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Neurotransmitter Interactions in the Stomatogastric System of the Spiny Lobster: One Peptide Alters the Response of a Central Pattern Generator to a Second Peptide

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Dickinson, Patsy S., Wesley P. Fairfield, John R. Hetling, and Jane Hauptman. Neurotransmitter interactions in the stomatogastric system of the spiny lobster: one peptide alters the response of a central pattern generator to a second peptide. *J. Neurophysiol.* 77: 599–610, 1997. Two of the peptides found in the stomatogastric nervous system of the spiny lobster, *Panulirus interruptus*, interacted to modulate the activity of the cardiac sac motor pattern. In the isolated stomatogastric ganglion, red-pigment-concentrating hormone (RPCH), but not proctolin, activated the bursting activity in the inferior ventricular (IV) neurons that drives the cardiac sac pattern. The cardiac sac pattern normally ceased within 15 min after the end of RPCH superfusion. However, when proctolin was applied within a few minutes of that time, it was likewise able to induce cardiac sac activity. Similarly, proctolin applied together with subthreshold RPCH induced cardiac sac bursting. The amplitude of the excitatory postsynaptic potentials from the IV neurons to the cardiac sac dilator neuron CD2 (1 of the 2 major motor neurons in the cardiac sac system) was potentiated in the presence of both proctolin and RPCH. The potentiation in RPCH was much greater than in proctolin alone. However, the potentiation in proctolin after RPCH was equivalent to that recorded in RPCH alone. Although we do not yet understand the mechanisms for these interactions of the two modulators, this study provides an example of one factor that can determine the ''state'' of the system that is critical in determining the effect of a modulator that is ''state dependent,'' and it provides evidence for yet another level of flexibility in the motor output of this system.

INTRODUCTION

Neuromodulators can activate, terminate, or alter the ongoing activity of rhythmic pattern generators, thereby allowing them to produce a variety of outputs. In many systems extensive reconfiguration is common. As a result, each pattern generator can produce a wide range of patterns that differ in frequency, phase relationships, and the number of participating neurons (Arshavsky et al. 1985, 1989; Benjamin and Elliott 1989; Flamm and Harris-Warrick 1986a,b; Getting and Dekin 1985; Hooper and Marder 1984, 1987; Marder 1987; Murphy et al. 1985; Nagy and Dickinson 1983; Nusbaum and Marder 1988; Quinlan and Murphy 1996; Satterlie 1991, 1993; Sherff and Mulloney 1991; Turrigiano and Selverston 1989; see also reviews by Harris-Warrick and Marder 1991; Katz 1995). Modulation of pattern generators has been seen in response to both identified neurons (Dickinson et al. 1988; Katz and Harris-Warrick 1989, 1990; Katz et al. 1989, 1994; Nagy and Dickinson 1983; Nusbaum and Marder 1989a,b) and bath-applied modulators (Blitz et al. 1995; Coleman et al. 1992; Flamm and Harris-Warrick 1986a,b; Hooper and Marder 1984, 1987; Kiehn and Kjaerulff 1996; Murphy et al. 1985; Nusbaum and Marder 1989a,b; Quinlan and Murphy 1996; Sherff and Mulloney 1991; Weimann et al. 1993). When bath applied at a given concentration and under defined conditions, each modulator generally results in a characteristic and recognizable pattern. For example, the same neurons are usually active. Those neurons have similar phase relationships, burst amplitudes, and spike frequencies. Additionally, cycle frequency is generally similar. Likewise, each modulatory neuron, when fired at a given frequency, tends to produce a characteristic pattern. However, the specific changes produced by a modulator can be ''state dependent''; that is, they may depend on the level and pattern of activity in the network at the time the modulator is applied. This has been seen, for example, in the changes evoked by both modulatory neurons (anterior pyloric modulator: Nagy and Dickinson 1983; modulatory proctolin-containing neuron: Nusbaum and Marder 1989b) and bath-applied transmitters (Blitz et al. 1995; Hooper and Marder 1987; Nusbaum and Marder 1988; Skiebe and Schneider 1994; Weimann et al. 1993) in the pyloric network of the stomatogastric system in crustaceans. In these cases, the extent to which cycle frequency changes is a function of the level of activity in the network when the modulatory input is activated or the modulator is applied. More complex state-dependent effects have been seen in the swimmeret and postural systems of the crayfish (Chrachri et al. 1994). In this case, the response of the swimmeret system to stimulation of a nerve root depends on the state of the postural system.

In many systems, large numbers of transmitters are present, and so networks can receive simultaneous or sequential input from a variety of neuromodulators. In the stomatogastric system of crustaceans, for example, ≥12 different transmitters have been localized to the stomatogastric ganglion (STG) (Blitz et al. 1995; Christie et al. 1994, 1995; Harris-Warrick et al. 1992; Marder 1987; Marder et al. 1995; Skiebe and Schneider 1994). Many of these are modulatory transmitters, which allow the stomatogastric networks to express a large number of patterns. Similar numbers of transmitters have been seen in other species, particularly vertebrates. In addition, single neurons often contain two or more different cotransmitters (e.g., crustaceans: Callaway et al. 1987; Christie et al. 1993, 1995; Cournil et al. 1984; Katz et al.

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1989; Nusbaum et al. 1989; Siwicki et al. 1987; Skiebe and Schneider 1994; *Aplysia* buccal neurons: Cropper et al. 1990; Weiss et al. 1992; Whim and Lloyd 1989; vertebrates: Bartfai et al. 1986; Campbell 1987; Ekblad et al. 1984; Morris 1993; Thorne et al. 1992). In vertebrates, for example, ''classical'' transmitters such as noradrenaline and acetylcholine are frequently found in the same neurons, and even in the same synaptic boutons, as neuropeptides (Bramham 1992; Ekblad et al. 1984; Fried et al. 1985; Hokfelt et al. 1980; Lundberg 1981; Lundberg et al. 1981).

Such a wealth of transmitters, contained both in the same neurons and in different neurons within the same region, generates an enormous potential for interactions among different transmitters. A number of specific interactions have been shown, particularly in vertebrate systems. These interactions include both potentiation and inhibition of responses to other transmitters, and they occur at both presynaptic and postsynaptic sites.

Interactions between two modulators, both of which increase heart rate, have also been reported in the tobacco hawkmoth, *Manduca sexta* (Prier et al. 1994). Both peptides [the cardioacceleratory peptides (CAP2s)] and amines (octopamine and serotonin) increase heart rate on their own, but subthreshold concentrations of the amines can substantially potentiate the response to the CAP2s.

These findings suggest that the many transmitters present in the stomatogastric system could interact in a variety of ways to increase the flexibility of these pattern generators. We have investigated the interactions of two neuropeptides that have been localized to the stomatogastric system of the spiny lobster, *Panulirus interruptus*, and have found that bath application of red-pigment-concentrating hormone (RPCH) dramatically alters the responses of the cardiac sac pattern generator to applications of proctolin. Some of these data have appeared previously in abstract form (Dickinson et al. 1990a).

METHODS

Experiments were performed on male and female California spiny lobsters (*P. interruptus*), weighing 150–600 g. Animals were purchased from Marinus (Longbeach, CA) or Don Tomlinson (San Diego, CA), and were kept in recirculating sea water at 12–15°C for up to 6 wk before use.

