pyloric activity when added to *in vitro* preparations (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a; Marder, 1987; Marder et al., 1987). Each of these neuromodulators evokes a different motor pattern from the same anatomically defined neural circuit by differentially affecting the intrinsic properties of each neuron in the circuit (Flamm and Harris-Warrick, 1986b; Harris-Warrick and Flamm, 1987; Hooper and Marder, 1987). Therefore, although the synaptic connections between cells in the circuit provide a framework for pattern generation, the modifiable properties of each component neuron provide the substrate for production of variable motor patterns.

The GPR cells provide the sole source of neuronally released serotonin to the STG of the crab (Beltz et al., 1984; Katz et al., 1989). Thus, examining the effect of the GPR cells on the entire pyloric CPG is a first step toward understanding the behavioral context for serotonergic modulation in this system.

Portions of this work have previously been reported in abstract form (Katz and Harris-Warrick, 1989b, c).

Materials and Methods

Animals. Jonah crabs, *C. borealis,* were obtained from suppliers in Maine and Massachusetts. The crabs were kept in artificial seawater aquaria at 12–14°C.

Anatomy. A schematic diagram of the stomatogastric nervous system as it appears in the animal and in the dish is shown in Figure 1. The STG is located on the dorsal side of the foregut. It is connected to the

rest of the nervous system through the stomatogastric nerve (*stn*). The majority of the motor axons exit the STG posteriorly and project bilaterally through the various motor nerves to their respective muscles.

The projection pattern of the GPR cells is shown in Figure 1*B* (for clarity only the right pair is shown; the left pair is symmetrical). There are 2 GPR cell types, GPR1 and GPR2, that are distinguished by their muscle innervation pattern and physiological response properties (see Katz et al., 1989). GPR1 has sensory dendrites in the gm8b muscle, while GPR2 extends its dendritic arbor to 2 different muscles, gm9a and cpv3a (muscle nomenclature follows Maynard and Dando, 1974). Both GPR cells project to the STG, where they arborize extensively before projecting to each of the paired commissural ganglia (COG).

Physiology. All experiments were performed on the isolated stomatogastric nervous system, consisting of the COG, the esophageal ganglion (OEG), and the STG (Fig. 1*B*). The stomatogastric nervous system was dissected from the animal as described by Selverston et al. (1976), placed in a Petri dish at 14–17°C, and superfused with oxygenated crab saline (composition: 440 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 26 mM $MgCl_2$, 8 mM glucose, 11 mM Trizma base, and 5 mM maleic acid, pH 7.4). The STG was surrounded by a vaseline barrier, creating a small (~0.5 ml) separate chamber for the ganglion (Fig. 1*B*). Drugs were applied to this chamber by switching perfusion lines. The flow rate was 4–6 ml/min, allowing rapid equilibration owing to the small volume of the chamber. The STG was desheathed and intracellular recordings were made with glass microelectrodes (4 M potassium acetate plus 0.3 M potassium chloride, resistance 15–50 MΩ). Extracellular recordings were made with platinum pin electrodes or bipolar suction electrodes. Motor neurons in the STG were identified by a one-for-one correlation of intracellularly recorded action potentials with spikes recorded extracellularly on the appropriate motor nerve (Fig. 1*B*; Maynard and Dando, 1974; Hooper et al., 1986).

Following the identification of STG motor neurons, a vaseline pool on the *stn* (Fig. 1*B*) was filled with an isotonic sucrose solution in 0.1 M Tris-maleate buffer, which prevented action potentials originating in the anterior ganglia from reaching the STG (Russell, 1979). We confirmed that transmission on the *stn* was blocked by stimulating the superior esophageal nerve (*son*) and failing to see effects in the STG, or activation of the GPR cells. After applying the sucrose, we waited at least 1 hr before attempting any recordings, to allow any lingering neuromodulatory effects of these descending inputs to disappear.

The GPR cells send axons anteriorly through the *stn* to the COGs, and may affect STG neurons indirectly by altering descending input from these ganglia. Blocking transmission on the *stn* prevents the GPR impulses from reaching the anterior ganglia. Therefore, we can be certain that the effects of GPR stimulation on cells in the STG that we observe are direct and not mediated by cells in other ganglia.

To determine whether an effect of GPR stimulation is due to the release of acetylcholine, we used the nicotinic antagonists *d*-tubocurarine and decamethonium, and the muscarinic antagonists scopolamine and pirenzepine (0.1 mM). Previous work (Katz and Harris-Warrick, 1989a) showed that rapid EPSPs in 2 gastric mill neurons are blocked by a variety of nicotinic antagonists, including *d*-tubocurarine, decamethonium, hexamethonium, and mecamylamine, with the first 2 compounds having the strongest effects. The muscarinic antagonists had no effect on GPR-evoked EPSPs in the gastric mill neurons, but were effective at blocking activation of the pyloric system by the muscarinic agonist pilocarpine (0.1 mM) and did not affect the response to exogenous serotonin (Katz and Harris-Warrick, 1989a). Unfortunately, there is no known serotonergic antagonist that is effective in this system, so we could not directly test whether a response was due to the release of serotonin by the GPR cells. Instead, we examined the effects of bath-applied serotonin (10 μM), to determine if they were similar to the effects of GPR stimulation.

Picrotoxin (5 μM) was applied for at least 1 hr to block inhibitory glutamate synapses between cells in the STG (Bidaut, 1980; Eisen and Marder, 1982; Marder and Eisen, 1984; Marder, 1987). Picrotoxin, *d*-tubocurarine chloride, pirenzepine dihydrochloride, scopolamine methyl bromide, and serotonin creatinine sulfate were purchased from Sigma Chemical Co. All solutions were made immediately before use in each experiment.

Stimulus paradigm. Single GPR cells were stimulated extracellularly near their somata as previously described (Katz and Harris-Warrick, 1989a). These neurons are located in fine nerve branches that also contain axons of identified STG motor neurons. Careful placement of the stimulating suction electrode resulted in the GPR cell being the lowest threshold unit. Contralateral motor nerves were monitored to assure that this stimulation did not directly excite the motor neuron axons running in the same nerves.

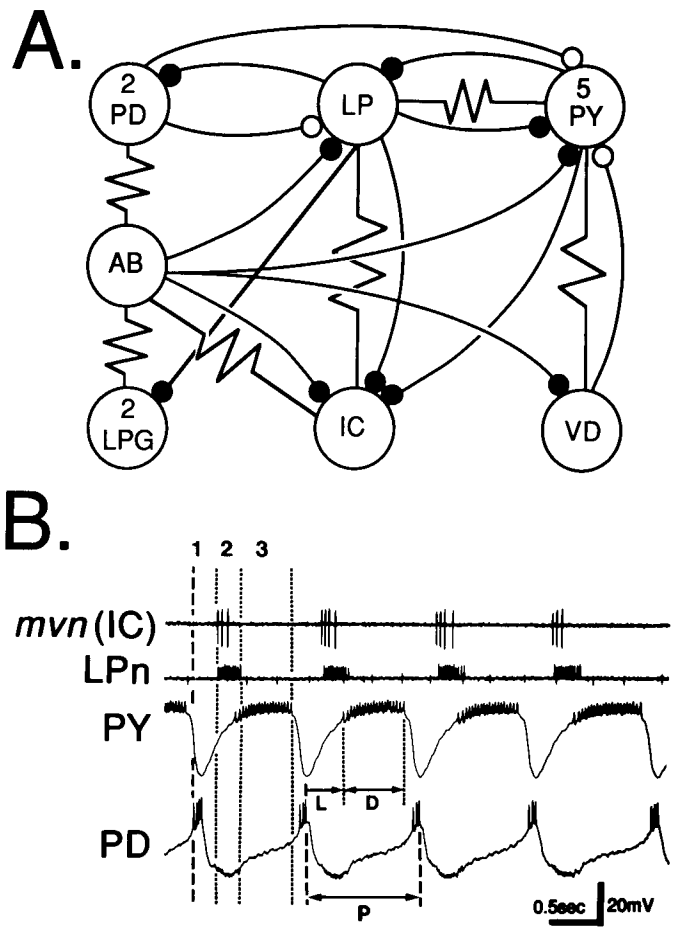

Figure 2. Wiring diagram and sample pattern for the pyloric CPG in the crab. *A*, This circuit diagram should be considered merely a functional circuit since the monosynapticity of some of these connections has not been verified (see text for discussion). Inhibitory chemical synapses are denoted by *circles*. *Filled circles* are PTX-sensitive glutamate synapses, and *open circles* are cholinergic synapses. Resistors indicate electrical coupling. Additional weak electrical coupling exists but is not shown here. The numbers above a cell name indicate how many cells there are of that cell type, if there is more than one. *LPG* is a gastric mill cell that is strongly coupled to *AB* and *PD* and thus behaves like a pyloric cell when the gastric mill is quiescent. *B*, Sample pyloric pattern. Extracellular recordings of *IC* (on the *mvn*) and *LP* (on the *LPn*) activity, combined with simultaneous intracellular recordings from *PY* and *PD* show the basic pyloric pattern (see text for details). VD is silent. The 3 phases of the cycle are separated by *dotted lines*. The various parameters describing the cycle are discussed in the text.

We stimulated the GPR cells in a manner that mimicked their normal activity. Recordings from semi-intact foreguts showed that the GPR cells fire in 2- to 5-sec volleys at frequencies between 10 and 20 Hz (Katz et al., 1989). Thus, to examine GPR effects on the pyloric pattern, we stimulated GPR cells at these frequencies *in vitro*. Note that we only stimulated a single GPR cell at a time, whereas in the animal 2–4 cells would probably be simultaneously active. Also, the effects of the 2 GPR cell types, GPR1 and GPR2, did not seem to be qualitatively different, so we will not discriminate between them.

Details about the pyloric circuit diagram. The pyloric circuit diagram (Fig. 2*A*) is based largely on the work done in the spiny lobster, *Panulirus interruptus* (Miller and Selverston, 1982; Miller, 1987), and work conducted in the crab by Marder and colleagues (J. Golowasch, P. Meyrand, M. Nusbaum, and J. Weimann, unpublished observations), as well as our own experiments.

There are some noteworthy differences between this circuit and the circuit in *Panulirus* that are important for understanding the results of

this paper. The first is the larger extent of electrical coupling in the crab. Because of the extensive electrical coupling between neurons in the crab STG, it is difficult in some cases to separate direct chemical synaptic effects from those occurring through an electrically coupled cell. This extensive electrical coupling also makes it extremely difficult to synaptically isolate pyloric neurons, as has been done in *Panulirus* (Miller and Selverston, 1982; Flamm and Harris-Warrick, 1986b; Hooper and Marder, 1987). Therefore, the indicated synapses should be viewed as functional connections rather than strict monosynaptic contacts. Electrical coupling between the lateral pyloric cell (LP) and inferior cardiac cell (IC), as well as between LP and the pyloric (constrictor) cell (PY), is important for determining the phase relationships of these cells. There is additional weak electrical coupling that is not shown in this circuit in order to simplify the diagram (Katz, unpublished observations; J. Weimann and E. Marder, personal communication). Second, inhibition of the PY cells by LP is weak. There are at least 5 PY cells in the crab, all of which fire in synchrony and appear to be electrically coupled (Hooper et al., 1986). There appear to be subgroups among the PY cells as there are in *Panulirus* (Hartline et al., 1987). The amount of inhibitory input from the LP cell differs among the PY cells (P. S. Katz, unpublished observations). Finally, Hooper et al. (1986) showed that some pyloric muscles in *Cancer,* which are homologous to PY muscles in *Panulirus* on the basis of position, are actually innervated by motor neurons that fire in phase with the 2 pyloric dilator cell (PD) motor neurons. These cells, which were previously named PD_{in} (Hooper et al., 1986) or s-PD (Hermann, 1979), seem to be homologous to the LPG motor neurons of *Panulirus* and will therefore be referred to as LPG (P. Meyrand, J. Weimann, and E. Marder, unpublished observations). To simplify the diagram, we have not included functional synaptic outputs of LPG; they are similar to those of PD.

Parameters used in analysis of the pyloric rhythm. Pyloric neurons fire repetitive bursts of action potentials with characteristic phase relations between the cells (Fig. 2*B*). Each cycle starts with the beginning of the PD anterior burster cell (AB) burst (Fig. 2*B*). The cycle period (*P*) is the time that elapses from one point in a cycle to the same point in the next cycle. In an ongoing pattern, the timing of the first spike of a PD burst is more variable than the timing of the last spike, because the slope of the ramp depolarization initiating a burst is less than the slope of the hyperpolarization terminating a burst (Fig. 2*B*). Therefore, the end of the PD burst provides the most precise time point from which to measure changes in cycle period (Fig. 2*B*). The cycle frequency is defined as the inverse of the cycle period. The burst duration (*D*) is the time from the first action potential to the last. The duty cycle, or fraction of the cycle occupied by a burst, is the burst duration divided by the cycle period.

