cAMP Elevation Modulates Physiological Activity of Pyloric Neurons in the Lobster Stomatogastric Ganglion

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SUMMARY AND CONCLUSIONS

1. We analyzed the physiological effects of the adenylate cyclase activator forskolin, and other adenosine 3',5'-cyclic monophosphate (cAMP)-elevating agents, on neurons of the pyloric circuit from the stomatogastric ganglion of the lobster Panulirus interruptus. Agents were bath applied to pyloric neurons either in the synaptically intact pyloric circuit or following isolation from all known synaptic input.

2. Several cAMP-elevating agents, including forskolin, 3-isobutyl-1-methylxanthine, Ro20-1724, and 8-bromo-cAMP, generated similar motor patterns from the pyloric circuit. The motor patterns exhibited an increased cycle frequency and enhanced spike activity from all classes of pyloric neurons. Since these agents differ both in structure and site of action in the CAMP pathway, their physiological effects on the motor pattern probably result from increased cAMP levels in pyloric neurons.

3. When forskolin was applied to synaptically isolated neurons, it caused a strong activation or enhancement of activity of all pyloric cells. However, it induced different types of activity in different cells, including the induction of bursting pacemaker potentials in one cell type, activation of plateau potentials in another, and depolarization with activation or enhancement of tonic spike activity in the remaining cells. Thus there is no single physiological response to cAMP elevation in the pyloric circuit; its effects can be quite diverse, mediating several activity states, in different cells.

4. Radioimmunoassays were performed on whole stomatogastric ganglia to determine whether known neuromodulators can affect cAMP concentrations. Both forskolin and octopamine increased cAMP levels, whereas dopamine, serotonin, proctolin, and FMRFamide did not appreciably affect cAMP levels. The physiological effects of octopamine and forskolin are similar in most, but not all, pyloric cells. Octopamine is thus a candidate neuromodulator whose actions may be mediated, at least in part, by increased cAMP in some pyloric cells; however, forskolin does not completely mimic the physiological effects of octopamine on all pyloric neurons, suggesting that octopamine can also act by other biochemical mechanisms.

INTRODUCTION

The modulation of simple centrally patterned behaviors has been under intensive study in recent years. Work has concentrated primarily on identifying neuromodulators and describing their physiological effects on motor patterns produced by central pattern generators (CPGs) (see 19 for review). Neuromodulators induce their effects on neurons by causing changes in biochemical processes and ionic conductances (see 30, 51 for reviews). These cellular actions are the ultimate basis for neuromodulator-induced plasticity of centrally patterned motor activity. However, the exact relation between cell and circuit modulation has not been studied in detail. Therefore, to better understand the
mechanisms by which a neuromodulator produces its effects on a motor pattern, one must determine the modulator's actions on the neurons in the circuit that generates the motor pattern.

We have been studying the pyloric circuit from the stomatogastric ganglion of the spiny lobster, Panulirus interruptus. The pyloric circuit is an intensively studied CPG in which the component neurons, their neurotransmitters (34), and synaptic connectivity of the circuit (14, 37, 38, 41) are all known. The physiological effects of several endogenous neuromodulators of the pyloric motor pattern have been described, including the amines dopamine, octopamine, serotonin (2, 15) and histamine (5), acetylcholine (35, 45), and the peptides proctolin and FMRFa-mide-like peptide (25, 25a, 36). The target neurons within the pyloric circuit that are directly modulated by dopamine, octopamine, serotonin, (16) and proctolin (26) have also been identified, and some of the physiological actions of these modulators have been characterized. This work has shown that a single neuromodulator can have diverse effects on the ionic currents and physiological activity of different pyloric neurons (20).

Second-messenger cascades usually mediate the physiological actions of neuromodulators. One compound, adenosine 3',5'-cyclic monophosphate (cAMP), is a ubiquitous mediator of a wide range of neurotransmitter-induced responses in the nervous system. cAMP is involved in modifications of the intrinsic activity of neurons (11, 12, 17, 26), as well as synaptic interaction between neurons (13, 18, 28). In this paper, we describe the physiological effects of the adenylate cyclase activator, forskolin, on pyloric neurons in the lobster stomatogastric ganglion. This is the first step in an analysis of biochemical mechanisms of neuromodulation of the pyloric CPG. We have also determined which known neuromodulators can enhance cAMP levels in the stomatogastric ganglion and compared their physiological effects with those of forskolin in this system.

METHODS

Pacific spiny lobsters (Panulirus interruptus) were purchased from Marinus, (Westchester, CA) and kept in marine aquaria at 15°C until use. The stomatogastric nervous system was removed from the animal, as described by Mulloney and Selverston (42) and was superfused at 3 ml/min with cold (14°C) oxygenated saline (saline composition in mM): NaCl 479, KCl 12.8, CaCl2 13.7, Na2SO4 3.9, MgSO4 10.0, glucose 2.0. Tris base 11.1, malic acid 5.1; pH 7.35.