Stomachs were removed from lobsters, and the complete stomatogastric system was dissected from the stomach (Selverston et al. 1976) and placed into cold *Panulirus* saline (composition, in mM/l: 479 NaCl, 12.8 KCl, 13.7 $CaCl_2$, 3.9 Na_2SO_4, 10 $MgSo_4$, 11 Trizma base, and 4.8 maleic acid, pH 7.5–7.6) in a Sylgard-lined petri dish. Included with the preparation were the four ganglia of the system [STG, 2 commissural (CoG), and 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. The STG was desheathed in all experiments to allow access to the cell bodies. In experiments in which the effects of peptides on the neuronal somata and/or terminals in the OG were examined, the OG was also desheathed. Additionally, the superior and inferior esophageal nerves were often desheathed so that conduction could be blocked with isotonic (750 mM) sucrose. In other experiments, the superior and inferior esophageal nerves were cut to block conduction irreversibly. A petroleum jelly wall was built across the dish so that the STG could be superfused separately from the other ganglia. In some experiments the OG was likewise isolated with petroleum jelly walls.

Standard electrophysiological techniques were used throughout the experiments. Nerves were recorded extracellularly with the use of 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 potassium acetate (resistances 10–30 MΩ), and WPI M707 microprobe systems or A-M Systems DC amplifiers. Data were recorded on a Gould TA4000 recorder and on video tape with a Vetter VCR adapter. Current for controlling intracellular membrane potentials as well as for extracellular stimulation was obtained from a Grass S88 stimulator with stimulus isolation units. In all experiments in which postsynaptic potential (PSP) amplitude was recorded, two electrodes were inserted into the cardiac sac dilator neuron CD2 and its membrane potential was held at a constant level by injecting current through the second electrode as needed.

Proctolin was purchased from Sigma, and RPCH from Peninsula. RPCH was dissolved in 5% dimethyl sulfoxide, and deionized water was added to make a 10^{-3} M solution. When subsequently diluted to 10^{-6} M, the concentration used in most experiments, dimethyl sulfoxide was at a final concentration of 0.005%, a concentration that had no effects in control experiments. Both peptides were kept as frozen stock solutions of 10^{-3} M and were diluted to the final concentration in *Panulirus* saline just before use.

Data were analyzed statistically with the use of a one-way analysis of variance and post hoc protected *t*-tests (Tukey's *t*-test) using GBStat (Dynamic Microsystems, Silver Spring, MD).

RESULTS

Pretreatment with RPCH alters the response of the cardiac sac network to proctolin

We have previously shown that the responses of the cardiac sac network to the peptides proctolin and RPCH are complex, and depend both on the part of the spatially distributed cardiac sac network to which the peptide is applied and on the connections the cardiac sac neurons make within the stomatogastric system (Dickinson and Marder 1989; Dickinson et al. 1990b, 1993). Thus both proctolin and RPCH can induce rhythmic activity in the cardiac sac network when applied to the entire stomatogastric system (all 4 ganglia; see Fig. 1), to the more anterior CoGs and OG, or to the STG when its connections to the anterior ganglia are intact. However, when the STG is isolated from the CoGs by blocking either the stomatogastric nerve or the superior and inferior esophageal nerves, proctolin no longer elicits cardiac sac activity. In contrast, RPCH elicits rhythmic cardiac sac activity in the isolated STG as well as in the anterior ganglia and in the intact system (Dickinson and Marder 1989).

Because the differences in the effects of proctolin and RPCH are much more dramatic in the isolated STG than in the intact system, and because the preparation has fewer uncontrolled variables, we chose to study the interactions of RPCH and proctolin in a simplified preparation, in which input from the CoGs was removed by either cutting or blocking conduction in both superior and inferior esophageal nerves. Under these circumstances, superfusion of the STG with 10^{-6} M proctolin did not activate cardiac sac bursting. It had no effect on the overall activity of either the inferior

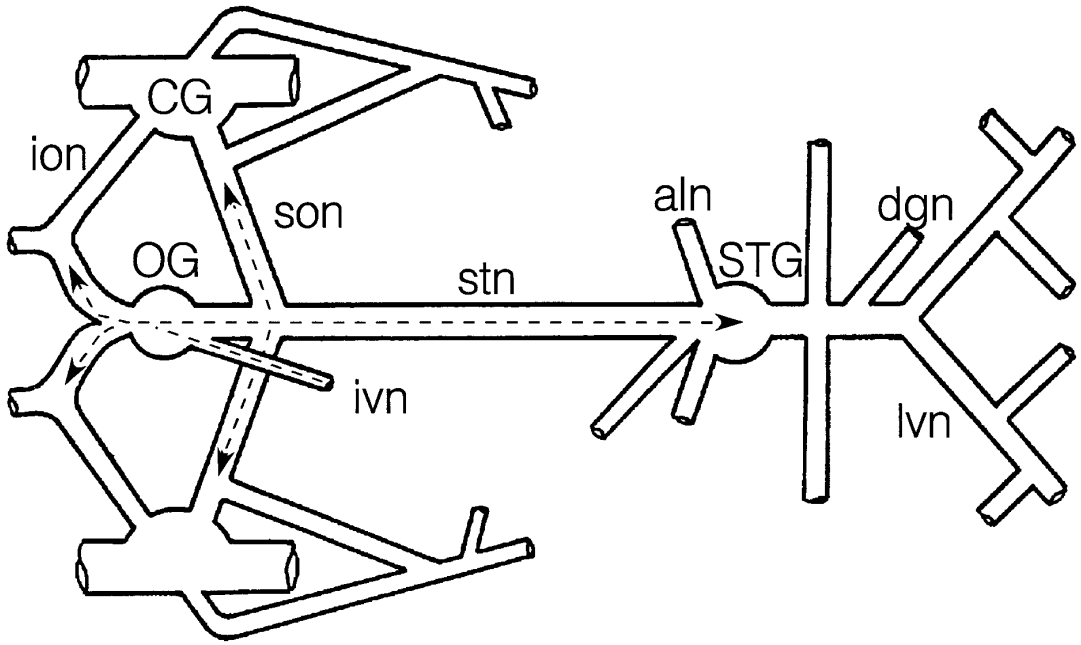

FIG. 1. Stomatogastric nervous system, showing axonal trajectories of inferior ventricular (IV) neurons (– – –). The IV cell bodies are located in the supraesophageal ganglion at the end of the IV nerve (ivn). aln, anterior lateral nerve; CG, commissural ganglion; dgn, dorsal gastric nerve; ion, inferior esophageal nerve; lvn, lateral ventricular nerve; OG, esophageal ganglion; son, superior esophageal nerve; STG, stomatogastric ganglion; stn, stomatogastric nerve.

ventricular (IV) neurons (*ivn* recording) or the cardiac sac dilator neurons CD1 or CD2 (Fig. 2*C*). In the same preparation, after a 1-h wash, bath application of 10^{-6} M RPCH initiated the synchronous bursting in CD1, CD2, and the IV neurons that is characteristic of the cardiac sac motor pattern (Fig. 2*D*). After a short wash (18 min in the preparation shown; Fig. 2*E*), all rhythmic activity had ceased, and proctolin (10^{-6} M) was once again applied. After such a pretreatment with RPCH, proctolin elicited strong cardiac sac activity (Fig. 2*F*). Similar results were seen in 49 of 53 experiments, in which the duration of the wash varied from 5 to 45 min. In an occasional experiment, proctolin continued to elicit cardiac sac activity even after >1 h of washing, but generally, after 45 min to 1 h, proctolin no longer elicited cardiac sac bursting.