The PD/AB group (which includes the AB interneuron and the PD and LPG motor neurons) inhibits all other cell types (Fig. 2*A*). Therefore, the end of the PD burst marks the end of the inhibition of the other cells in the circuit. The latency (*L*) of a cell is the time from the end of the PD burst to the beginning of that cell's burst and is an important factor in understanding the relative phasing of cell activity. Since phases are normally expressed with reference to the beginning of the cycle (that is, the beginning of the PD/AB burst), the onset phase was calculated as the latency divided by cycle period plus the PD duty cycle.

In some cases (such as Fig. 4), the pyloric cycle is interrupted by a GPR stimulus. In such cases, the time points until the next PD burst were excluded from measurements of duration, latency, and phase. Also, in Figure 4*A* (left) individual PD action potentials that did not cause a full LP hyperpolarization and were not followed by a rebound to an LP burst were not considered PD bursts.

Results

Description of the pyloric motor pattern

The basic pyloric motor pattern, seen when the STG is receiving input from the anterior ganglia via the *stn,* is essentially a 3-part rhythm (Fig. 2*B*). The AB interneuron and the PD and LPG motor neurons begin the cycle. They are followed after a short latency by the IC and LP motor neurons. The PY and ventral dilator cell (VD) motor neurons fire immediately after IC and LP.

This motor pattern arises from a combination of the intrinsic properties of the cells and the synaptic connectivity of the circuit. The cells in the circuit diagram (Fig. 2*A*) are drawn in the same order (from left to right) as the basic pyloric rhythm (Fig. 2*B*); the first cells active in the cycle are PD, AB, and LPG. They are strongly electrically coupled to each other and thus form a tight group which we will refer to as the PD/AB group. The PD/AB cell group is the major pacemaker of the pyloric circuit, primarily due to the intrinsic oscillatory properties of the AB interneuron. Together, these cells inhibit all the other cell types, thereby resetting the cycle and controlling its frequency. The IC and LP motor neurons are the first cells to rebound from PD/AB inhibition. This is due mainly to the intrinsic properties of these cells which cause rapid postinhibitory rebound (PIR; Hartline, 1979; Miller, 1987). After rebounding, LP inhibits the PD/AB group, prolonging the hyperpolarization of these cells and helping to regulate the cycle frequency. The PY and VD cells are the last to rebound from PD/AB inhibition and fire in phase with each other. The PY latency is partially caused by LP inhibition, but is mainly the result of an intrinsic "delaying conductance" that resembles the transient potassium current, I_A, (Hartline, 1979; Harris-Warrick, 1989). The PY cells then inhibit the LP and IC motor neurons, thereby terminating the second phase of the cycle and removing the inhibition of the PD/AB group by the LP cell. The PY cells continue to fire until they fatigue or are inhibited by the PD/AB group and the cycle begins again.

Thus, the intrinsic membrane properties of the cells (bursting in the PD/AB group, strong PIR in LP and IC, and delaying conductance in PY), combined with the synaptic connectivity between the cells, produce the basic pyloric motor pattern. Cells that are electrically coupled and have similar membrane properties fire in phase, whereas those that are mutually inhibitory fire out of phase.

Effects of GPR stimulation on the pyloric motor pattern

The basic pattern shown in Figure 2*B* is produced when spontaneous neuromodulatory inputs from other ganglia to the pyloric CPG are active. In all of our experiments, action potential propagation on the *stn* was blocked with an isotonic sucrose solution, eliminating these descending modulatory inputs. Under these conditions, rhythmic pyloric activity usually became slow and irregular (Fig. 4*A*, left) and occasionally ceased completely (Fig. 8*A*). Stimulation of the GPR cells with the *stn* blocked thus allowed us to examine their effects when the circuit was not being simultaneously modulated by other inputs and allowed us to be certain that the effects we observed were due to direct effects of the GPR cells on neurons in the STG.

GPR stimulation superimposes a gastric mill-like rhythm on the ongoing pyloric pattern

In a semi-intact foregut preparation, the GPR cells are responsive to gastric mill movements (Katz et al., 1989). Thus, multisecond volleys of GPR spikes normally arrive every 10–15 sec in phase with the repetitive gastric mill rhythm. In the isolated crab STG preparation, the gastric mill is always inactive (P. S. Katz, unpublished observations; E. Marder, personal communication). We therefore mimicked the effect of gastric mill movements in the *in vitro* preparation by directly stimulating a single GPR cell in repetitive 3-sec trains every 10 sec (Fig. 3).

Figure 3 shows the effect of repetitive GPR trains on an actively cycling pyloric rhythm. Periodic GPR activity had several effects on the ongoing pyloric pattern which occurred only during the GPR train: (1) IC and PY neurons transiently stopped

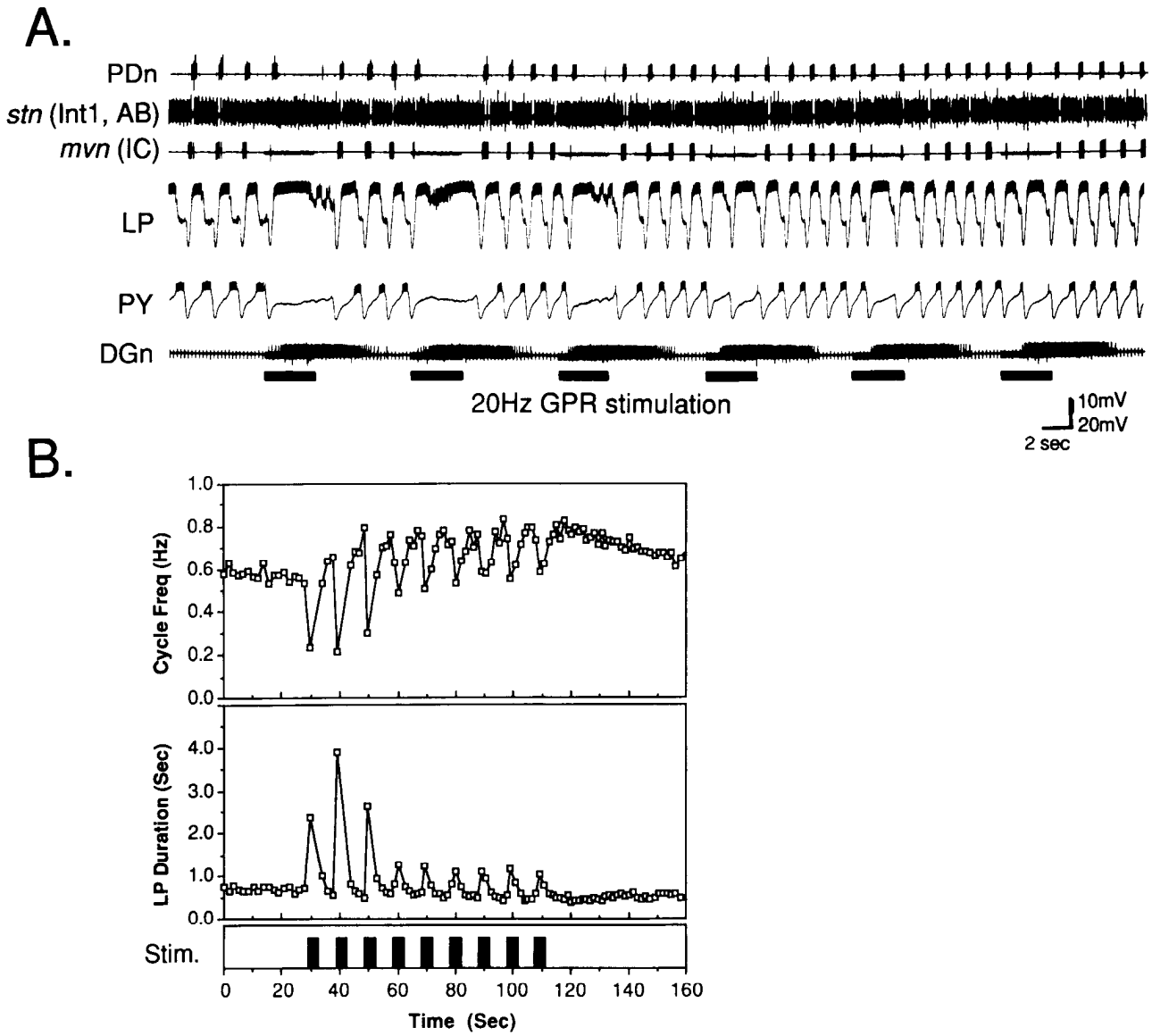

Figure 3. Periodic GPR stimulation causes repetitive modulation of the pyloric rhythm. *A*, A GPR cell is stimulated for 3 sec at 20 Hz every 10 sec (*bars*). This results in a periodic inhibition of *IC* and *PY*, a periodic excitation of *LP*, and a periodic pause in the pyloric cycle. Each GPR train triggers a plateau potential in the gastric mill neuron, *DG* (Katz and Harris-Warrick, 1989a). *B*, Cycle frequency and *LP* duration are plotted versus time for the example in *A*. The GPR stimulus trains are shown as *bars* above the time axis. The traces in *A* show only the first portion of the trial that is displayed graphically in *B*.

firing; (2) the LP motor neuron was transiently excited; and (3) the pyloric pattern periodically paused during each GPR stimulus train. In addition to these transient effects, there were also longer-term cumulative effects of GPR stimulation: (1) the cycle frequency gradually rose, and only slowly declined after the cessation of GPR stimulation; and (2) the transient effects during each GPR train became less pronounced with repetition. As the cycle frequency increased with repeated GPR stimulus trains, the pause induced by each GPR train became less pronounced (Fig. 3*B*, top). At the same time, the increase in LP duration during the later stimulus trains became less pronounced than during the first 3 trains (Fig. 3*B*, bottom). In addition, IC and PY ceased to be completely inhibited during the GPR train; instead of dropping out of the pattern, they merely fired fewer spikes during the later GPR stimulus trains. The magnitude of the GPR effect on the pyloric pattern also depended on the spike frequency of the GPR train itself. A 10-Hz GPR train was less effective at causing each of the transient effects than a 20-Hz train (not shown). Thus, in the intact animal, the effect of rhythmic GPR input may be strongly dependent on both the strength of the GPR activity and the level of ongoing pyloric activity.

GPR stimulation changes many parameters in the pyloric pattern

We further characterized the effects of GPR stimulation on the pyloric pattern by examining the effects of individual stimulus trains. The effect of GPR stimulation on the pyloric pattern in a weakly active preparation is shown in Figure 4 and diagrammatically in Figures 5 and 6. In this experiment, two 5-sec GPR