Physiological effects of cAMP-elevating agents on pyloric neurons

Data collection and analysis have been previously described (15). Neuronal activity was recorded with glass suction electrodes to record extracellular action potentials from motor roots and with glass microelectrodes to record intracellularly from the somata of identified neurons. A second intracellular microelectrode (20–30 MΩ) was often used for current injection. Amplification and recording were conventional.

Experiments were performed on the effects of cAMP-elevating agents on single pyloric neurons that had been isolated from all known synaptic input in order to simplify analysis. The synaptic isolation procedure was a modification of the one described by Flamm and Harris-Warrick (16). Vaseline bridges were used to separate the stomatogastric nervous system into three compartments (Fig. 1A). Descending modulatory inputs from the commissural (CG) and oesophageal (OG) ganglia were blocked by bathing the compartment containing these ganglia and associated nerves in saline containing 1 PM tetrodotoxin (TTX). The two commissural ganglia were desheathed to aid in penetration of TTX. Also, action-potential conduction through the stomatogastric nerve (STN), which carries input axons from the OG and CG to the stomatogastric ganglion (STG), was blocked by the replacement of saline with 5 M sucrose and 5 μM TTX in the middle compartment (sucrose/TTX) block. The STN was desheathed to aid in penetration of sucrose and TTX. Two procedures were used to block pyloric circuit synaptic connections within the STG. 1) Saline containing 5 μM picrotoxin was bath applied throughout the experiment to the compartment containing the STG and its associated motor nerves to block synaptic input from glutamatergic pyloric neurons (34) (Fig. 1B). 2) Within the STG, synapses from cholinergic neurons (Fig. 1B) and synapses from electrotonically coupled pyloric neurons, (with the exception of weak electronic connections among pyloric cells), were eliminated by photoinactivation of the presynaptic cells (16, 39, 40). To do this, the tip of a low-resistance microelectrode (15 MΩ) was filled with 5,6-carboxyfluorescein (Eastman Kodak 3% in double distilled water, pH 7) and backfilled with 4 M potassium acetate. The dye was iontophoresed
FIG. 1. A: stomatogastric nervous system (STNS). The STNS is composed of 4 ganglia and motor nerves, which innervate the stomach. The stomatogastric ganglion (STG) contains the neurons of the pyloric network and receives modulatory input from the 2 commissural ganglia (CGs) and oesophageal ganglion (OG) via the stomatogastric nerve (STN). We divided the STNS into 3 compartments, defined by shaded lines, to aid in synaptic isolation of pyloric neurons. See text for details. MVN, median ventricular nerve; PDN, pyloric dilator nerve; PYN, pyloric nerve; LPN, lateral pyloric nerve.

B: pyloric network. The pyloric circuit consists of 14 neurons in 6 classes. Five classes are motorneurons and one, the AB cell, is an interneuron. Synaptic connections are either chemical inhibitory (filled circles) or electrotonic (resistors). The 2 PD neurons are electrotonically coupled as are the PY cells. Strong synaptic connections are represented by solid lines; weak connections are represented by dashed lines. The PD and PD are cholinergic (shaded) and the other cells are glutamatergic (striped). AB, anterior burster; PD, pyloric dilator; IC, inferior cardiac; LP, lateral pyloric; PY pyloric.

into selected neurons with 6-nA hyperpolarizing current pulses of 600-ms duration at a frequency of 1 Hz for 10–15 min. After a 10-min wait to allow for diffusion of the dye, the cells were exposed to bright blue light (Leitz epifluorescence attachment to Wild dissecting microscope, wave-length = 390–490 nm, 50-W mercury lamp) for ~10 min until inactivated as observed by elimination of resting potential, extracellularly recorded action potentials, and synaptic potentials onto follower cells. Following synaptic isolation, neurons were either inactive or exhibited weak tonic spike activity (16). We waited at least 2 h following the synaptic isolation procedure before starting an experiment.

Cyclic nucleotide-elevating agents were bath applied at 3 ml/min to the compartment containing the STG and its associated nerves. The small volume of this compartment (2–3 ml) allowed us to add and remove these agents rapidly during the experiment. Forskolin (Calbiochem) was dissolved in absolute ethanol or dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO) at a concentration of 50 mM and stored at 5°C. It was diluted in lobster saline, just prior to use, to make concentrations from 5 to 50 μM. Neither solvent alone (up to 0.1% by volume) had a detectable effect on neuronal activity. 3-Isobutyl-1-methylxanthine (IBMX, Aldrich Chemical) and 8-bromo-cAMP (Sigma Chemical) were dissolved in lobster saline just prior to use. Ro20-1724 (gift from Hoffman-La Roche) was dissolved in DMSO at concentrations of 35 mM then diluted with lobster saline to a final concentration of 10-35 μM just prior to use.