There was not a unique threshold at which RPCH alone induced cardiac sac bursts in all preparations. Instead, the threshold, which was $\sim 10^{-8}$ M, varied somewhat from preparation to preparation. However, the percentage of preparations that showed rhythmic activity in response to RPCH increased as a function of peptide concentration (Fig. 3), with >95% of preparations exhibiting rhythmic activity in 10^{-6} M RPCH. In contrast, only 6% of preparations showed rhythmic activity in 10^{-6} M proctolin without RPCH pretreatment. However, after pretreatment with RPCH, almost all preparations (92%, $n = 53$) showed rhythmic cardiac sac activity in 10^{-6} M proctolin.

Both the duration and the intensity of bursting in proctolin after RPCH varied somewhat. In some cases, such as that shown in Fig. 4, burst frequency was higher than that previously seen in RPCH, and bursting continued throughout the duration of the proctolin application. At the other extreme, burst frequency was lower than that recorded in RPCH, and bursting ceased during the proctolin application. The intensity of the proctolin response depended to some extent on the time after RPCH, but there was considerable variability even with the same duration of wash.

We wished to examine in more detail the mechanisms responsible for these interactions of proctolin and RPCH. However, the cardiac sac bursting that is recorded in RPCH appears to be generated in the terminals of the IV neurons in any of the ganglia of the stomatogastric system (Dickinson et al. 1993). Because the IV somata were not present in these preparations, and because bursting appears to be intrinsic to the IV neurons, we suspect that the interactions of RPCH and proctolin likewise occur in the IV terminals. Because it has thus far not been possible to record from these terminals, we have been able to address these questions only indirectly. First, we wondered whether the IV terminals would respond to proctolin only after they had been exposed to concentrations of RPCH high enough to induce rhythmic activity, and whether prior rhythmic activity itself might be necessary. To test this hypothesis, we determined the threshold in a given preparation, then bath applied RPCH at a concentration below threshold (10^{-10} M in the experiment shown in Fig. 5*A*). As predicted, no rhythmic activity resulted. Similarly, no rhythmic activity was recorded when proctolin was applied at 10^{-6} M (Fig. 5*B*). However, when 10^{-6} M proctolin was applied simultaneously with subthreshold RPCH (10^{-10} M), bursting was induced (Fig. 5*C*), indicating that neither previous cardiac sac activity nor previous exposure to high concentrations of RPCH were required. Similar results were obtained in all nine preparations in which subthreshold RPCH was applied together with 10^{-6} M proctolin, although the RPCH concentrations required to activate bursting varied from 10^{-7} to 10^{-10} M.

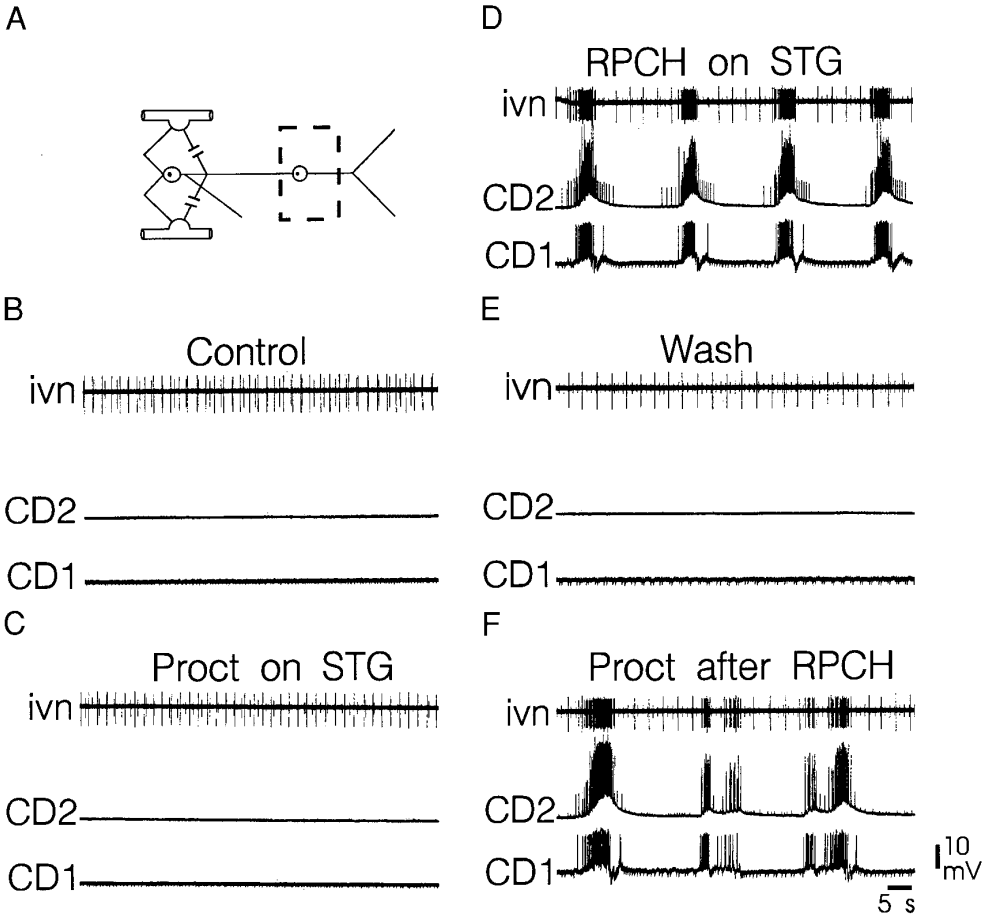

FIG. 2. Proctolin alone did not activate cardiac sac bursting, but proctolin after red-pigment-concentrating hormone (RPCH) did so. *A*: schematic of experimental arrangements: superior esophageal nerves were blocked or cut to decrease commissural ganglion (CoG) input into the STG. Superfusion of peptides was restricted to the STG by a Vaseline wall. *B*: control, showing no cardiac sac activity. *C*: proctolin (10^{-6} M) on the STG did not activate the cardiac sac pattern. *D*: RPCH (10^{-6} M) superfused over the STG activated the cardiac sac pattern, seen here as synchronous bursts on the *ivn* (from the IV neurons) and in the 2 cardiac sac dilator motor neurons CD1 and CD2. *E*: after a short (20 min) wash, cardiac sac activity had ceased. *F*: proctolin (10^{-6} M) applied after RPCH (10^{-6} M) activated the cardiac sac pattern.