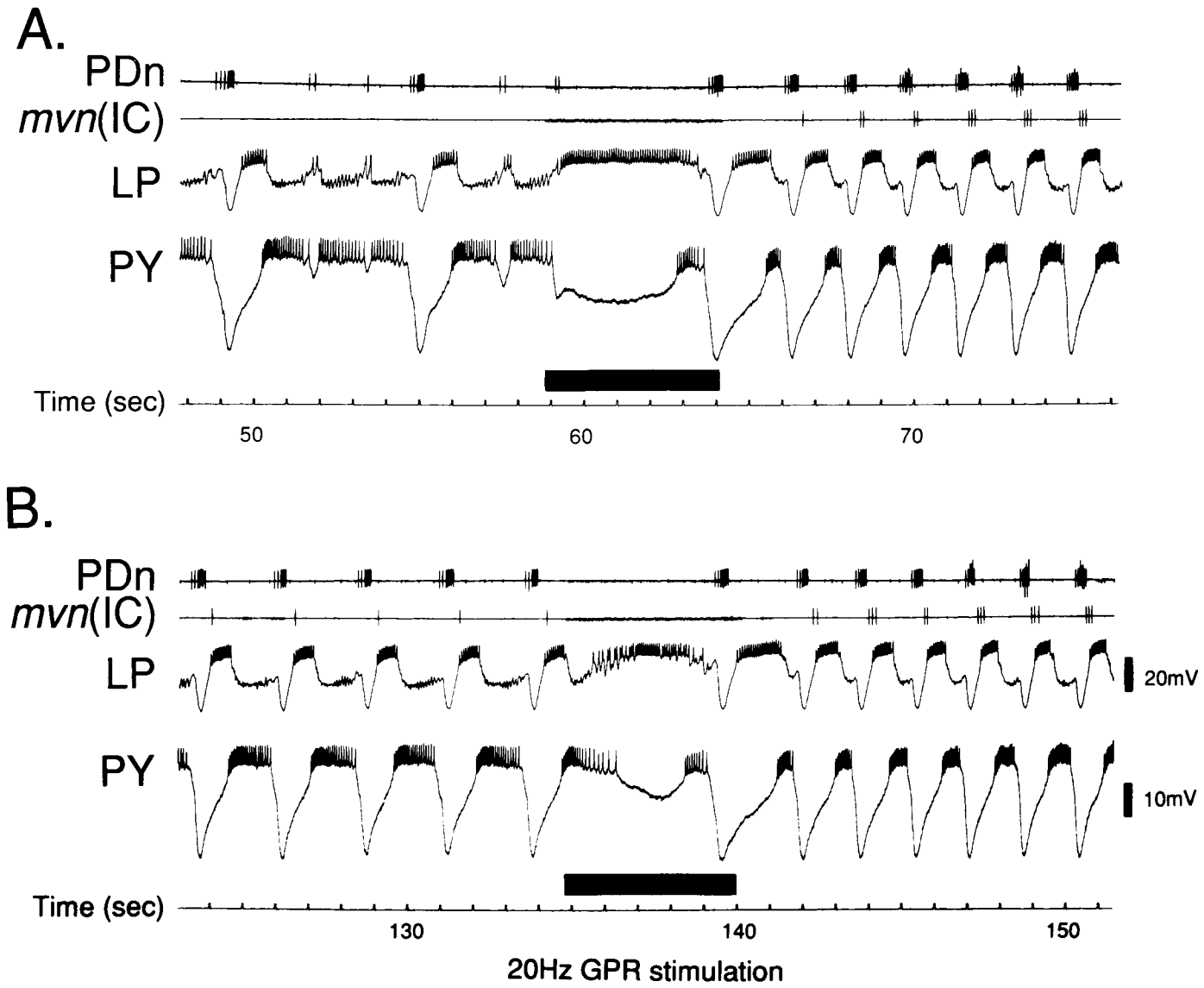

Figure 4. Effects of GPR stimulation on the pyloric motor pattern. *A*, The pyloric pattern is initially slow and irregular. One 5-sec, 20-Hz GPR stimulation (*bar*) causes a pause during which *LP* is excited and *PY* is inhibited. Following the stimulation, cycle frequency and regularity increase. The *IC* neuron (spikes on *mvn*), which was silent prior to GPR stimulation, fires bursts of action potentials in advance of each *LP* burst. *LP* burst duration is transiently increased, while *PY* burst duration is transiently decreased. *B*, One minute following the first GPR stimulus train, the cycle frequency and cell activity are still elevated. A second 20-Hz GPR stimulus train interrupts the ongoing cycling, then enhances it. The time line for *A* and *B* is continuous and corresponds to the time scale in Figure 5.

stimulus trains were presented 76 sec apart, as shown by the time scale beneath the traces. The effects on the pyloric pattern in this example illustrate the types of changes that GPR stimulation induces.

An afferent volley of GPR impulses had 6 major effects on the pyloric pattern:

1. The cycle frequency and regularity increased for over a minute following each GPR stimulus train (Figs. 4, 5*A*). Prior to the first stimulus, the pattern was slow and irregular. Following the first stimulus train, the pyloric rhythm became very regular and the cycle frequency increased to 0.6 Hz. The cycle frequency gradually declined, but remained above prestimulus levels for over 1 min. Thus, at the time of the second stimulus train, the pattern was still cycling regularly at 0.4 Hz. The second stimulus train again elevated the cycle frequency to 0.6 Hz. The cycle frequency reached its peak within 5 cycles after each GPR stimulus train. The activity of the cells in the pattern became more uniform as the cycle frequency increased, particularly after the first stimulus.

2. In many preparations, there was a disruption of the ongoing pattern during the GPR stimulus train (Fig. 4). This was particularly evident during the second stimulus because the activity prior to stimulation was more regular than it was prior to the first stimulus (Fig. 4*B*). During the GPR stimulus train, the LP motor neuron was highly excited and the PD/AB burst was delayed. At the same time, the PY motor neurons were inhibited. The appearance of this disruption was strongly dependent on the state of the motor pattern and the frequency of the GPR stimulus (as discussed below). In preparations where the PD/AB group was more strongly active than the LP neuron, GPR stimulation did not disrupt or slow the pattern (see Fig. 9*A*).

3. The latency of PY bursts transiently increased following

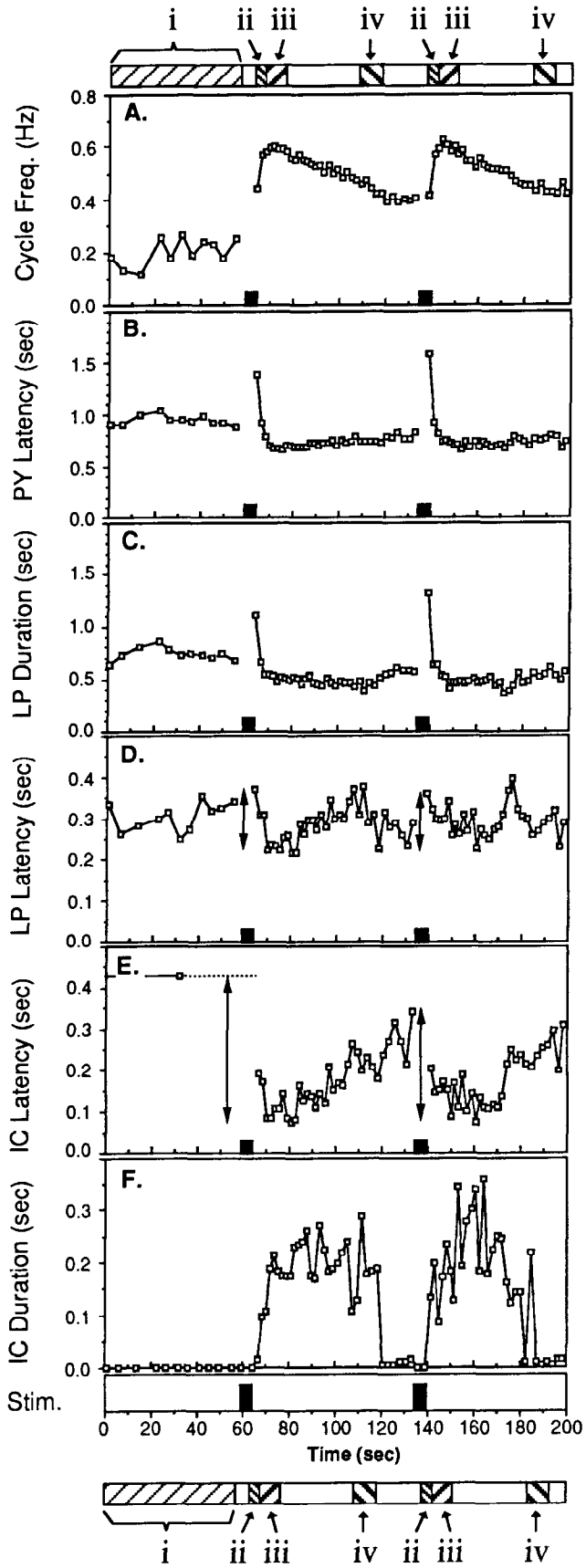

Figure 5. Diagrammatic representations of parameters that change due to GPR stimulation (from the experiment shown in Fig. 4). *A*, The 2, 5-sec, 20-Hz GPR trains are shown as *solid bars* over the *time scale* at the bottom of each figure. The time shown on the *time scale* corresponds to the time shown in Figure 4. The *striped bars* labeled i–iv at the top and bottom of the figure indicate the time periods that were included in the averages shown in the phase diagrams in Figure 6. A discontinuity in the graph indicates times when the cycle was interrupted during GPR stimulation. *A*, Cycle frequency increases following each GPR train. The cycle frequency remains elevated for over a minute following each stimulus train. *B*, PY onset latency transiently increases following the 2 GPR trains. *C*, LP burst duration transiently increases following GPR stimulation. *D*, There is no consistent change in the latency of LP bursts following GPR stimulation. The *double arrows* show the magnitude of the maximum difference in LP latency. This difference is within the variation seen without GPR stimulation. *E*, The latency of IC bursts decreases following each GPR stimulation. The magnitude of the maximum change in latency following GPR stimulation is shown by the *double arrows*. The latency of IC prior to the first GPR train is based on a single spike that occurred in this time period, but is consistent with measurements made in other weakly active preparations where IC exhibited some spiking prior to GPR stimulation. *F*, GPR stimulation causes IC to fire multispike bursts. Prior to GPR stimulation, IC is silent or firing a single spike per cycle. The duration of IC bursts remained elevated for up to 1 min after each GPR stimulus train.

each GPR stimulus train (Fig. 5*B*): following the GPR stimulus, it took longer for PY to rebound from PD/AB inhibition. The PY latency returned to a steady state after 1–3 cycles. The steady-state latency following each stimulus was slightly shorter than the latency measured before the initial stimulus.

4. LP burst duration transiently increased (Fig. 5*C*). As with PY latency, LP burst duration rapidly shortened within 2 or 3 cycles following GPR stimulation to a level slightly below pre-stimulation levels.

5. The effect of GPR stimulation on LP latency depended on the initial state of LP. In this preparation (Fig. 4), the LP latency did not show a consistent change following GPR stimulation (Fig. 5*D*); LP followed PD with a relatively constant latency (between 0.25 and 0.35 sec) regardless of the cycle period. However, in other preparations, where LP was initially less excited, there was a substantial decrease in LP latency following GPR stimulation (not shown).

6. IC responded in a biphasic manner to GPR stimulation. Following GPR stimulation, IC took less time to rebound from PD/AB inhibition, as seen by the significant decrease in IC latency following each GPR train (Fig. 5*E*). In addition, GPR stimulation caused IC to fire multispike bursts for over a minute (Figs. 4, 5*F*). However, when IC was active prior to GPR stimulation, GPR stimulation inhibited IC during the stimulus (Fig. 4*B*).

In contrast to the other pyloric cells, under our experimental conditions VD was not affected by trains of GPR impulses. When the *stn* was blocked, VD always fell silent and was only passively inhibited by the rhythmically active PD/AB group. GPR stimulation did not change VD activity (not shown).

GPR-induced changes in phase relationships

The GPR-induced changes in the parameters discussed above caused changes in the phase relationships within the pyloric pattern (Fig. 6*A*). Some of these phase changes were merely consequences of the shorter cycle period resulting from the GPR-induced increase in cycle frequency. This is apparent when the activity is plotted against real time (Fig. 6*B*). For instance, the PD duty cycle increased after GPR stimulation (compare Fig. 6*A*, i, ii), but the actual PD burst duration did not lengthen during this time; in fact, it shortened slightly (Fig. 6*B*, i, ii).