Pyloric neurons exhibit a variety of intrinsic membrane properties, such as plateau potentials (47, 48), conditional bursting (40), and postinhibitory rebound (PIR: 50). We studied plateau potential capabilities with short (50 ms) depolarizing current pulses. Plateau potential properties are active if a short current pulse elicits a prolonged depolarization that outlasts the duration of the current pulse (47). Endogenous bursting may be directly observed in pyloric neurons during superfusion of cAMP-elevating agents. However, bursting could be masked by another activity state, such as tonic spike activity. Therefore, we injected both depolarizing and hyperpolarizing DC currents to determine whether rhythmic activity could be induced at different membrane potentials during forskolin application. cAMP-induced changes in PIR could be measured as changes in the rebound depolarization and spike activity following hyperpolarizing current pulses (500 ms).

Radioimmunoassay of cAMP levels in intact STG

A radioimmunoassay (RIA) procedure was used to determine the effects of several compounds on cAMP levels in the STG. The STG was dissected out with at least 1 cm each of the two major nerves still connected to the ganglion (see
Fig. 1A), desheathed, and superfused with chilled (15°C) saline for 30–60 min. The desired drug was bath applied for 10 min at a concentration shown to have very strong or physiologically saturating effects on the pyloric motor pattern (15, 36). The ganglion, with one ganglion’s length of the two major nerves still attached, was frozen in 50 μl of 6% trichloroacetic acid (TCA) on dry ice. After thawing, the sample was homogenized with an additional 25 μl of 6% TCA and centrifuged at 10,000 g. The supernatant was assayed for cAMP using materials and procedures provided by a cAMP, [125I]-RIA kit (New England Nuclear). The TCA-precipitable pellet was assayed for protein content using the Amidoschwarz assay of Schaffner and Weissman (49). Ganglia each contained between 2 and 10 μg of TCA-precipitable protein using this assay.

RESULTS

Physiological effects of cAMP-elevating compounds on the pyloric motor pattern

Figure 2 shows the physiological responses of a basal cycling pyloric motor rhythm (15) to agents that increase intracellular cAMP levels by acting at several different sites in the cAMP cascade. Bath application of the adenylate cyclase activator forskolin, the phosphodiesterase inhibitor IBMX, and the cAMP analogue 8-bromo-cAMP all produced similar physiological effects. Spike activity from all six classes of pyloric neurons was either initiated [lateral pyloric (LP), pyloric (PY), inferior cardiac (IC)] or enhanced [pyloric dilator (PD), ventral dilator (VD)].
and the cycle frequency of the motor pattern was increased. Bursting in the PD [and the strongly electrotonically coupled anterior burster (AB)] was enhanced, primarily by an increased amplitude of the membrane potential oscillation and by an increased spike activity during each burst. Similar effects were also observed during bath application of the nonmethylxanthine phosphodiesterase inhibitor Ro20-1724 (10–35 μM). Since we observed similar responses of the circuit to each of these compounds it is likely that these effects were primarily due to increased cAMP levels.

**Effects of forskolin on synaptically isolated pyloric neurons**

Even in a fairly simple neuronal circuit such as the pyloric CPG, synaptic interactions among neurons could mask the direct physiological effects of cAMP in individual cells (15, 16). Therefore, it was necessary to

![Image of data showing effects of forskolin on pyloric neuron activity.](image-url)

**Fig. 3**. Effects of 50 μM forskolin on the anterior burster neuron. A: the cell is inactive after synaptic isolation. Irregular burst activity is initiated by forskolin (2 min) and suddenly becomes regular with a large amplitude (arrow, 3 min). After reaching a maximum amplitude of 20 mV (5 min), the bursting pacemaker potentials (BPPs) gradually decline in amplitude primarily by depolarization of the trough phase (9 min). Early in the wash (10-min wash) the cell generates mostly tonic spike activity and recovers to control levels in ~120 min. Arrows to the right of the traces: ~63 mV, the preforskolin membrane potential. B: membrane potential of cell shown in A. The membrane potential of the peak and trough of the membrane potential oscillations are represented on the graph. Twelve min after addition of forskolin, bursting became irregular and is not shown. *Upward arrow*, forskolin addition; *downward arrow*, wash. Each point is the mean of 15–25 BPPs. Standard deviations are not shown but all are <1 mV.
investigate the physiological effects on cAMP on each pyloric neuron without the complications of synaptic input. To do this, we isolated each pyloric neuron from all known synaptic input (16). We could then observe the direct effect of forskolin on each neuron’s physiological activity.