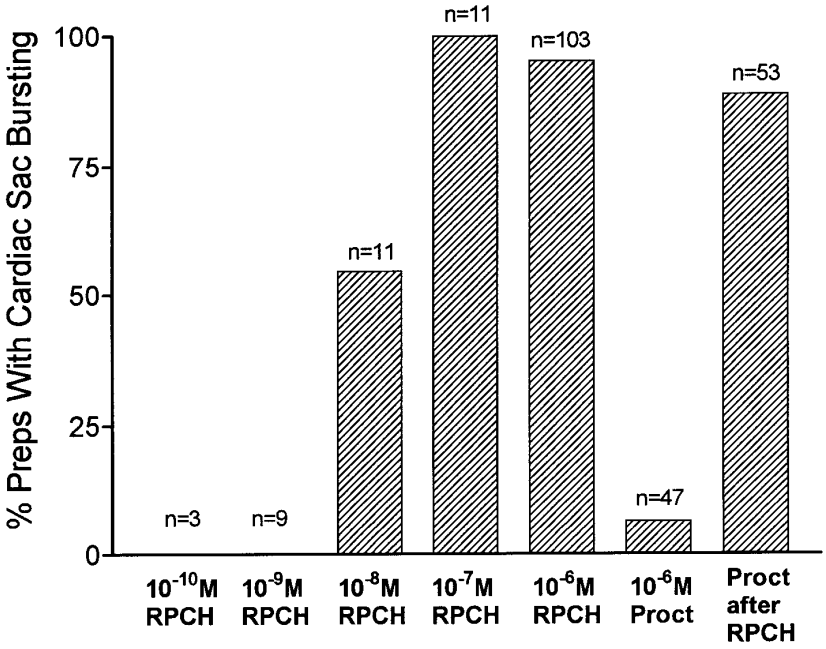

FIG. 3. Response to RPCH was dose dependent, with more preparations showing cardiac sac activity at higher concentrations, so that virtually all preparations showed bursting in 10^{-7} or 10^{-6} M RPCH. Proctolin (10^{-6} M) alone initiated bursting in very few preparations, but did so in nearly all preparations when applied shortly after an application of RPCH (10^{-6} M).

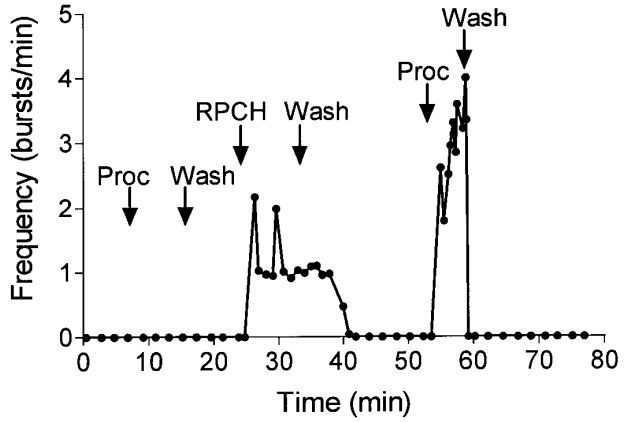

FIG. 4. Cardiac sac burst frequency as a function of time, as proctolin (10^{-6} M) and RPCH (10^{-6} M) were applied. In this case, the bursting due to RPCH took ~7 min to wash out, whereas bursting ceased almost immediately on washout from proctolin. Burst frequency was also considerably higher in proctolin after RPCH than in RPCH alone in this preparation, although that was not a consistent finding.

Next, we wondered whether the relatively long-lasting effects of high (10^{-7}–10^{-6} M) concentrations of RPCH were due simply to interactions of low concentrations of RPCH and higher concentrations of proctolin at the level of the receptors. For example, proctolin may induce rhythmic activity in the IV neurons for many minutes after exposure to RPCH, because low levels of RPCH are still present. If that were the case, then one would expect the duration of the effect (i.e., the amount of time after washout of RPCH during which proctolin will activate the cardiac sac pattern) to be proportional to the perfusion rate during the wash. Thus we both increased and decreased the speed of the superfusion (from 10–12 ml/min to 5–6 ml/min or 18–20 ml/min). Although it is possible that RPCH in tightly bound spaces was not washed away more rapidly by this treatment, in no case did we see any correlation of the duration of the interactive effects with the perfusion speed, suggesting that the interaction between RPCH and proctolin is not due solely to lingering (and unbound) RPCH. We also noted that there was not a strong correlation between the concentration of RPCH used and the duration of the effect. There was a loose correlation, with the effect generally lasting longer after higher RPCH concentrations, but even at a single concentration the duration varied considerably. However, such a relationship could equally well be explained by increased alteration of receptors or by increased second-messenger production in the higher RPCH concentrations.

RPCH and proctolin interact in the OG as well as the STG

Because the cardiac sac network is distributed, and because the IV neurons appear to initiate bursting in response to RPCH in each of the four ganglia (Dickinson et al. 1993), we wondered whether proctolin and RPCH interacted in a similar way in each of the ganglia. To answer this question, we first examined the responses of the cardiac sac network to proctolin superfused separately over the CoGs and the OG. When bath applied to the CoGs, proctolin alone was generally sufficient to induce cardiac sac activity, so we did not continue to look for interactions in the CoGs. In the OG, however, the response to proctolin alone was inconsistent. In 67% of the preparations tested ($n = 12$), proctolin did not activate the cardiac sac network (Fig. 6, *B* and *C*), but

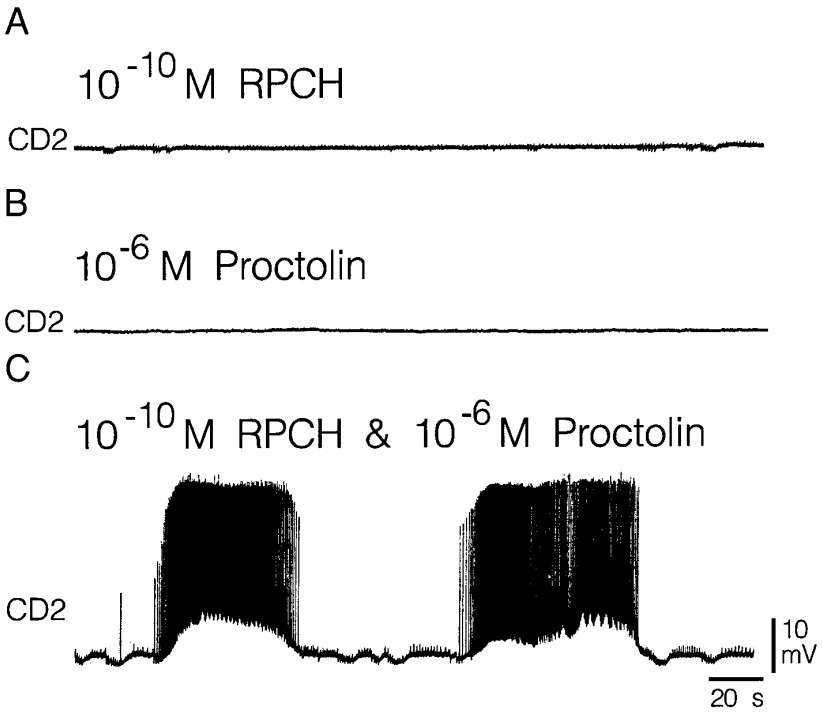

FIG. 5. Proctolin and subthreshold RPCH can induce cardiac sac bursting. *A*: 10^{-10} M RPCH, which was always subthreshold (see Fig. 3), alone had no visible effect. *B*: proctolin alone had no effect on CD2 activity. *C*: when applied together, 10^{-6} M proctolin and 10^{-10} M RPCH induced strong cardiac sac bursting.