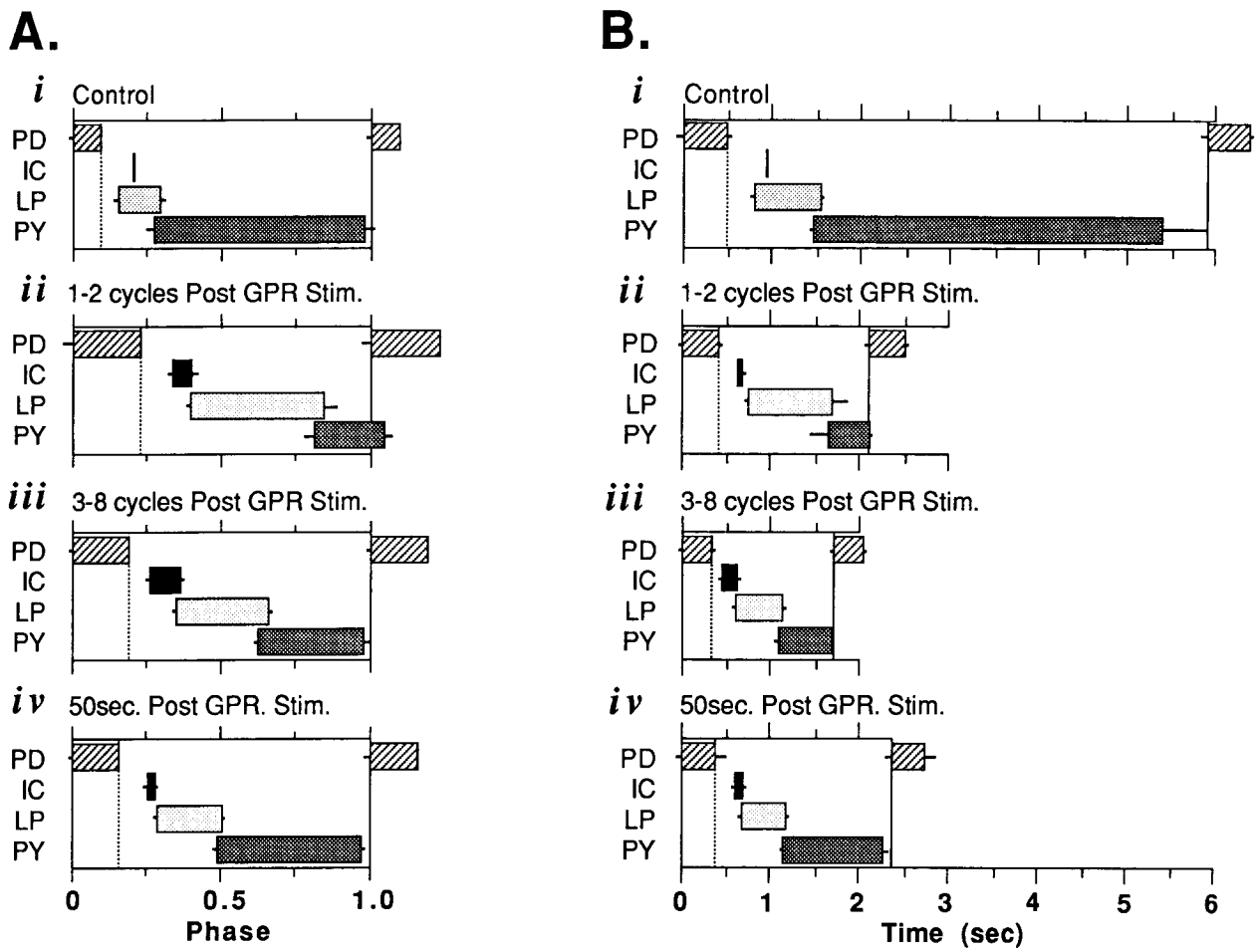

Figure 6. GPR stimulation induces changes in the phasing of the activity of different cells in the pyloric pattern. The time periods used for the averages are marked *i–iv* as in Figure 5 and come from the experiment shown in Figure 4. *Error bars* at the beginning of a burst denote standard error for the onset phase or latency, while those at the end of a burst show the standard error for duty cycle or burst duration. A *dotted line* is drawn from the end of each PD burst to allow comparisons of changes in phase and latency. *A,* The cycle periods have been normalized to create phase diagrams of the pyloric pattern. *Bars* represent the mean phase of the spike bursts for each of the cell groups. The phase relationships of different cells in the pattern change following GPR stimulation. *B, Bar graphs* show the average timing and duration (in sec) of each of the components of the pyloric pattern. Notice that following GPR stimulation, there is a prolonged decrease in cycle period.

However, since the cycle period shortened to an even greater degree, the calculated PD duty cycle lengthened.

Other GPR-induced phase changes resulted both from the shortened cycle period and from GPR-evoked changes in the parameters in Figure 5. For instance, GPR stimulation shortened the PY duty cycle as a consequence of both the shorter cycle period and a real prolongation of PY latency. As described previously, after rebounding from PD/AB inhibition, PY continued to spike until it was inhibited by the next PD/AB burst. Thus, shortening the time between PD/AB bursts (i.e., the cycle period) also shortened the PY bursts by *terminating* them sooner. GPR stimulation additionally decreased PY duration by transiently increasing the PY latency, thus also causing the PY bursts to *start* later (Fig. 6*B,* ii). This increase in PY latency lasted only 1–3 cycles (Figs. 5*B;* 6*B,* iii); thus, for the next few cycles, the PY duty cycle increased (Fig. 6*A,* iii) although the average cycle period decreased (Fig. 6*B,* iii). As the cycle period lengthened, PY duration lengthened further (Fig. 6*B,* iv) and the PY duty cycle increased (Fig. 6*A,* iv). Notice that the onset phase of the PY burst continued to decrease 50 sec after the GPR stimulation although the actual latency remained constant; again, this was an indirect effect of the change in cycle period.

GPR stimulation lengthened the LP duty cycle (Fig. 6*A*). The duty cycle lengthened in the first cycles following GPR stimulation as a consequence of both the decreased cycle period and a transient increase in LP duration (Fig. 6*B,* ii). After the first few cycles, the LP burst duration shortened to a fairly constant length. The cycle period increased slowly (Fig. 6*B,* iii, iv), and the percentage of the cycle occupied by the LP burst decreased correspondingly (Fig. 6*A,* iii, iv).

In contrast to its effects on PD, PY, and LP phasing, GPR stimulation had prolonged effects on IC phase that were relatively independent of cycle period. In the control period, IC was generally silent. In the one cycle in which it fired action potentials, its spike occurred after the onset of LP spiking (Fig. 6*B,* i). Immediately after each GPR stimulus train, the IC latency decreased, causing IC to lead LP (Fig. 6*B,* ii). Over the next 5 cycles, IC maintained its phase lead over LP (Fig. 6, *A,* iii, *B,* iii). Fifty seconds after a GPR train, IC only slightly preceded LP (Fig. 6, *A,* iv, *B,* iv). Thus, the activation of IC and its concurrent decrease in latency changed the phasing of IC activity independently of cycle frequency.

Cellular targets of GPR input

In order to understand the mechanisms underlying the GPR-induced alteration of the pyloric motor pattern, we sought to separate direct effects of the GPR cells on target neurons in the pyloric circuit from effects that were indirect consequences of pyloric synaptic interactions. For example, is the decrease in PY activity due to direct inhibition of PY by the GPR cells or is it due to GPR-evoked excitation of LP, which then inhibits PY? Since the GPR cells contain both ACh and 5-HT as co-transmitters (Katz et al., 1989), we also wished to distinguish the components of an effect that were due to release of ACh from those due to release of 5-HT.

GPR cells evoke discrete nicotinic synaptic potentials in only 2 pyloric cells (IC and VD)

GPR stimulation evoked constant latency postsynaptic potentials (PSPs) in IC and VD, but not in the other pyloric motor neurons (Fig. 7). (AB was not tested because of the difficulty in recording from it in the crab STG). IC received a biphasic PSP, with an initial depolarization and a more prolonged hyperpolarization. The inhibitory component of this response was blocked by bath application of picrotoxin (PTX, Fig. 7*A*), which blocks inhibitory glutamatergic synapses from several other neurons within the STG. Both the excitatory and inhibitory components were blocked by the nicotinic antagonist *d*-tubocurarine (Fig. 7*A*). While we are not yet certain of the sources of this biphasic response, our data are consistent with the hypothesis that GPR directly excites IC through a nicotinic receptor and indirectly inhibits IC through nicotinic excitation of an unidentified glutamatergic neuron.

In over 7 preparations examined, VD always exhibited a very small, rapid GPR-evoked EPSP (Fig. 7*B*). This was reversibly blocked by the nicotinic antagonists decamethonium (0.1 mM; Fig. 7) and *d*-tubocurarine (not shown). This EPSP was never sufficient to cause VD to spike, even when GPR was stimulated at high frequency.

The other pyloric neurons, LP, PY, PD, and LPG, usually did not receive detectable GPR-evoked rapid PSPs (Fig. 7*C*). In 4 of 5 LP neurons tested, no GPR-evoked PSP was observed; a small (<0.5 mV) EPSP was recorded in only one preparation. In 8 of 10 PD neurons tested, no discrete potential was seen; a very-low-amplitude (<0.5 mV), long-duration (>0.4 sec) EPSP was recorded in 2 PD neurons in response to GPR stimulation (Katz and Harris-Warrick, 1989a). Thus, GPR stimulation could cause large changes in the activity of these cells without evoking discernible rapid PSPs.

GPR stimulation directly enhances PD/AB bursting

We have previously shown that GPR stimulation directly evokes or enhances bursting in the PD/AB group (Fig. 8*A;* Katz and Harris-Warrick, 1989a). Bath application of PTX removes all chemical synaptic input to the PD/AB group from cells within the STG. In the presence of PTX, GPR stimulation continued to enhance bursting in the isolated PD/AB group (Fig. 8*B*). This burst enhancement was not blocked by cholinergic antagonists but was mimicked by 5-HT, consistent with the hypothesis that 5-HT released from the GPR cells causes PD/AB burst enhancement (Katz and Harris-Warrick, 1989a).

GPR stimulation excites LP

GPR stimulation caused LP to increase its burst duration as well as its spike frequency (Figs. 4, 5). We attempted to determine whether the GPR-evoked excitation of LP was direct or resulted from synaptic interactions with other pyloric neurons. Inspection of LP activity under different conditions strongly suggested that GPR input directly excites LP.

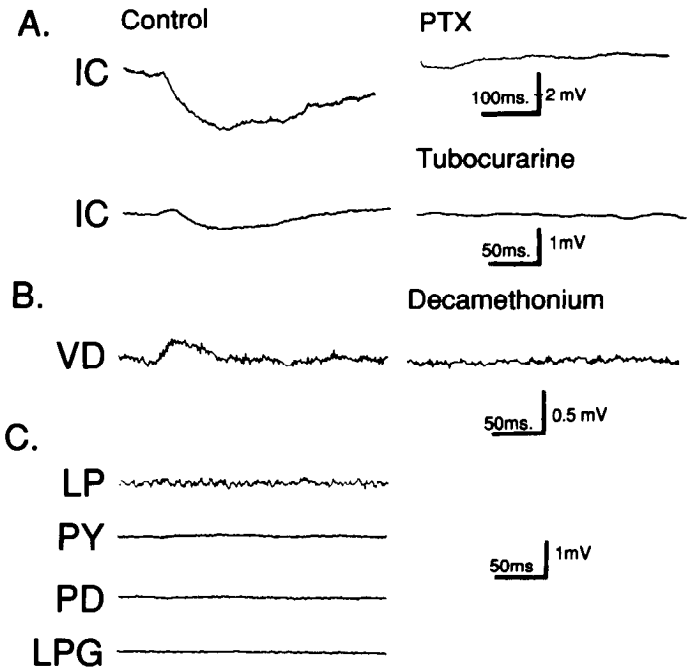

Figure 7. GPR-evoked nicotinic postsynaptic potentials in *IC* and *VD* and the absence of evoked synaptic potentials in the other pyloric cells. *A*, GPR stimulation evokes a biphasic synaptic potential in *IC*: 5 μM PTX blocks the inhibitory component, leaving a purely excitatory synaptic potential. Both the excitatory and inhibitory components are reversibly blocked by 0.1 mM *d*-TC. *B*, GPR stimulation evokes a short duration EPSP in VD, which is reversibly blocked by 0.1 mM decamethonium. *C*, GPR stimulation does not evoke a detectable rapid synaptic potential in LP, PY, PD, or LPG. All traces are averages of between 16 and 32 repetitions of single GPR stimuli presented at 0.3 Hz.

Four cell types within the STG synapse on LP: the glutamatergic AB and PY neurons and the cholinergic PD and LPG neurons. All of these neurons inhibit LP. Thus, GPR-induced excitation of LP is either (1) direct, (2) mediated by relief from inhibition arising from one or more of these neurons, or (3) a consequence of enhanced PIR resulting from increased inhibition from the PD/AB group. Input to LP from the glutamatergic AB and PY neurons can be blocked by bath application of picrotoxin (Fig. 8*B*). Under these conditions, LP is still highly excited by a train of GPR stimuli, demonstrating that its excitation does not derive secondarily via a relief from inhibition by these neurons.