**AB neurons**

The AB was usually inactive in synaptic isolation (Fig. 3A, “control”), and short depolarizing or hyperpolarizing current pulses resulted in only passive membrane responses (Fig. 4A). The AB responded to forskolin with the induction of endogenous bursting pacemaker potentials (BPPs; 6/6 experiments). The initial BPPs during addition of forskolin were very irregular, exhibiting rapid depolarizing jumps in membrane potential (5/6 experiments) (Fig. 3A, “2 min”). At this time, BPPs could be induced by short depolarizing current pulses and terminated by short hyperpolarizing current pulses (Fig. 4B). Within 3 min after forskolin addition, bursting suddenly became regular, with larger amplitude BPPs (4/6 experiments) (Fig. 3A, arrow in “3 min”, “5 min”). The trough of the BPP was hyperpolarized from the control membrane potential (Fig. 3B, arrows at right of traces in Fig. 3A). Bursting continued with a constant frequency for the time that forskolin remained in the bath. However, after reaching a maximum of 20 mV the BPP amplitude gradually started to decrease, mainly due to a depolarization of the trough phase and some hyperpolarization of the peak phase (Fig. 3A, “9 min”, Fig. 3B). During washout of forskolin, bursts were replaced by tonic spike activity in 4/6 preparations (Fig. 3A, “10 min wash”), with short periods of spontaneous damped oscillations. A few bursts could be induced during this period by injection of a short hyperpolarizing pulse (Fig. 4C). During forskolin washout in the other two preparations, bursting continued but became progressively weaker and less frequent. The AB cell recovered to control levels after ~2 h. (Fig. 3A, “120 min wash”).

The irregular burst activity observed early during forskolin superfusion (Fig. 3A, “2 min”) suggested that the AB could burst in two different states, dependent on the cAMP concentration; low cAMP concentrations early during forskolin application may induce a conductance state resulting in small amplitude BPPs, whereas higher cAMP concentrations generated by a longer period of forskolin addition modulate another set of ion conductances to generate large-amplitude BPPs. The irregular bursting at early times could result from the transition between the low-cAMP and high-cAMP states. Alternatively, only one set cAMP dependent burst-generating conductances may be present in the AB cell, and the irregular bursting activity could result from an unstable intermediate state during a continuous

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**Fig. 4.** Current pulses (50 ms) can evoke bursting pacemaker potentials in the anterior burster (AB) cell during forskolin application. A: only passive responses to current pulses are elicited in the AB cell. B: bursts can be induced by depolarizing current pulses (left) or terminated with hyperpolarizing current pulses (right). C: during tonic activity observed early in the wash, a short hyperpolarizing current pulse elicits a series of increasingly damped oscillations that are similar to the bouts of burst activity occurring spontaneously at this time (see Fig. 3A). i, current.
change in the density of these conductances (4). As cAMP levels are increased in the cell, the density of the cAMP-dependent conductances would increase. During the transition from an inactive to a bursting state, the cell would pass through an unstable activity state until the density of the conductances are high enough to support stable oscillations (4). To study this question, we applied varying concentrations of forskolin to a synaptically isolated AB cell (Fig. 5). Bursting induced by a low concentration of forskolin (5 
\mu M) was not stable (Fig. 5A). Large-amplitude BPPs were often followed by BPPs of decreasing amplitude, even after 18 min of forskolin application. The lack of stable bursting throughout the application of 5 
\mu M forskolin was similar to the irregular bursting observed after 2 min in 50 
\mu M forskolin (Fig. 3A). BPPs were stable in 20 
\mu M forskolin (Fig. 5B). They reached a maximal amplitude of \sim 10 mV and remained so for 2–3 min, then started to decrease, primarily by a depolarization of the trough phase. Fifty micromolar forskolin produced results that were identical to those shown in Fig. 3 (Fig. 5C). The fact that low concentrations of forskolin did not stabilize a “small-burst” state, but generated irregular oscillatory activity, and only higher concentrations (\geq 20 
\mu M) produced stable burst activity, supports the second hypothesis.

We have recently shown that different combinations of ionic conductances appear to underly BPPs induced in the AB cell by dopamine, octopamine, and serotonin (21, 22). For example, \textit{10}^{-4} M dopamine induces rhythmic BPPs without action potentials in the presence of \textit{10}^{-7} M TTX, whereas \textit{10}^{-5} M octopamine and \textit{10}^{-2} M serotonin do not. Similar to octopamine and serotonin, forskolin (50 
\mu M) produced only a small reversible depolarization in \textit{10}^{-7} M TTX. No endogenous bursting was observed, and BPPs could not be induced by depolarizing current injection.