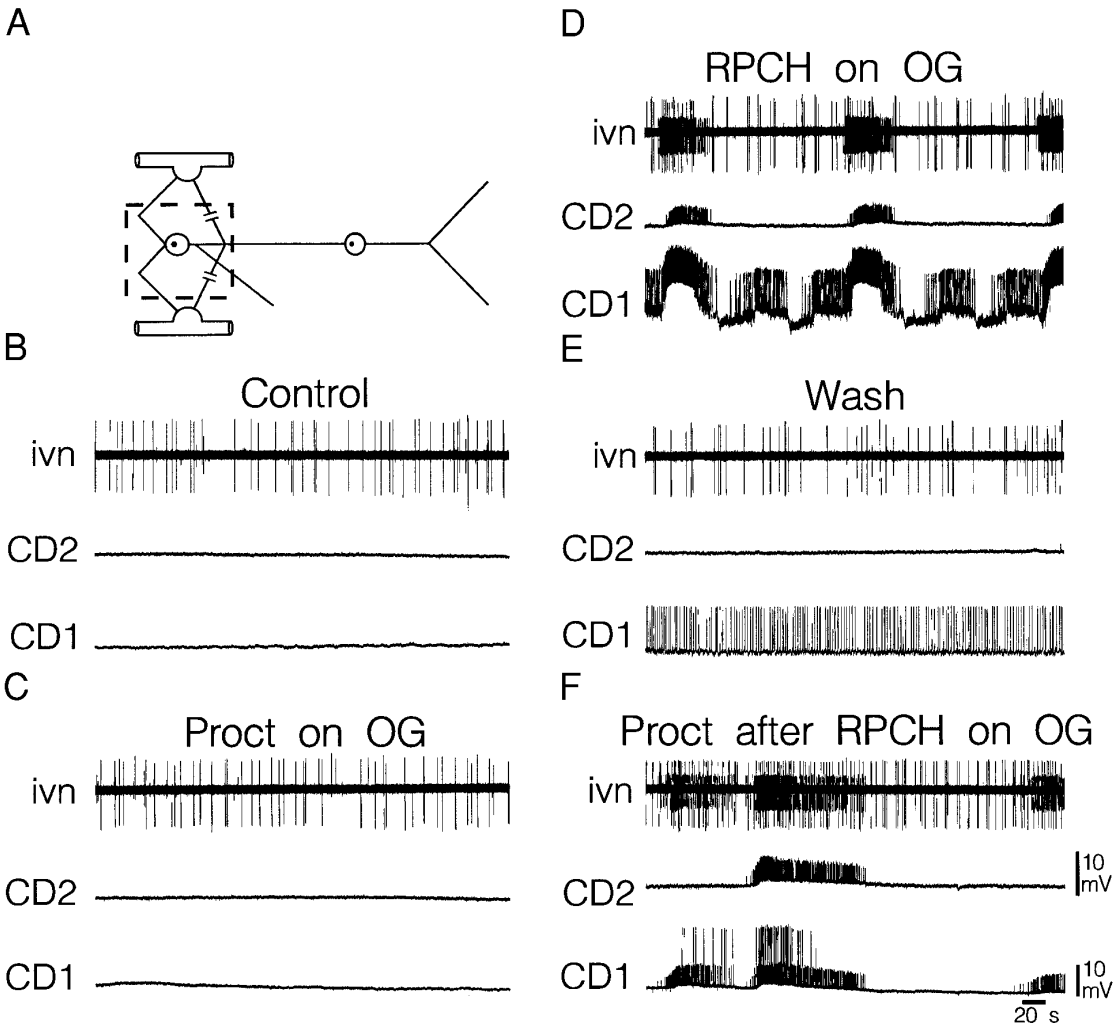

FIG. 6. Effects of RPCH and proctolin superfused over the OG were similar in some cases to the effects recorded when peptides were applied to the STG. *A*: input from the CoGs was minimized by blocking or cutting the superior esophageal nerves, and peptides were bath applied to the OG only. *B*: control, showing no cardiac sac activity. *C*: proctolin (10^{-6} M) did not elicit cardiac sac bursting in this preparation. *D*: RPCH (10^{-6} M) elicited strong cardiac sac activity, seen as synchronous bursts in the IV neurons recorded on the IV nerve and in CD1 and CD2. In addition, less intense bursts, whose origin was not clear, were recorded in CD1. *E*: effects of RPCH readily washed out, although CD1 remained somewhat depolarized and continued to spike tonically. *F*: proctolin applied 25 min after the start of the wash induced clear cardiac sac activity, including synchronous bursts of action potentials in CD1, CD2, and an IV neuron. In this case, a 2nd type of burst was recorded on the IV nerve, with associated bursts in CD1 but not CD2. These most likely represent nonsynchronous firing of the 2 IV neurons, with 1 of the IVs activating CD1 but not CD2. It is possible that the axon from the 2nd IV to CD2 was damaged in desheathing, or it is possible that 1 of the IVs makes synaptic contact with CD1 but not CD2. This is not usually seen, because the IV neurons most commonly burst synchronously.

RPCH did so (Fig. 6*D*). In 87.5% of those preparations (7 of 8), proctolin after RPCH did induce rhythmic cardiac sac activity (Fig. 6, *E* and *F*). In the 33% of preparations in which proctolin alone was sufficient to induce bursting (Fig. 7, *A* and *B*), burst frequency was generally higher when proctolin was applied after RPCH (Fig. 7).

RPCH and proctolin interact to potentiate an identified EPSP

The onset of rhythmic activity is not all or none. Although there are differences in frequency and intensity, as well as in the duration of the effect, which are loosely correlated with RPCH concentration, there is considerable variability, and so these differences are difficult to quantify. We therefore decided to examine another effect of RPCH, one that would be more readily quantified and that might allow us to see interactions at concentrations lower than those that induce bursting. Additionally, this would allow us to look at a more graded response, rather than simply at an ON/OFF response. We had previously shown that the amplitudes of excitatory PSPs (EPSPs) from the IV neurons to CD2 increase in the presence of RPCH (Dickinson et al. 1990b, 1993). Part of this increase results from facilitation, but the PSP amplitude increased before the onset of bursting, indicating a direct effect of