It is also unlikely that LP excitation arises solely by enhanced PIR after PD and LPG inhibition because in both normal saline (Fig. 8*A*) and PTX-saline (Fig. 8*B*), GPR-evoked LP excitation is seen to *precede* GPR activation of PD and LPG. In the experiment shown in Figure 8*A*, the AB/PD group was quiescent, and LP was only weakly active before GPR stimulation. LP showed an immediate increase in spike frequency upon the commencement of GPR stimulation, preceding the first PD burst by over 2 sec. In PTX-saline (Fig. 8*B*), where the only input to LP is from the cholinergic PD and LPG cells, LP activation again preceded PD activation. These results strongly support the hypothesis that the GPR cells directly excite LP. However,

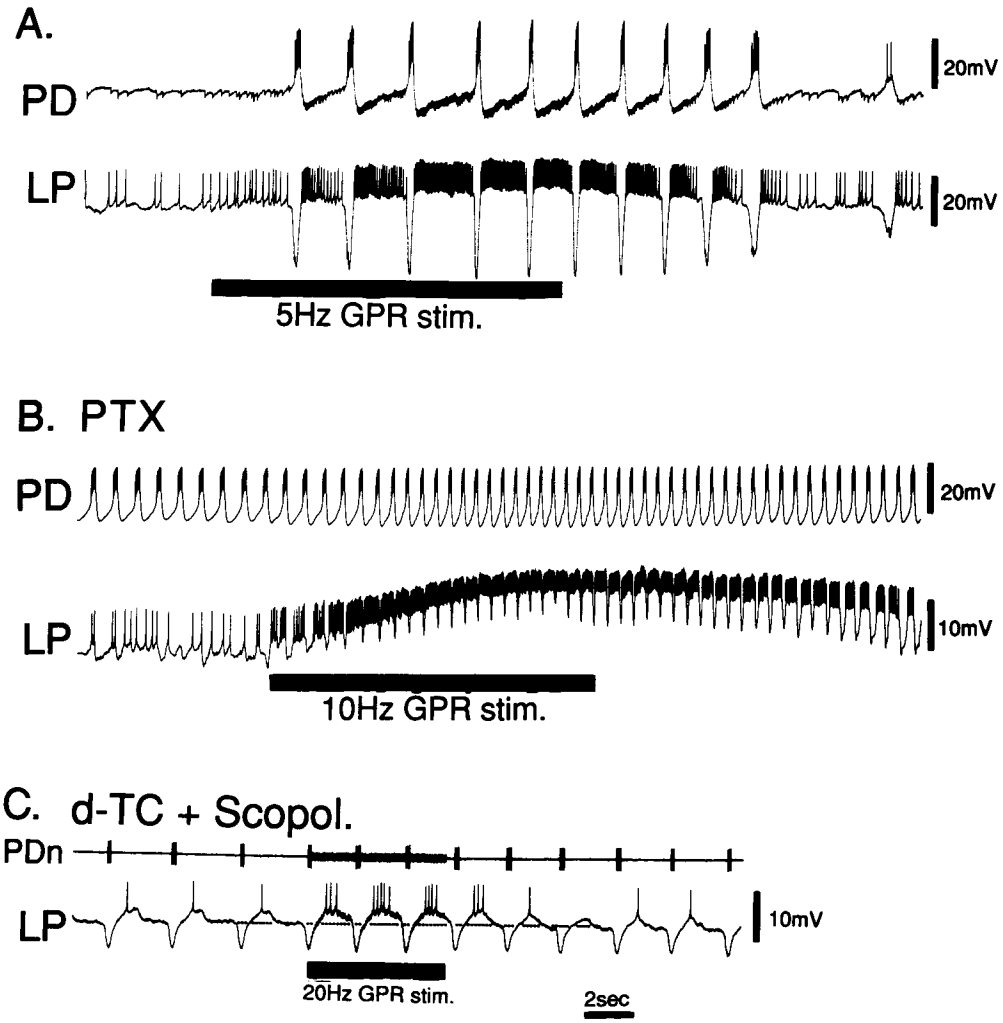

Figure 8. GPR stimulation directly excites LP and the PD/AB group. *A*, (Control) Prior to GPR stimulation, this preparation is quiescent. A 14-sec, 5-Hz GPR stimulus train (*bar*) causes a progressive depolarization of LP, accompanied by an increase in spike frequency. In addition, the PD neuron begins to oscillate, periodically inhibiting LP. *B*, 5 μM PTX blocks the inhibitory glutamate synapses within the STG (see Fig. 2*A*). Thus PY and AB no longer inhibit LP, whereas the cholinergic PD cells still cause periodic LP inhibition. In addition, LP no longer inhibits the PD/AB group. A 13-sec, 10-Hz GPR stimulus train (*bar*) causes a dramatic depolarization of LP that gradually subsides after cessation of the stimulus. PD burst frequency and burst amplitude also increase during the GPR stimulation. This example shows our strongest LP response to GPR stimulation. *C*, GPR-evoked LP excitation is not blocked by cholinergic antagonists. Bath application of the nicotinic blocker, *d*-TC (0.1 mM), and the muscarinic blocker, scopolamine (Scopol., 0.1 mM) does not prevent a 5-sec, 20-Hz GPR stimulus train (*bar*) from causing a transient excitation of LP, as well as an increase in cycle frequency.

enhanced PIR following each AB/PD burst may also play a role in the high level of LP activity, since we see a strong increase in LP spike frequency immediately following each AB/PD burst in normal saline (Fig. 8*A*). Such an increase is not seen in PTX (Fig. 8*B*), suggesting that the PIR may specifically follow the glutamatergic inhibition from the AB cell.

GPR-evoked excitation of LP was not blocked by either the nicotinic cholinergic antagonist *d*-tubocurarine or the muscarinic antagonists scopolamine and pirenzepine (Fig. 8*C*). Thus, this response is probably not mediated by ACh released from the GPR terminals. Bath-applied 5-HT mimics this excitation (see below).

GPR stimulation inhibits PY

The PY neurons receive inhibitory synaptic input from the glutamatergic LP and AB neurons and the cholinergic PD, LPG, and VD neurons. GPR-induced PY inhibition could therefore be either direct or mediated indirectly via excitation of one or more of these presynaptic inhibitory neurons. The most likely cell to mediate an indirect effect is LP, which is highly activated over the same time course as the PY inhibition (Figs. 3, 4, 9*A*).

We performed 2 experiments to demonstrate that LP does not mediate the inhibitory action of GPR on PY. First, we injected hyperpolarizing current into the single LP neuron, bringing its membrane potential below the threshold for release of neurotransmitter (Fig. 9*B*). This also reduced spiking activity in the PY cells due to weak electrical coupling between LP and PY (Fig. 2*A*). Under these conditions, where LP could not mediate the inhibition, GPR stimulation still inhibited PY. Second, we stimulated the GPR cells in the presence of PTX, which eliminates synaptic input from LP and AB to PY (Fig. 9*C*). The PY cells were still inhibited and silenced by the GPR train, suggesting that LP and AB do not mediate the inhibitory response. It is unlikely that the cholinergic VD, PD, and LPG neurons mediate the response, since VD is not activated at all by GPR stimulation, and in many preparations PD and LPG are inhibited (by LP) at the same time that PY is silenced during the GPR train (e.g., Figs. 3, 4). We conclude that GPR exerts a direct action to inhibit the PY cells. This inhibition is not blocked by the nicotinic antagonist *d*-tubocurarine (*d*-TC) nor by the muscarinic antagonists scopolamine and pirenzepine (not shown), suggesting that it does not result from acetylcholine released from GPR terminals. Bath-applied 5-HT does mimic this inhibition (see below).

GPR stimulation causes a prolonged excitation of IC

IC has a complex biphasic response to GPR stimulation (Fig. 10). At high stimulus frequencies (10 Hz or greater), IC was

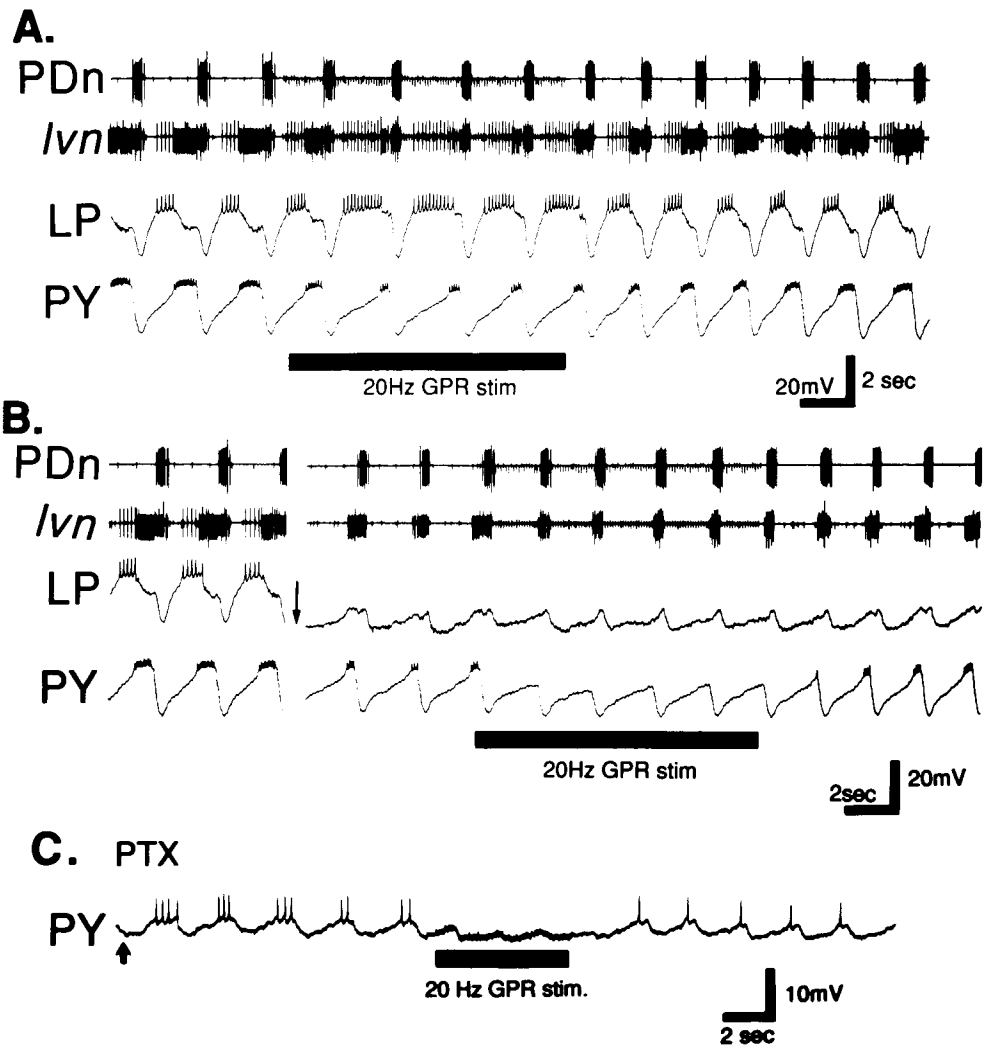

Figure 9. GPR stimulation directly inhibits PY. *A*, A 10-sec, 20-Hz GPR stimulation (*bar*) causes LP burst duration to expand and PY duration to decrease. LP, PY, and PD spikes are visible on the *lvn* trace. *B*, PY inhibition during GPR stimulation is not due to inhibition from LP. Hyperpolarization of LP (*downward arrow*) causes an immediate decrease in PY spiking due to the electrical coupling between the 2 cells. A 10-sec GPR stimulus train (*bar*), applied while LP was hyperpolarized, results in an inhibition of PY. The membrane potential of the LP cell is not precisely known because it was hyperpolarized using a single electrode and a balanced bridge, but it is beyond the reversal potential for PD/AB inhibition, as evidenced by the upward moving potential during PD/AB bursts. *C*, Bath application of 10 μM PTX does not block GPR-evoked PY inhibition. The PY neuron was depolarized prior to the beginning of this trace (*upward arrow*) to cause it to spike.

transiently inhibited during the GPR train. Following the stimulus train, IC became more excited, firing more spikes per burst (Fig. 10*A*). When a GPR cell was stimulated at lower frequencies (less than 5 Hz), IC did not exhibit any substantial inhibition, but displayed a gradual increase in excitation that outlasted the stimulus train (Fig. 10*B*).

The inhibition of IC during high-frequency stimulation appears to be due to the inhibitory component of the biphasic GPR-evoked synaptic potential. This inhibition can be blocked by bath application of PTX (Fig. 11*A*). Under these conditions, IC was excited during the GPR stimulus trains due to the uncovering of the GPR-evoked EPSP (Fig. 7*A*). In addition to the transient excitation of IC during each GPR train, there was a prolonged depolarization that was independent of the rapid EPSPs; this prolonged depolarization continued to be elicited by GPR stimulation when the GPR-evoked EPSPs were blocked by the nicotinic antagonist *d*-tubocurarine (Fig. 11*B*). Thus, GPR stimulation has a least 3 types of effects on IC: (1) transient inhibition that is probably polysynaptic and is mediated by PTX-sensitive synaptic potentials; (2) transient excitation that is uncovered by PTX and is mediated by nicotinic EPSPs; (3) prolonged excitation that is not blocked by nicotinic antagonists.