\textit{PY} neurons

There are about eight PY cells in the pyloric circuit. These cells are divided into two subgroups, early (PEs; 2–4 cells) and late (PLs; 3–5 cells), determined by their time of firing in the pyloric cycle (24). Both subgroups responded to forskolin in the same manner.

With our synaptic isolation procedure, the PY cells were freed from synaptic input from all other cell types. However, they remain weakly electrotonically coupled to each other (24), and this must be taken into account in

![Figure 5](image-url)

**Fig. 5.** Concentration-dependent effects of forskolin on burst activity of the anterior burster cell. Bursting pacemaker potentials (BPPs) induced by 5 (A), 20 (B), and 50 \(\mu M\) (C) forskolin. BPPs were irregular with a low forskolin concentration and more stable at higher concentrations. All traces are from the same preparation.
interpreting our results with forskolin. After the isolation procedure, PY cells were inactive (Fig. 6A, "control"). Within 5 min after addition of 50 μM forskolin, the cells depolarized and exhibited spontaneous plateau potentials (Fig. 6A, "5 min"; Fig. 6B). These consisted of a rapid rise in membrane potential to a plateau lasting over 1 s and then a gradual repolarization. As bath application proceeded, the cells continued to depolarize and the plateau potentials became more frequent, often reaching a point where each PY cell was almost continuously active (Fig. 6A, "8 min").

Injection of hyperpolarizing DC current disclosed further information about these plateau properties (Fig. 7A). Plateau potentials appeared as large-amplitude bursts. With sufficient hyperpolarization, spontaneous plateau potentials were abolished, al-

**FIG. 6.** Effect of 50 μM forskolin on pyloric (PY) neurons. A: forskolin induces spontaneous plateau potentials in a previously inactive PY cell. The maximum response occurs at 8 min. Plateau potential activity is replaced, early in the wash, by tonic spike activity (30-min wash) before the cell recovers (120 min). Arrow to the right of each trace: -69 mV, the preforskolin membrane potential. B: membrane potential of cell shown in A. The membrane potential of the peak (measured from the base of the action potentials) and trough of the plateau potentials are recorded. Upward arrow, forskolin addition; downward arrow, wash. Membrane potential measurements during plateau potentials represent the mean of 4-8 plateaus. Standard deviations are not shown but are <1 mV. C: during entry of forskolin, bursts of action potentials from many PY neurons recorded extracellularly in the PY nerve (pyn, top trace) occur in synchrony with subthreshold depolarizations and plateau potentials in an intracellularly recorded PY cell (bottom trace). Time scale: A = 2 s; C = 4 s.
FIG. 7. Plateau potentials in electrotonically coupled PY cells. A: a continuous record of a pyloric (PY) cell showing accentuation in amplitude of plateau potentials by injection of DC hyperpolarizing current. Increasing the hyperpolarizing current decreases the occurrence of the plateau potentials until they do not occur spontaneously. Underlying the plateau potentials are small transient depolarizations. Short (50 ms) depolarizing current pulses can occasionally evoke a plateau potential. The dotted line above the bottom two current traces indicate 0 nA. B: during washout of forskolin, injection of hyperpolarizing DC current during tonic spike activity results in the abolition of tonic spike activity. No depolarizing steps are seen. Short depolarizing current pulses do not evoke plateau potentials. The membrane potential during short depolarizing current pulses in B are clipped by the chart recorder. i, current.

though they could still be induced by short depolarizing current pulses. At this point, small (3–4 mV) depolarizing steps underlying the plateau potentials became apparent (Fig. 7A). These depolarizations probably resulted from plateau potentials from other PY cells that were weakly electrotonically coupled to the cell being recorded from, because 1) the depolarizations occurred in synchrony with bursts of action potentials recorded extracellularly in other PY cells (Fig. 6C) and 2) the amplitude of these depolarizations did not change with increasing hyperpolarization, as would be expected if they were chemically mediated postsynaptic potentials. Thus a low-amplitude step, arising from a plateau potential in one PY cell, could act as a trigger for the generation of larger plateau potentials in other PY cells.

Upon removal of forskolin, plateau potentials were eventually replaced by tonic spike activity (Fig. 6, “wash, 30 min”). Injection of hyperpolarizing DC current abolished spike activity (Fig. 7B). No depolarizing steps or
plateau potentials were seen or could be induced by the injection of depolarizing current pulses during this period. Thus, although the PY cell still showed tonic activity due to forskolin, its ability to generate plateau potentials was lost. The cell slowly hyperpolarized and eventually became inactive (Fig. 6A, "120 min").