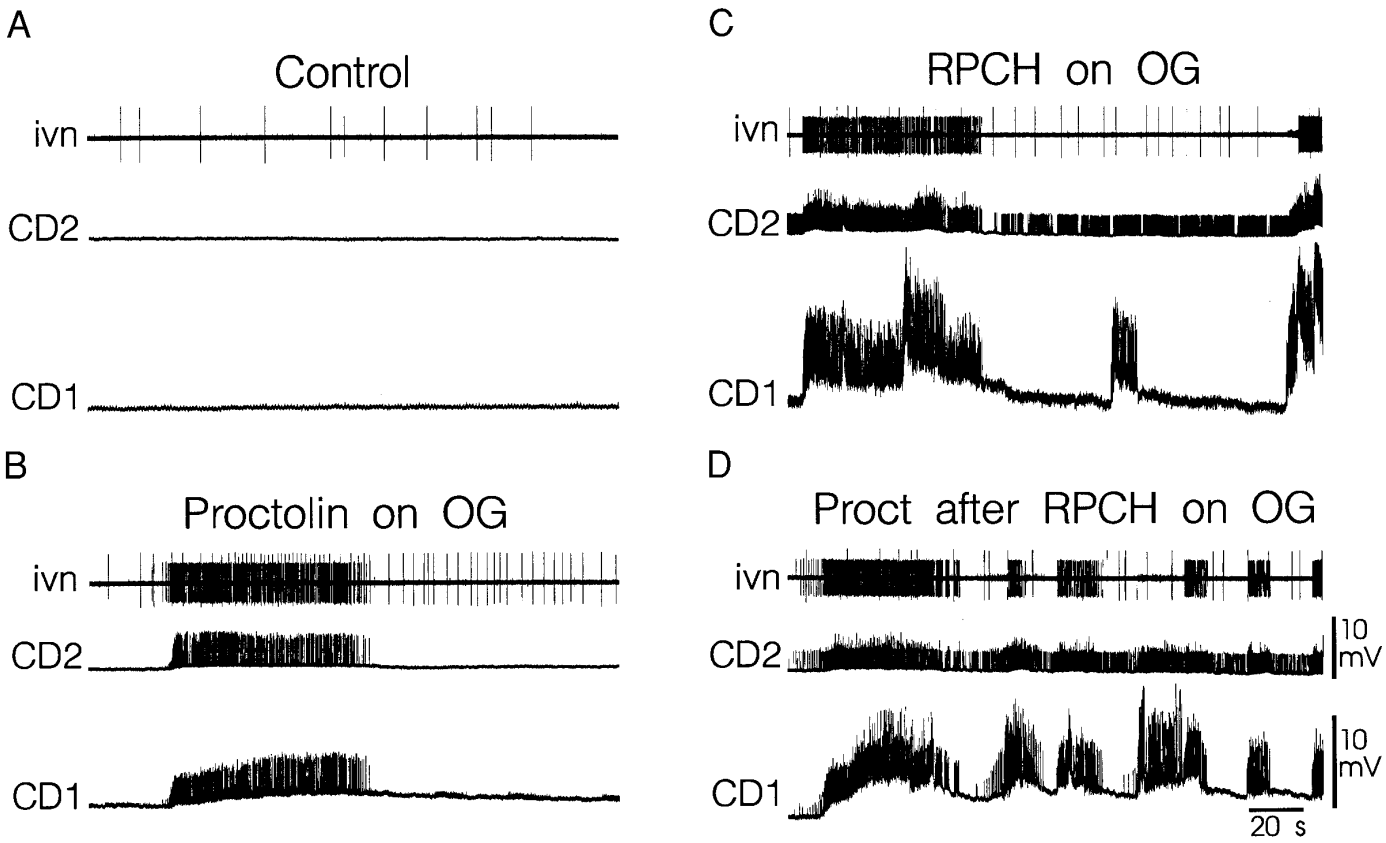

FIG. 7. In some preparations, proctolin alone was able to elicit cardiac sac activity when superfused over the OG; in these cases, activity levels increased in proctolin after RPCH. *A*: control, showing no spontaneous cardiac sac activity. *B*: proctolin (10^{-6} M) bath applied to the OG elicited a slow cardiac sac pattern, with longer but infrequent bursts. *C*: RPCH likewise provoked cardiac sac activity. *D*: in proctolin after RPCH, cardiac sac bursting was more intense and had a higher frequency.

RPCH on the PSP as well (Dickinson et al. 1993). We therefore examined the effects of proctolin, of different concentrations of RPCH, and of proctolin after RPCH on the amplitude of the EPSP in CD2.

Bath application of either 10^{-6} M RPCH or 10^{-6} M proctolin caused an increase in the amplitude of the EPSP elicited in CD2 by stimulation of the *ivn*, which activates the IV neurons (Fig. 8). In neither case was there a change in the postsynaptic membrane resistance, as reflected in the current-voltage relationships of CD2 (proctolin, data not shown; RPCH, Dickinson et al. 1990b). The increased PSP amplitude in proctolin was much smaller than that recorded in RPCH. Mean PSP amplitude increased to 150% of control values in proctolin, and to 400% of control in RPCH. Although the PSP returned to the initial control value in the wash after proctolin, it returned only to a higher level, 150% of control, in the shorter wash after RPCH. This value was not significantly different from the value recorded for proctolin alone. However, when proctolin was applied shortly after RPCH (to the already somewhat larger PSP), its amplitude increased to ~600% of control, a value not significantly different from that recorded in RPCH alone. Thus, whereas proctolin alone did potentiate the IV PSP in CD2, proctolin after RPCH caused further potentiation. The time course of the increase in PSP amplitude in proctolin after RPCH was consistently faster than that of the increase in the preceding RPCH perfusion (Fig. 9). In part, this may reflect the more rapid onset of bursting in proctolin after RPCH. The largest increase in PSP amplitude in both RPCH and proctolin after RPCH took place just after the first burst, suggesting that a major component of the increased PSP amplitude may have been facilitation resulting from the cardiac sac bursts. Like the cardiac sac burst frequency, the PSP amplitude in proctolin after RPCH sometimes increased to a size greater than that recorded during the preceding RPCH treatment, but was sometimes a bit smaller. Similarly, in some cases it decreased early during the perfusion (Fig. 9*B*), whereas in other cases (Fig. 9*C*) it remained potentiated throughout most or all of the duration of the proctolin (after RPCH) perfusion. In general, the initial rapid decrease in PSP amplitude (Fig. 9*B*) correlated well with the cessation of bursting. Thereafter, PSP amplitude returned very gradually toward control levels. This slower decrease in amplitude reflected the time course of the return of PSP amplitude to control after RPCH alone, which was very slow, and frequently took >1 h.

We also examined the amplitude of the IV PSP in CD2 in a number of concentrations of RPCH, both sub- and suprathreshold for induction of rhythmic cardiac sac bursting. In the experiment from which these data were taken, the thresh-