GPR stimulation enhances the plateau potential capability of IC

To test whether GPR stimulation enhanced active properties in IC, we pharmacologically blocked all of the inhibitory chemical synapses to IC from cells in the STG with picrotoxin (Fig. 2*A*). When IC was hyperpolarized by current injection, GPR stimulus trains often evoked rapidly depolarizing prolonged plateau potentials in an all-or-none fashion (Fig. 12*A*). These plateau potentials were not the result of coupling to PD (note *PDn* in Fig. 12*A*) or LP, but were independently triggered in IC.

In some trials, GPR stimulation did not trigger an IC plateau potential. However, in the period following GPR stimulation, IC showed an enhanced ability to produce a plateau potential in response to a short current pulse (Fig. 12*B*). A short depolarizing current pulse that was subthreshold for plateau potential production before GPR stimulation successfully triggered an IC plateau potential several seconds after a train of GPR impulses. The enhancement of plateau capability by GPR stimulation was not blocked by *d*-TC or the muscarinic antagonist scopolamine, applied either separately or together (Fig. 12*C*). Thus, GPR stimulation enhanced the ability of IC to produce active plateau

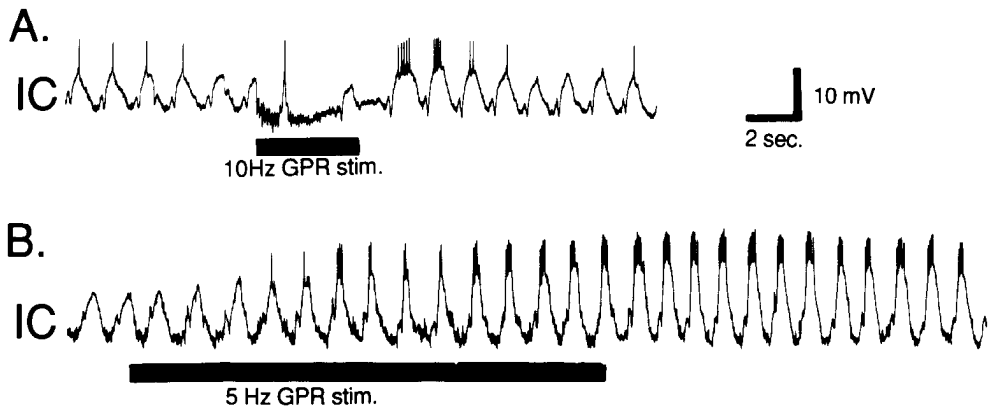

Figure 10. GPR stimulation has a biphasic effect on IC. *A*, A short, 10-Hz GPR stimulus train (*bar*) causes an immediate inhibition of IC. Upon cessation of stimulation, IC shows enhanced activity. *B*, Lower-frequency GPR stimulation (5 Hz) does not cause the initial inhibition but elicits a gradual excitation of IC that continues after the cessation of stimulation.

potentials through a mechanism that does not appear to involve acetylcholine receptors. It is possible that the enhancement of plateau potentials and the prolonged depolarization of IC by GPR stimulation are 2 manifestations of the same ionic mechanism; this has yet to be examined.

State-dependent effects of GPR stimulation on cycle frequency

The GPR cells generally increased the cycle frequency of the pyloric pattern, due in large part to a direct enhancement of bursting in the PD/AB group (Katz and Harris-Warrick, 1989a). However, there was an apparent upper limit to the enhancement; when a single GPR cell was fired at 20 Hz, the resulting pyloric rhythm never exceeded much more than 1 Hz, regardless of the initial cycle frequency. The pyloric rhythm is capable of cycling faster, often reaching 2 Hz with the *stn* intact. The GPR-induced enhancement of cycle frequency decreased as the initial cycle frequency increased (Fig. 13). For example, if the cycle frequency was initially about 0.2 Hz (Fig. 5*A*), a 5-sec, 20-Hz GPR stimulus train increased it by 0.4 Hz, to about 0.6 Hz. If the cycle frequency was already 1 Hz, the same stimulus train raised the cycle frequency by only 0.1 Hz, to 1.1 Hz. It is not clear why there is a ceiling near 1 Hz, but it has been observed with other modulatory inputs to the pyloric CPG (Nagy and Dickinson, 1983; Nusbaum and Marder, 1989).

One factor that regulates cycle frequency is the inhibition of the PD/AB group by LP. LP provides the only chemical synaptic input to the PD/AB group (Fig. 2*A*). When LP inhibition was removed by adding PTX, basal PD/AB cycle frequency often increased. However, when the PD/AB cycle frequency data obtained in the presence of PTX were plotted, they generally fell within the scatter of points for normal saline, even though some preparations exhibited a basal cycle frequency in PTX that was greater than 1 Hz (Fig. 13, open squares). This indicates that the ceiling effect is not solely due to GPR-evoked LP excitation and the attendant inhibition of the PD/AB group, but reflects some property within the PD/AB group itself.

LP/PD mutual inhibition affects GPR-induced cycle frequency changes

Although LP inhibition of the PD/AB group does not limit the maximum cycle frequency that can be achieved following GPR stimulation, GPR-evoked LP excitation can cause a momentary decrease in cycle frequency. GPR stimulation causes an increase in PD/AB cycle frequency that can last for over a minute; however, the GPR-evoked excitation of LP is often much briefer, lasting just a few seconds (Fig. 5, *A*, *C*). During high-frequency GPR stimulation there was sometimes a delay during which cycle frequency transiently declined before accelerating (Figs. 3, 4); however, this delay was not always seen (Fig. 9). The ability of GPR stimulation to cause the pyloric pattern to pause momentarily was strongly dependent on the state of the system at the time. Our data suggest that the pause results from a combination of the LP inhibition of the PD/AB group and the ceiling to GPR-induced enhancement of PD/AB cycle frequency described above. Thus, changes in the relative activity of LP or PD/AB might alter the effect of GPR stimulation.

This interplay between LP and the PD/AB group was studied in the experiment shown in Figure 14. In the control situation (Fig. 14, *A1, B1*), LP was strongly active, preventing the PD/AB group from bursting regularly. A 2-sec, 20-Hz GPR train caused LP to be even more excited and this completely blocked PD/AB bursts for a short period of time. Following the GPR stimulus, the PD cells increased their burst frequency to about 0.7 Hz for a few cycles (Fig. 14*B1*). After the preparation had

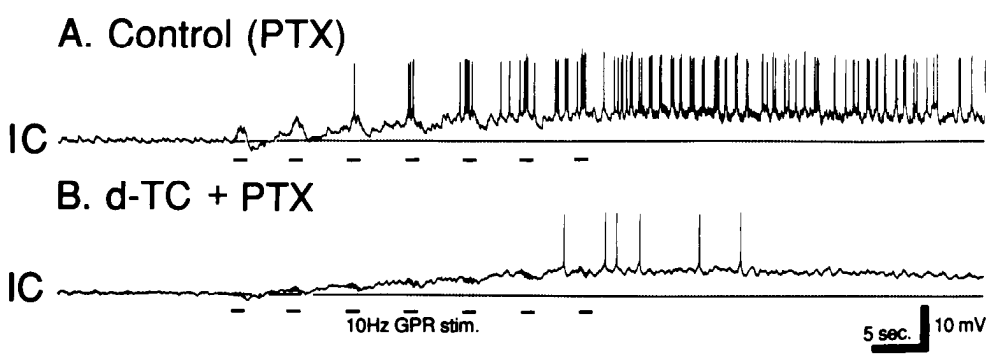

Figure 11. GPR stimulation evokes nicotinic EPSPs and a prolonged, tubocurarine-insensitive depolarization of IC. *A*, In the presence of 5 μM PTX, repetitive, 1-sec, 10-Hz GPR trains (*bars*) evoke EPSPs in IC that summate and cause transient depolarizations. In addition, there is a cumulative, long-lasting depolarization that persists for over 30 sec after the last GPR stimulus train. *B*, Bath application of 0.1 mM *d*-TC, in the continued presence of PTX, blocks the rapid synaptic potentials but does not eliminate the prolonged depolarization.

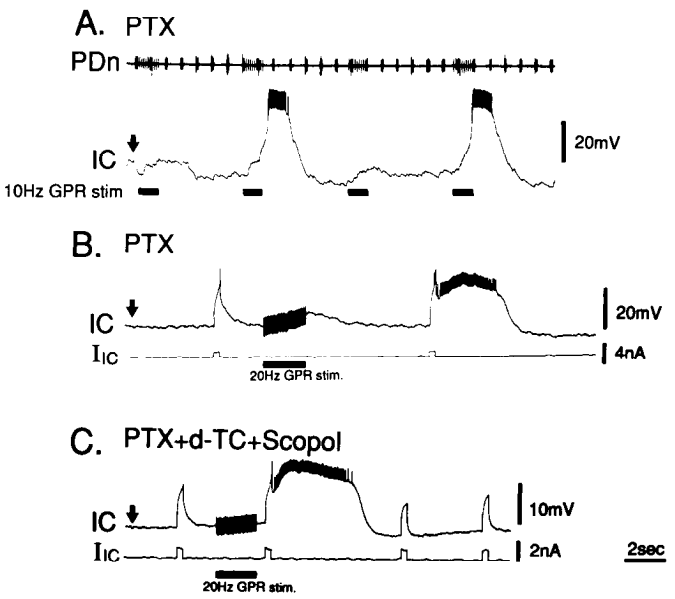

Figure 12. GPR stimulation enhances endogenous IC plateau properties. In each example, IC was hyperpolarized by current injection prior to the beginning of the trace to prevent it from spiking spontaneously. *A,* Repeated 10-Hz GPR stimulus trains (*bars*) trigger IC plateau potentials in an all-or-none fashion. The bath contains 5 μM PTX. *B,* GPR stimulation enhances the ability of IC to produce plateau potentials in the presence of 10 μM PTX. Prior to GPR stimulation, a short depolarizing current pulse causes IC to fire a single action potential. A 20-Hz GPR train (*bar*) evokes nicotinic EPSPs in IC but does not trigger a plateau potential. Following the stimulus, the same current pulse elicits a prolonged IC plateau potential. *C,* GPR stimulation enhances the ability of IC to produce plateau potentials in the presence of 10 μM PTX, 0.1 mM *d*-TC, and 0.1 mM Scopolamine HCl. *d*-TC blocks the GPR-evoked nicotinic EPSPs in IC, but together with scopolamine has no effect on burst enhancement. A short depolarizing current pulse that is subthreshold prior to GPR stimulation triggers a prolonged plateau potential after GPR stimulation.

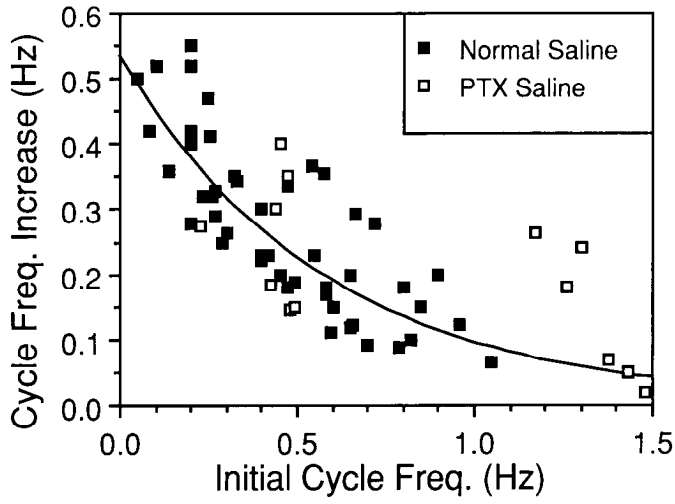

Figure 13. The maximum change in cycle frequency following 5-sec, 20-Hz GPR stimulus trains vs average cycle frequency prior to GPR stimulation (*Initial Cycle Freq.*). Data from many preparations are pooled in this figure. Measurements from preparations bathed in 5 μM PTX (*open squares*) show that at low initial cycle frequencies, the points fall within the scatter from preparations in normal saline. The line drawn is an exponential function which was fit to the data from normal saline using a least-squares method ($r = 0.82$).

recovered, we injected hyperpolarizing current into LP, reducing the inhibition of the PD/AB group (Fig. 14*A2*). This resulted in an increase in basal PD/AB cycle frequency to 0.6 Hz (Fig. 14*B2*). Under these conditions, the same GPR stimulation resulted in only a modest increase in cycle frequency to 0.75 Hz following cessation of the GPR stimulus. The LP cell was also excited, causing a slight delay before the increase in cycle frequency. Next, we released LP from hyperpolarization and instead depolarized PD to produce a cycle frequency of 0.8 Hz (Fig. 14, *A3, B3*). In this state, the same GPR stimulus caused a momentary decrease in cycle frequency during the GPR stimulus, followed by only a very slight increase to 0.85 Hz after the GPR train. The decrease in cycle frequency was clearly associated with an elongation of the LP burst. Finally, when PD was depolarized and LP hyperpolarized to produce a cycle frequency of 0.8 Hz (Fig. 14, *A4, B4*), there was a clear excitation of both LP and PD, yet there was no change in cycle frequency.