**PD, VD, LP, and IC neurons**

Each of these cell types responded to bath application of 50 μM forskolin in the same manner. Forskolin produced a strong activation or enhancement of tonic spike activity concomitant with a depolarization of several millivolts (Fig. 8, Table 1). Neither plateau potentials nor BPPs could be induced by current injection either before or during forskolin application (Fig. 9A, PD used as an example; Fig 9B, LP used as an example). Postinhibitory rebound was enhanced in the LP (Fig. 9C) and IC neurons. Hyperpolarizing current pulses (500 ms), which elicited few or no rebound spikes and little postpulse depolarization in controls, caused a transient

**Table 1. Forskolin-induced changes in membrane potential and action-potential activity in the PD, VD, LP, and IC neurons**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Membrane Potential, mV</th>
<th>Spike Frequency, spikes/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Forskolin</td>
</tr>
<tr>
<td>PD</td>
<td>-60.3 ± 1.3 (4)</td>
<td>-56.8 ± 0.7 (4)*</td>
</tr>
<tr>
<td>VD</td>
<td>-65.0 ± 0.7 (3)</td>
<td>-59.7 ± 0.4 (3)*</td>
</tr>
<tr>
<td>LP</td>
<td>-61.0 ± 2.1 (3)</td>
<td>-54.3 ± 2.0 (3)*</td>
</tr>
<tr>
<td>IC</td>
<td>-67.3 ± 3.8 (4)</td>
<td>-55.5 ± 2.0 (4)*</td>
</tr>
</tbody>
</table>

Values are means ± SF; no. of cells used for each measurement are in parentheses. PD, pyloric dilator; VD, ventral dilator; LP, lateral pyloric; IC, inferior cardiac. * P < 0.005, Student's t test comparing control and forskolin values.
FIG. 9. Analysis of membrane properties in the pyloric dilator (PD) and lateral pyloric (LP) neurons. A: plateau potentials could not be evoked in the PD cell. Control: PD was inactive and responded only passively to a short (50 ms) current pulse. 50 μM Forskolin: the cell was hyperpolarized below the spike threshold and a short depolarizing current pulse, up to 10 nA, elicited only a passive response. Similar results were recorded in the ventral dilator (VD), LP, and inferior cardiac (IC) neurons (not shown). B: bursting pacemaker potential could not be unmasked in the LP cell by injection of DC current. Injection of DC current to vary the membrane potential over a similar voltage range, before and during forskolin addition, resulted in activation or enhancement of tonic spiking activity (see Control) or abolition of spiking activity (see 50 μM Forskolin). The same results were observed in the PD, VD, and IC cells (not shown). C: postinhibitory rebound was enhanced in the LP neuron. Control: postinhibitory rebound was absent at the resting potential (left) and only faintly detectible during injection of DC depolarizing current above the spike threshold. 50 μM Forskolin: postpulse depolarization and a transient increase in spike activity were observed. Similar results were observed in the IC cell (not shown). Left trace: 0 nA DC; middle trace: −0.5 nA DC; right trace: −1.25 nA DC. Dotted line: 0 nA. The membrane potential during the current pulse was clipped by the chart recorder. Vertical scales: voltage, A = 20 mV, B and C = 10 mV; current, A = 10 nA, B and C = 5 nA; horizontal scales: A = 1 s, B (control) = 5 s, B (50 μM forskolin) = 12.5 s, C = 2.5 s.

increase in spike activity and a greater postpulse depolarization during forskolin superfusion. No reproducible changes in PIR were observed in the PD and VD neurons (not shown). All four cell types recovered from forskolin application by a gradual hyperpolarization of membrane potential and reduction in spike frequency, reaching control levels in about 2 h. (Fig. 8).

Effects of neuromodulators on STG cAMP levels

We and others have studied the physiological effects of three amines, dopamine, octo-
TABLE 2. Effects of neuromodulators on cAMP levels in whole STG

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>cAMP Level pmol/μg protein</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.11 ± 0.05 (7)</td>
<td>100</td>
</tr>
<tr>
<td>Forskolin</td>
<td>50 μM</td>
<td>1.17 ± 0.23 (6)*</td>
<td>1,070</td>
</tr>
<tr>
<td>Octopamine</td>
<td>100 μM</td>
<td>0.53 ± 0.28 (9)*</td>
<td>480</td>
</tr>
<tr>
<td>Dopamine</td>
<td>100 μM</td>
<td>0.16 ± 0.09 (8)</td>
<td>140</td>
</tr>
<tr>
<td>Serotonin</td>
<td>10 μM</td>
<td>0.13 ± 0.05 (7)</td>
<td>120</td>
</tr>
<tr>
<td>FMRFamide</td>
<td>1 μM</td>
<td>0.07 ± 0.03 (3)</td>
<td>64</td>
</tr>
<tr>
<td>Proctolin</td>
<td>1 μM</td>
<td>0.16 ± 0.10 (3)</td>
<td>145</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>1 mM</td>
<td>0.06 ± 0.01 (3)</td>
<td>53</td>
</tr>
</tbody>
</table>