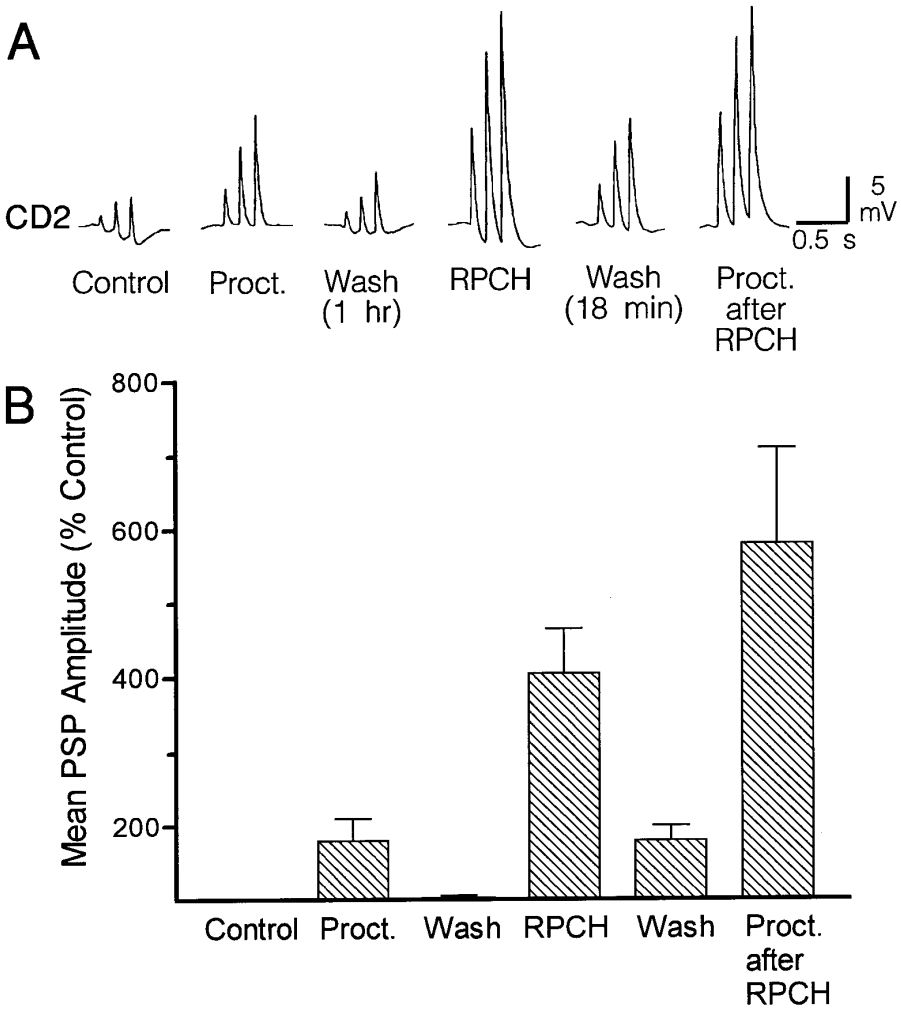

FIG. 8. Amplitude of CD2 excitatory postsynaptic potentials (EPSPs) from the IV neurons is increased in proctolin, RPCH, and proctolin after RPCH. Membrane potential of CD2 was −78 mV at the start of each train of postsynaptic potentials (PSPs). *A*: recordings of trains of 3 PSPs each, provoked by stimulation of the IV nerve. *B*: mean PSP amplitude doubled in proctolin alone, whereas it increased to ~400% of control in RPCH (10^{-6} M) alone and to nearly 600% in proctolin (10^{-6} M) after RPCH (10^{-6} M). During the wash from RPCH, PSP values fell only to ~200% of control, not significantly different from the value recorded in proctolin alone. (Given long enough, it would have returned to control levels.) The mean values of PSPs in RPCH and proctolin after RPCH were not significantly different. Means and SEs of average PSP amplitudes from 10 experiments.

old for cardiac sac bursting was 10^{-7} M RPCH (Fig. 10), and PSP amplitude increased rapidly with increasing RPCH concentration. Additionally, it was clear that the induction of cardiac sac bursting and the potentiation of the PSP are not tightly correlated. In RPCH, cardiac sac bursting and PSP potentiation were both seen at concentrations of 10^{-7} M. However, proctolin (10^{-6}) alone also caused a potentiation of the PSP equal to that seen in 10^{-7} M RPCH, yet no cardiac sac rhythm was induced.

DISCUSSION

The effects of a given modulator on a rhythmic motor pattern are determined not only by the nature and concentration of the modulator and by the system on which it acts, but also by the ''state'' of that system. We have shown here that both the presence of other modulators and the system's history of exposure to modulators, even at subthreshold concentrations, are among the crucial determinants of this state. In the stomatogastric system, the response of the cardiac sac pattern generator to the peptide proctolin is qualitatively as well as quantitatively different when the peptide is applied in the absence of other modulators than when it is applied either shortly (5–20 min) after the rhythm induced by RPCH has ceased or during the application of subthreshold RPCH. Thus, when proctolin was applied after RPCH, the IV neurons were able to generate the bursting activity that underlies the cardiac sac pattern. Additionally, the strength of the synaptic interactions between the IV neurons and the cardiac sac dilator neuron CD2 was enhanced. Although proctolin alone enhanced PSP amplitude to an extent equal to that seen in low concentrations of RPCH, it did not induce the rhythmic bursting in the IV neurons that characterizes the cardiac sac pattern, as did low concentrations of RPCH. This suggests the possibility that the induction of bursting and the enhancement of the PSP may not be controlled by the same mechanism.

The fact that PSP amplitude increased in proctolin alone, whereas the postsynaptic membrane resistance remained unchanged, suggests that proctolin itself was affecting the IV neuron terminals. However, the PSP in proctolin alone is still severalfold smaller than in proctolin after RPCH. This might be due in part to direct interactions of the two peptides at the terminals. However, much or even all of this increase may have been due to facilitation, for the IV neurons show extensive and long-lasting facilitation (Dickinson et al. 1993), which would have been activated by the bursting induced in proctolin after RPCH.

Functional interactions between transmitters have been

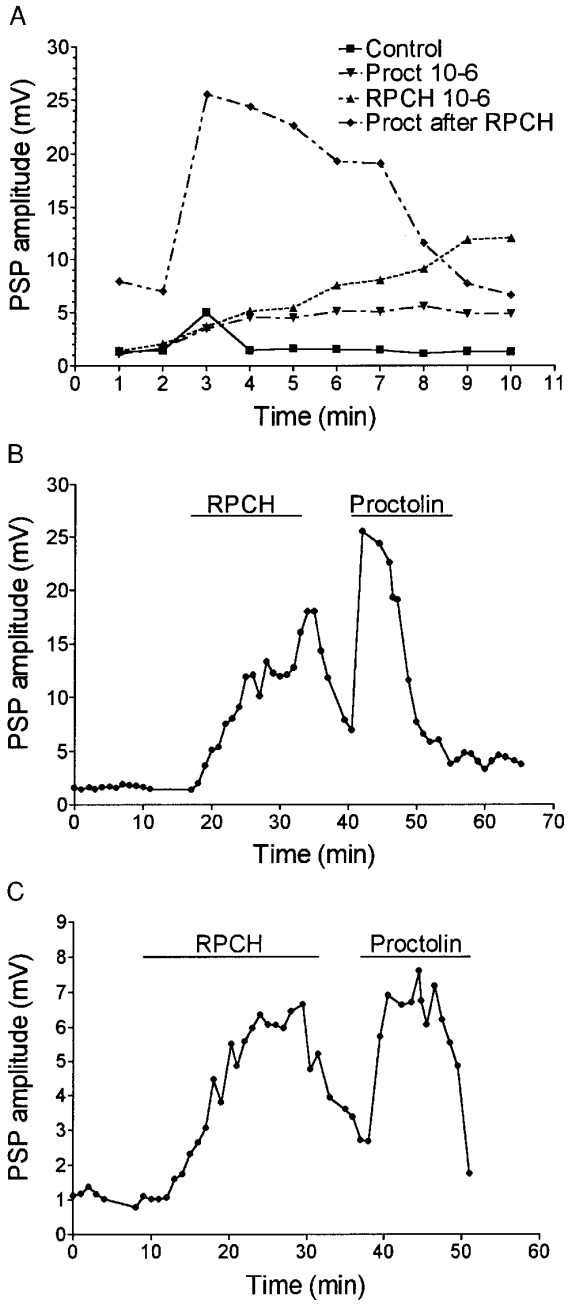

FIG. 9. Time course of changes in PSP amplitudes as a function of time after proctolin (10^{-6} M) and RPCH (10^{-6} M) application. PSPs measured were the 1st in each of a series of 4 PSPs provoked by stimulation of the *ivn* at 4 Hz. Single PSPs showed similar responses. *A*: 1st 10 min of each peptide application show that the initial increase in PSP amplitude is slow in both proctolin (alone) and in RPCH, but is very rapid in proctolin after RPCH. *B* and *C*: PSP amplitude increased slowly in RPCH, but rapidly in proctolin after RPCH. PSP amplitude generally remained elevated throughout the duration of the RPCH application. In some preparations (*B*) PSP amplitude decreased during proctolin application, whereas in other preparations (*C*) it remained elevated during the entire application.