Thus, LP excitation and PD/AB burst enhancement form a balance. Under certain conditions, LP excitation predominates, causing the cycle to slow down momentarily. Under other conditions, there is only a short pause, or no pause at all, before the cycle frequency increases. Finally, under some conditions, GPR stimulation may not cause *any* change in cycle frequency, although it clearly excites both LP and PD.

Bath application of serotonin mimics many of the effects of GPR stimulation

Bath application of 10 μM 5-HT had effects on many cells in the pyloric pattern that resembled the prolonged modulatory effects of GPR stimulation. 5-HT caused the PY motor neurons to hyperpolarize and stop firing, while it had the opposite effect on LP, causing it to depolarize and spike more strongly (Fig. 15*A*). In the presence of PTX to block glutamate synapses within the STG, 5-HT caused an increase in PD/AB burst frequency (Katz and Harris-Warrick, 1989a) and continued to inhibit PY and enhance LP tonic firing (not shown). Thus, the effects of bath-applied 5-HT strongly resembled many of the effects of GPR stimulation. All of the effects of 5-HT reversed after a 30-min wash in normal saline.

When 10 μM 5-HT was applied for more than a minute, a new motor pattern usually emerged, as first reported by Beltz et al. (1984). This pattern consisted of high-frequency PD/AB and IC bursting interrupted by long LP plateau potentials; the PY and VD neurons were silent (Fig. 15*B*). The cause of this novel pattern is explainable based on our knowledge of the effects of 5-HT on the different cell types described above and the pattern of synaptic connectivity (Fig. 2*A*). Normally, PY bursts inhibit LP, thereby terminating LP spike activity and causing the shoulder on the LP potential [seen before 5-HT has an effect (Fig. 15*A*)]. When the PY motor neurons are inhibited by serotonin, LP continues to spike until it fatigues or terminates on its own. LP and the PD/AB group are both excited by serotonin, but are mutually inhibitory. Thus, when the LP plateau potential terminates, the PD/AB group is freed to begin oscillating at a high frequency while LP is refractory from its plateau. LP gradually recovers and eventually rebounds from PD/AB inhibition to a new plateau potential, again inhibiting the PD/AB group. This interplay between LP and the PD/AB group during serotonin application is reminiscent of the pauses and increases in cycle frequency seen during GPR stimulation.

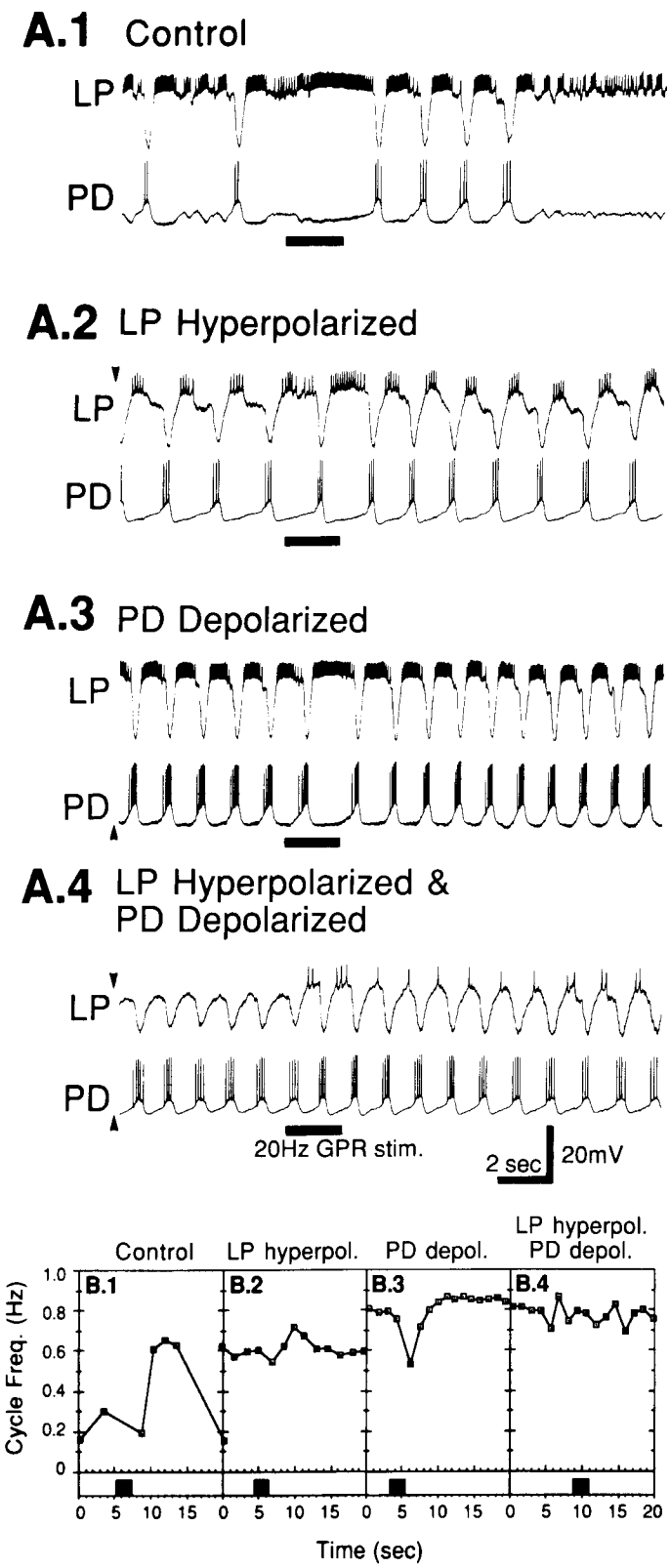

Figure 14. The effect of GPR stimulation on cycle frequency depends on the balance between LP and PD/AB excitation. *A(1–4)*, Intracellular recordings from LP and PD neurons. *B(1–4)*, Graphic representation of the cycle frequency from the recordings in *A*. A 2-sec, 20-Hz GPR stimulus is shown as a *bar* in each panel. See text for explanation.

IC was affected by bath-applied 5-HT in a manner that resembled some of the GPR effects. Prior to the application of serotonin to the bath, IC was only weakly active. When 5-HT first arrived in the bath, IC was transiently inhibited (Fig. 15*A*), but after 70 sec in 5-HT, IC was strongly active, firing at a very short latency after PD bursts (Fig. 15*B*).

VD was unaffected by 5-HT. It remained passively inhibited by PD/AB bursts, its excitability unchanged (not shown). Depolarization of VD by constant current pulses produced the same number of spikes during serotonin as before 5-HT, demonstrating that there are no voltage-dependent serotonergic responses in the VD cell (not shown). This is consistent with the lack of a noncholinergic effect of GPR stimulation.

Discussion

A periodic envelope of GPR-evoked modulatory effects

The GPR cells are a set of proprioceptive cells that use both acetylcholine and serotonin as cotransmitters (Katz et al., 1989). These muscle receptor cells sense movements related to the gastric mill musculature in the foregut of the crab (Katz et al., 1989). In semi-intact foregut preparations, the GPR cells fire in repeated barrages about 5 sec in length every 10–15 sec in response to movements of the gastric mill (Katz et al., 1989). When a single GPR cell is stimulated in this way *in vitro*, we see a rhythmic envelope of transient GPR effects superimposed on the ongoing faster pyloric motor pattern. This envelope is produced by inhibition of IC and PY and excitation of LP (Fig. 3). Furthermore, when the pyloric pattern is cycling weakly, LP excitation is often so strong as to cause the cycling to pause momentarily.

Our results suggest that the pyloric pattern can be subject to periodic modulation by the gastric mill via a peripheral feedback loop involving the GPR cells. In this way, sensory input directly orchestrates the coordination between the 2 ongoing motor patterns by sensing the movements caused by one motor circuit and changing the activity of cells in the other motor circuit at appropriate times. The effects of GPR input on pyloric cells may be important for coordinating the pyloric pattern with ongoing gastric mill movements; by transiently exciting the LP motor neuron and inhibiting the PY motor neurons, GPR activity would transiently constrict the entrance to the pyloric chamber and relax the posterior pylorus. The pause that sometimes occurs in the pattern would accentuate these effects by holding the muscles in this position at the proper time during the gastric mill movements. The function of this change in motor output for the processing of food in the foregut has yet to be analyzed.

GPR cells have many synaptic effects on pyloric neurons

The changes in the pyloric pattern caused by GPR stimulation can be understood in terms of the effects of GPR stimulation on individual cells in the pyloric circuit. The effects of GPR stimulation are summarized in Table 1. The cells that receive GPR-evoked nicotinic PSPs are listed. In addition, the prolonged modulatory effects of high-frequency GPR stimulation are summarized and compared to the effects of bath-applied 5-HT.

GPR stimulation causes neuromodulatory effects without discrete synaptic potentials

Several cells whose activities are modulated by GPR input do not receive detectable discrete GPR-evoked rapid synaptic potentials. The GPR cells enhance the burst capabilities of the

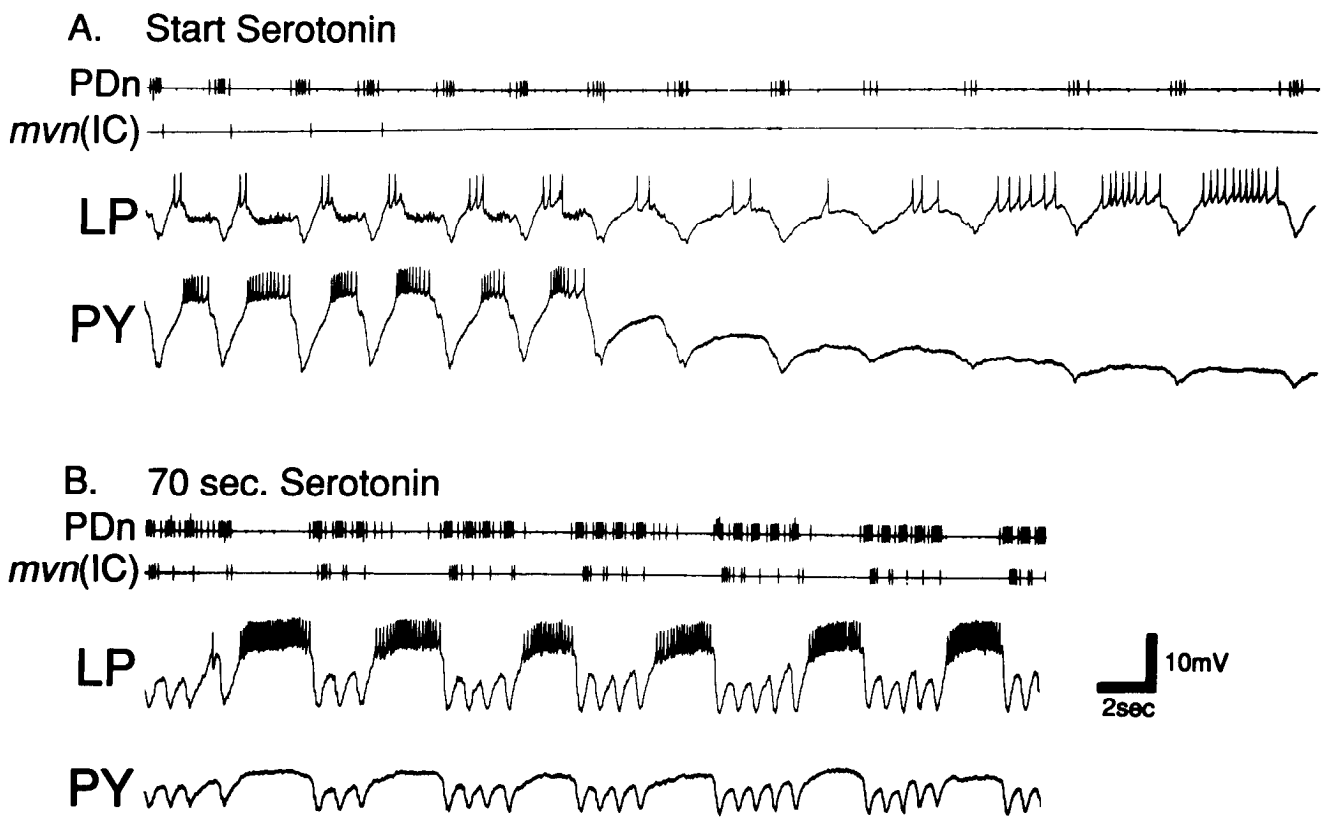

Figure 15. The effect of bath-applied serotonin on pyloric cells. *A*, 10 μM serotonin is applied to the bath at the beginning of the trace. IC (spikes on the *mvn*) ceases to fire action potentials. PY becomes hyperpolarized. LP first loses the inhibition from PY, but does not expand its burst duration until it gradually depolarizes and begins to spike at a higher frequency. PD bursts initially become slower and weaker. *B*, After 70 sec in serotonin, a new burst pattern has emerged in the same preparation. PD bursts at high frequency, followed at short latency by IC bursts. These high-frequency bursts are periodically interrupted by long LP bursts. PY remains hyperpolarized and is periodically inhibited by the PD bursts.