Values are means ± SE; sample sizes for each measurement are in parentheses. CAMP, adenosine 3',5'-cyclic monophosphate; STG, stomatogastric ganglion. * P < 0.002; Student's t test comparing drug and control values.

pamine and serotonin (15, 16) and two peptides, FMRFamide and proctolin (25, 25a, 36), on the motor pattern and isolated neurons of the pyloric CPG. As a first step in determining whether these modulators might use cAMP as a second messenger in the STG, we used a RIA procedure to measure their effects on cAMP levels in whole STG (Table 2). Each modulator was added at concentrations that induce very robust physiological effects on the pyloric motor pattern (15, 36). Both forskolin and octopamine caused highly significant increases in cAMP levels over controls. Dopamine, serotonin, FMRFamide, proctolin, and pilocarpine had very modest effects, which were not statistically significant in our experiments.

DISCUSSION

Forskolin induces a dramatic activation of the pyloric motor pattern, due to a direct excitation of all of the pyloric CPG neurons. For several reasons, we believe that these effects stem from forskolin's direct activation of adenylate cyclase to elevate cAMP levels (53) and not from some other side effect of the drug (7). First, forskolin evoked a 10-fold increase in cAMP in the STG. Second, we examined the physiological effects of a variety of cAMP-elevating agents on the pyloric motor pattern. Although they have very different structures and act by totally different mechanisms to enhance cAMP levels, all these compounds had similar effects on the pyloric motor pattern and on the neurons within the pyloric circuit. Thus we can interpret our results with forskolin as the effects of elevated cAMP in pyloric neurons.

Although forskolin directly excites every cell in the pyloric circuit, the effects are different for different cells. Bursting pacemaker potentials are activated in the AB cell. Plateau potentials are induced in the PY cells. Depolarization and tonic spike activity are produced in the PD, VD, LP, and IC neurons. Thus increases in intracellular cAMP result in a variety of physiological effects that are different for different cells in a small motor circuit.

Mechanisms of cAMP-mediated modulation of pyloric neuron activity

Forskolin's changes in the activity of pyloric neurons are likely to be caused by changes in one or more ionic conductances (see 32 for review). We do not yet know the ionic mechanisms underlying the variety of cAMP-mediated effects on different pyloric neurons; however, we can speculate on general mechanisms.

Activation of BPPs in the AB neuron

In other systems, alteration of cAMP levels can lead to the activation (11, 17), abolition (12), or alteration (31) of BPP activity. Forskolin could activate bursting in the AB cell by at least two different ionic mechanisms. First, forskolin could directly activate burst-generating conductances that are not expressed under control conditions. Alternatively, forskolin could inactivate a tonic hyperpolarizing conductance(s) that prevents burst-generating conductances from being expressed. In fact, several potassium channel blockers including tetracethylammonium (TEA), 4-aminopyridine, and apamin, can unmask endogenous burst activity in previously inactive AB neurons (23, 44). A reduction in a tonic hyperpolarizing conductance could also explain the slow depolarization of the trough phase of the membrane potential oscillations after several minutes of forskolin superfusion.
Activation of plateau potentials in the PY neurons

All pyloric neurons can exhibit plateau potentials when exposed to the appropriate neuromodulatory environment (40, 47). However, the PY cells were the only ones to generate plateau potentials in response to forskolin. This suggests that the cellular mechanisms that enable plateau potential properties are at least partially different in the PY cells compared with other pyloric neurons.

The eight PY neurons are electrotonically coupled to each other and these electronic connections were still intact during our experiments. This caused plateau potentials to occur synchronously in many PY cells. We have considered the possibility that these are not true plateau potentials, but result from positive feedback interactions among tonically firing, electrotonically coupled PY cells. We do not believe that this is true for two reasons. First, plateau potentials can be triggered by injections of brief (50 ms) current pulses into a hyperpolarized PY cell (Fig. 8A). Second, dopamine and octopamine activate tonic activity, but never plateau potentials in PY cells following an identical synaptic isolation procedure (16).

The ionic mechanisms underlying forskolin-induced plateau potentials in PY neurons are not known. In other crustaceans, a voltage-sensitive calcium current appears to underly the sustained depolarization during a plateau potential (43, 55). Plateau potentials activated in pyloric neurons in Jasus lalandii by stimulation of the anterior pyloric modulator (APM) neuron or by application of the cholinergic agonist pilocarpine (10) may be caused, at least in part, by a decrease in a resting potassium conductance that normally prevents this calcium current from being expressed (44). Bath-applied TEA can also activate or enhance driver potentials in lobster cardiac ganglion neurons (55). cAMP-mediated activation of plateau potentials in PY cells could occur by a similar mechanism.