shown in a number of cases. In many of these cases, at least a part of the mechanism by which the transmitters interact is also known. The mechanisms involved are quite diverse, and include both pre- and postsynaptic mechanisms, as well as indirect effects, and intracellular interactions mediated by second messengers. For example, neuropeptide Y in the sympathetic nervous system can either increase or decrease the effects of catecholamines, depending on the relative concentration of two receptor types. One is presynaptic, causing inhibition of release, and one is postsynaptic, causing potentiation of the postsynaptic response (Colmers et al. 1987, 1988; Ekblad et al. 1984; Martire and Pistritto 1992). In the hippocampus, neuropeptide Y appears to suppress the release of catecholamines by decreasing presynaptic Ca^{2+} influx (Colmers et al. 1987, 1988). Much the same mechanism appears to account for the inhibition by neuropeptide Y of transmitter release in sympathetic nerve terminals (Toth et al. 1993). The potentiation by neurotensin of dopamine inhibition in the neostriatum likewise appears to be presynaptic, although the mechanism is not known in more detail (Beauregard et al. 1992). Similarly, P2 purinoceptors mediate a prejunctional negative feedback in which ATP inhibits noradrenaline release in sympathetic neurons of the mouse vas deferens (Von Kuegelgen et al. 1993).

The interactions between dopamine and neurotensin are mediated by postsynaptic mechanisms in addition to the presynaptic mechanisms described above, both in the neostriatum and in the prefrontal cortex (Beauregard et al. 1992). The alteration of binding constants as well as changes in the number of receptors have been implicated in a number of systems (Fuxe and Agnati 1985; Lundberg et al. 1982).

A potentially widespread mechanism by which two or more modulators could interact is through second-messenger pathways. Two modulators may activate the same pathway, or one may inhibit the activation of the pathway activated by the second, as has been shown for dopamine and carbachol in rat brain membrane preparations (Salles et al. 1993; Wallace and Claro 1990). Two or more pathways could also converge at other intracellular sites, as appears to be the case in the buccal ganglion of *Aplysia*, where two peptides ulti-

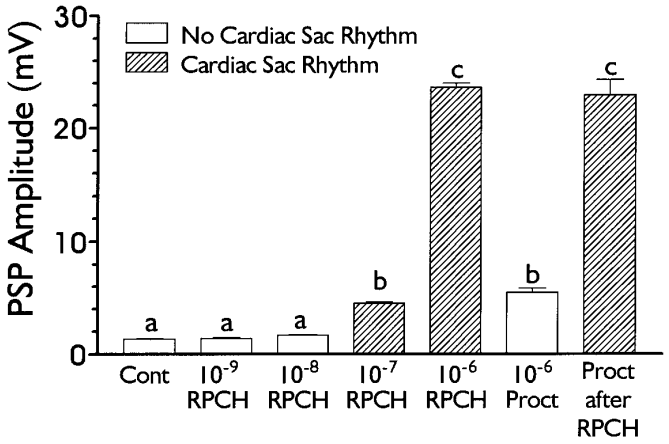

FIG. 10. Threshold for initiation of cardiac sac bursting and an increase in the amplitude of the CD2 PSP from the IV neurons were not always the same. In this preparation, cardiac sac activity (filled bars) was initiated in 10^{-7} M RPCH, and PSP amplitude began to increase in 10^{-7} M RPCH. However, when the STG was superfused with proctolin alone, a similar increase in PSP amplitude was seen, but cardiac sac bursting was not elicited. Means and SEs of the amplitudes of the 1st PSP in each of 4 trains from a single preparation are shown. Letters atop bars: groups that are not significantly different from 1 another. Groups (a–c) are significantly different ($P < 0.01$).

mately promote phosphorylation of the same membrane protein (Weiss et al. 1992).

The effects of RPCH on the cardiac sac rhythm are mediated largely, or perhaps entirely, by their effects on the terminals of the IV neurons in all the ganglia of the stomatogastric system (Dickinson et al. 1993), so we would predict that the interactions of RPCH and proctolin take place at these same sites. Because of this, it has been impossible to determine directly the mechanisms by which proctolin and RPCH interact. However, we have considered a number of possibilities, including an interaction at the level of the receptors and an interaction of second messengers within presynaptic terminals. RPCH might, for example, ''prime'' a receptor, with one of two results: proctolin is able to bind to and activate a receptor that is otherwise unavailable to it, or the binding of proctolin to its receptor is enhanced. On the other hand, RPCH may very well work by activating a second-messenger pathway. The effects of RPCH alone are relatively long lasting modulatory effects (Dickinson and Marder 1989; Dickinson et al. 1990b, 1993), and such effects are frequently mediated by second messengers. Additionally, although proctolin alone does not activate the cardiac sac pattern in an isolated STG, it is clear that proctolin by itself does affect the IV neurons: in the isolated STG, proctolin by itself causes an increase in the amplitude of PSPs from the IV neurons, and in the intact system, proctolin can activate cardiac sac bursting (Dickinson and Marder 1989). If both the proctolin and the RPCH effects are mediated by second messengers, then there is a myriad of ways in which those messengers could interact to alter the effect of the proctolin after RPCH. We do know that the IV neurons need not be exposed to RPCH before proctolin, because proctolin in the presence of subthreshold RPCH can provoke cardiac sac activity. Additionally, we know that even much higher concentrations of proctolin alone (e.g., 10^{-4} M) do not initiate cardiac sac bursting (unpublished observations).

A similar interaction of second messengers has been suggested as a possible mechanism for the interactions of the CAPs and biogenic amines in *Manduca.* In this case, the peptides and the amines each activate a separate second-messenger pathway. However, the mechanism responsible for the potentiation of the CAP response by the amines is not yet understood (Prier et al. 1994).

It is also possible that the interactions of proctolin and RPCH are indirect, as has been suggested for the interactions of opioids and glutamate in promoting long-term potentiation in the hippocampus. In this case, opioids may suppress inhibitory interneurons, allowing pyramidal and granule cells to depolarize further than normal, thereby resulting in stronger activation of NMDA receptors and an enhancement of LTP (Bramham 1992).

Functionally, we would predict that the interactions between proctolin and RPCH would add to the flexibility of the stomatogastric system. It is not clear from work to date that the cardiac sac pattern itself is different when induced by RPCH alone or by proctolin after RPCH. However, we know from previous work that the activation of the cardiac sac pattern by RPCH can also alter the interactions of the cardiac sac network with other networks of the system (Dickinson et al. 1990b), so it will be of interest to examine the modulation of the gastric mill and pyloric patterns in proctolin after RPCH. Furthermore, in the presence of appropriate inputs from the more anterior ganglia, proctolin alone is able to induce cardiac sac activity (Dickinson and Marder 1989), suggesting the possibility that multiple mechanisms may enable the IV neurons to generate a bursting pattern in response to proctolin.

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