PD/AB group (Katz and Harris-Warrick, 1989a), excite the LP motor neuron, and inhibit the PY motor neurons, yet they do not produce detectable fast postsynaptic potentials in these cell types. None of these modulatory effects are blocked by nicotinic or muscarinic antagonists, but most are mimicked by 5-HT, indicating that these effects are probably not due to the release of ACh from the GPR cells, but may result from the release of 5-HT.

The GPR cells are the only neuronal source of 5-HT for the STG in the crab (Beltz et al., 1984; Katz et al., 1989), and many of the modulatory effects of GPR stimulation are mimicked by bath application of 5-HT. However, we do not yet have an effective 5-HT antagonist for this system, so we have been unable to test directly whether the slow modulatory effects of GPR stimulation are due entirely to the release of 5-HT. It is conceivable that the GPR cells contain additional neurotransmitters that could mediate some of these modulatory effects. The homologous cells in the lobster, *Homarus americanus*, display FMRFamide-like and CCK-like immunoreactivity as well as 5-HT immunoreactivity (P. Meyrand and G. Turrigiano, personal communication). However, the similarity of the effects of bath-applied 5-HT to GPR stimulation indicates that the most parsimonious explanation is that the modulatory effects of GPR stimulation result from the release of 5-HT.

There are a number of possible explanations for the lack of a detectable discrete PSP in PD, LP, and PY. First a PSP may arise at synapses on distal dendritic branches, but be undetectable in our soma recordings. This is unlikely, due to the mag-

Table 1. Summary of the direct effects of GPR stimulation on the pyloric circuit with the *stn* blocked

Pyloric cell name	GPR-evoked nicotinic PSP	Prolonged effect of GPR trains	Effect of bath-applied serotonin
AP, PD, and LPG	None	Burst enhancement	Burst enhancement
LP	None	Excitation	Excitation
PY	None	Inhibition	Inhibition
IC	Biphasic EPSP/IPSP	Excitation and plateau enhancement	Excitation
VD	EPSP	None	None

nitude of the change in membrane potential evoked by GPR stimulation and the fact that EPSPs can be recorded in other cells such as VD. Second, it is possible that these cells lack nicotinic cholinergic receptors at the GPR synapses, so they respond only to the released 5-HT and not to ACh. This is also unlikely since PD, LP, and PY have large nicotinic depolarizing responses to iontophoresed ACh (Marder and Paupardin-Tritsch, 1978). Conceivably (though unlikely), the GPR cells violate Dale's principle and release only 5-HT, and not ACh, from the synaptic terminals that contact these cell types. A fourth hypothesis is that the GPR cells may not synapse directly onto these cells, but only nearby, onto other cells. The coreleased ACh would be rapidly inactivated by cholinesterases in the synaptic cleft, but the 5-HT could diffuse long distances as a "local hormone" to reach these cells, as has been proposed in other systems (Beaudet and Descarries, 1978; Vizi, 1981a; Jan and Jan, 1982; Piccolino et al., 1987).

GPR stimulation evokes nicotinic PSPs in 2 pyloric neurons

Three gastric mill neurons receive discrete nicotinic synaptic potentials from the GPR cells (Katz and Harris-Warrick, 1989a, unpublished observations), yet among the pyloric cells, only IC and VD exhibit discrete nicotinic GPR-evoked PSPs. The IC and VD motor neurons are more strongly affected by gastric mill cycling than the other pyloric neurons (P. S. Katz, unpublished observations), and the muscles they innervate lie in the gastric mill region. Thus, the presence of GPR-evoked nicotinic EPSPs in these cells is consistent with their close ties to the gastric mill.

The response of IC to GPR stimulation has many components. There is a biphasic excitatory/inhibitory PSP that causes the transient inhibition of IC during a GPR train. The inhibitory component of this PSP may be disynaptically mediated because its presence or absence is correlated with the activity of other neurons (Katz, unpublished observations) and it can be blocked by PTX, which antagonizes many synapses from other STG neurons onto IC. During high-frequency GPR stimulation, the inhibitory synaptic potential overwhelms the EPSP, hyperpolarizing IC during the stimulus. Following GPR stimulation, IC undergoes a prolonged period of excitation, including an enhancement of plateau potential capabilities. The prolonged excitation is not blocked by nicotinic or muscarinic blockers, but is partially mimicked by 5-HT.

Unlike the other pyloric cells, VD does not exhibit any modulatory response to GPR stimulation or to bath-applied 5-HT, although it receives a small GPR-evoked nicotinic EPSP. The small EPSP is insufficient to depolarize VD even when GPR is stimulated at high frequency. This lack of response could be a consequence of the quiescent state of the VD cell following blockade of the *stn*. It is conceivable that if the VD were in another more active state, it might be affected by GPR stimulation and/or by bath-applied 5-HT.

Thus, IC appears to be the only pyloric cell to receive both nicotinic cholinergic and serotonergic input from the GPR cells: VD appears to receive only nicotinic cholinergic input, while LP, AB, PD, and LPG appear to receive only serotonergic input.

None of the GPR effects are blocked by muscarinic antagonists. However, muscarinic agonists are known to enhance bursting and have various other effects on cells in the pyloric circuit (Marder and Paupardin-Tritsch, 1978; Nagy et al., 1984, 1985; Marder and Hooper, 1985), and muscarinic antagonists do block the modulatory actions of another cholinergic input cell, APM (Nagy and Dickinson, 1983). It would be interesting to know whether this indicates a physical segregation of muscarinic receptors away from GPR release sites.

Interpreting the effects of GPR stimulation on the pyloric pattern

Three main aspects of the rhythmic pyloric motor pattern are altered by GPR stimulation. These alterations in the motor pattern can be explained by the synaptic effects of the GPR cells on each of the pyloric cells.

1. GPR stimulation increases the pyloric cycle frequency. This is due to GPR enhancement of PD/AB bursting through a noncholinergic mechanism that is mimicked by serotonin (Katz and Harris-Warrick, 1989a). Although we have treated the PD/AB group (the 2 PD and 2 LPG motor neurons and the AB interneuron) as a single cell group, it is possible, and even likely, that the GPR cells have different effects on the 3 cell types. Using the photoinactivation technique (Miller and Selverston, 1979) to kill cells selectively, Flamm and Harris-Warrick (1986b) showed that, in the spiny lobster, *Panulirus,* 5-HT directly enhances bursting in the AB cell, but has no effect on the PD neurons. We have been unable to perform similar experiments in the crab because of the extreme difficulty in locating the AB neuron in the crab. More experiments need to be performed in order to determine the exact locus for 5-HT's action on the PD/AB group in the crab.

2. The pyloric pattern often pauses during GPR stimulation. This appears to be due to GPR-evoked LP excitation and PY inhibition. The highly excited LP neuron inhibits the PD/AB group, preventing it from bursting and causing the rhythm to pause. Normally, PY activity follows LP, terminating its activity and thereby relieving the PD/AB group from LP inhibition. However, GPR-evoked inhibition of PY allows LP to remain depolarized, thereby extending the inhibition of the PD/AB group. If LP inhibition of the PD/AB group is pharmacologically blocked, the pyloric pattern does not pause during GPR stimulation (Fig. 8*B*). When LP inhibition of the PD/AB group is intact, the presence or absence of a pause is strongly dependent on the relative excitation of LP versus the PD/AB group; when the PD/AB group is bursting strongly, it can overcome LP inhibition, and no pause is seen.

3. The phase relationships of cells within the pyloric pattern change due to GPR stimulation. Some of the changes arise secondarily from the shortened cycle period. Other changes result from GPR input combined with intrinsic synaptic interactions in the pyloric circuit. For instance, the LP duty cycle increases and the PY duty cycle decreases due to direct effects of GPR input. These effects are further enhanced by the mutual inhibition between these 2 cell types and the shortened cycle period. The IC neuron has a complicated response to GPR stimulation that appears to be independent of changes in cycle period. During a GPR train, IC is inhibited by GPR-evoked inhibitory synaptic potentials. Following GPR stimulation, IC is strongly activated and phase-advanced relative to LP. This may be due to the prolonged GPR enhancement of IC plateau potential production.

State dependence of GPR-induced burst enhancement

The pyloric CPG in the crab is capable of oscillating at frequencies greater than 2 Hz, yet stimulation of a single GPR cell never elevates the cycle frequency far above 1 Hz. Stimulation of more than one GPR cell, or stimulation at a higher frequency,

might increase the cycle frequency above 1 Hz. However, it is clear that, at a set stimulus frequency and duration, the relative GPR enhancement of cycle frequency declines as the initial cycle frequency increases. This may be due to a limitation in the intrinsic bursting properties induced in AB, PD, and/or LPG by GPR stimulation. An alternative explanation is that the GPR cells may enhance bursting only in the AB cell, while the cells that are electrotonically coupled to AB (PD and LPG) act as a drag on it. In similar experiments, Hooper and Marder (1987) showed in *Panulirus* that when the AB neuron is isolated from electrically coupled neurons, it will reach a burst frequency of about 2 Hz when the peptide proctolin is applied, whereas prior to isolation, proctolin only increased the burst frequency to a ceiling of 1 Hz. They demonstrated that this ceiling was due to a drag on AB by the electrically coupled cells which were not excited by proctolin. Similarly, stimulation of the modulatory proctolin-containing neuron with the AB connected to other cells will not increase cycle frequency far above 1 Hz (Nusbaum and Marder, 1989).

Sensory cells can have prolonged modulatory effects

The role of phasic sensory input to CPGs has been investigated in many systems (Andersson et al., 1981a; Grillner et al., 1981; Pearson et al., 1983). There is little doubt that phasic synaptic input can change the phase relationships of neurons involved in the generation of motor patterns (Pearson and Reye, 1987; Wolf and Pearson, 1988). However, an important point to note in the case of the GPR effects on the pyloric motor pattern is that these phase changes do not require GPR stimulation at a particular phase of the pyloric pattern. That is, they are not accomplished by direct phasic synaptic input occurring on each cycle of activity, but rather through prolonged modulatory changes in the excitability and intrinsic properties of each pyloric cell type. This modulatory input alters the overall physiological organization of the circuit without being involved in cycle-by-cycle synaptic integration. The same is true of the increase in pyloric cycle frequency observed following GPR stimulation, which is not due to phasic entrainment, but rather to a prolonged enhancement of the intrinsic bursting abilities of cells within the circuit. There are effects of the GPR cells that *are* phase-related; these are mostly concerned with the operation of the gastric mill (Katz and Harris-Warrick, 1989a, and unpublished observations). However, the primary effects of the GPR cells on the pyloric CPG involve neuromodulatory actions of 5-HT, not phase-dependent rapid synaptic effects.

Interestingly, there is only one other known putative sensory cell that synapses directly on cells in the pyloric CPG, the hepatopancreatic duct neuron (Hartline et al., 1987). This neuron evokes discrete synaptic potentials in some pyloric cells and also has prolonged modulatory effects. The fact that 2 different sensory cell types in the stomatogastric system have modulatory effects on the pyloric pattern generator does not necessarily indicate that the stomatogastric system is inherently different from other systems. Rather, we believe that because of the small size of the stomatogastric system and the fact that the components of the circuits are known, modulatory effects can be correctly identified as such.

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