Possible role of cAMP in the actions of identified neuromodulators of the pyloric CPG

Our RIA measurements showed that forskolin caused a 10-fold increase in the level of cAMP in the pyloric CPG.
cAMP MODULATION OF PYLORIC CIRCUIT NEURONS 1383

TABLE 3. Summary of the physiological effects of identified neuromodulators on the 6 classes of pyloric neurons in P. interruptus

<table>
<thead>
<tr>
<th>Modulator</th>
<th>AB</th>
<th>PD</th>
<th>VD</th>
<th>LP</th>
<th>PY</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td>BPP</td>
<td>tonic</td>
<td>tonic</td>
<td>tonic</td>
<td>plateaus</td>
<td>tonic</td>
</tr>
<tr>
<td>Octopamine*</td>
<td>BPP</td>
<td>tonic</td>
<td>tonic</td>
<td>tonic</td>
<td>tonic</td>
<td>tonic</td>
</tr>
<tr>
<td>Dopamine*</td>
<td>BPP</td>
<td>hyperpol</td>
<td>hyperpol</td>
<td>tonic</td>
<td>tonic/NE</td>
<td>tonic</td>
</tr>
<tr>
<td>Serotonin*</td>
<td>BPP</td>
<td>NE</td>
<td>hyperpol</td>
<td>hyperpol</td>
<td>NF</td>
<td>tonic</td>
</tr>
<tr>
<td>Proctolin†</td>
<td>BPP</td>
<td>NE</td>
<td>NE</td>
<td>tonic</td>
<td>tonic</td>
<td>NE</td>
</tr>
<tr>
<td>Pilocarpine‡</td>
<td>BPP</td>
<td>BPPs</td>
<td>$</td>
<td>$</td>
<td>$</td>
<td>$</td>
</tr>
</tbody>
</table>

AB, anterior burster; PD, pyloric dilator; VD, ventral dilator; LP, lateral pyloric; PY, pyloric; IC, inferior cardiac; BPP, bursting pacemaker potential; hyperpol, hyperpolarization and inhibition of spike or burst activity; tonic, activation or enhancement of tonic spike activity; NE, no effect observed. *16; †26; ‡36; § effect of modulator on isolated cell not reported.

cAMP in the intact STG. Octopamine, which is an excitatory modulator of most pyloric neurons (15, 16), also caused a significant fivefold increase, whereas the other modulators did not significantly change cAMP from control levels. These results must be interpreted with caution for several reasons. First, our measurements were made on whole STG and could include modulator-induced changes in cAMP in glial cells, axons, and nerve terminals of cells entering the ganglion from elsewhere, and nonpyloric neurons in the STG, as well as from cells of the pyloric CPG. Second, a number of neuromodulators appear to act by inhibition of adenylate cyclase, lowering cAMP levels (8). If serotonin or dopamine were to increase cAMP in some cells but decrease cAMP in others, the net effect might be little or no change in the whole ganglion. Third, modulator-induced increases or decreases in cAMP levels in a very small number of cells may not be detected by the assay.

Despite these reservations, it is useful to compare the physiological effects of forskolin with those of the amines and other known neuromodulators of the pyloric CPG (Table 3). Significantly, the greatest similarity is between forskolin and octopamine, which caused the greatest increase in cAMP in the intact STG. Both forskolin and octopamine (21) induce TTX-sensitive bursting in isolated AB cells; however, forskolin-induced bursting is more variable and complex than octopamine-activated bursts (see below). Like forskolin, octopamine induces depolarization and tonic firing in LP, VD, PD, and IC cells (16). The only major difference between octopamine and forskolin is with the PY cells, where octopamine induces either tonic activity or has no effect (16), whereas forskolin induces plateau potentials. These results suggest that octopamine may act by a cAMP-dependent mechanism in several pyloric neurons, but its actions on some cells are not completely mimicked by forskolin. In these cells, octopamine may act by other biochemical mechanisms, either alone or in addition to cAMP, to produce unique variants of cell activity.

Forskolin mimics the effects of the other modulators on some cells while having markedly different actions on other cells (Table 3). For example, while forskolin’s effects are purely excitatory, several of the modulators excite, inhibit, or have no apparent affect on different pyloric neurons. If any of these modulators use cAMP mechanisms in pyloric cells, they can do so only in a subset of neurons. This is consistent with their modest effects on cAMP levels in the STG (Table 2). Note that several neurons respond identically to forskolin and to several neuromodulators (Table 3). While each modulator could act by an independent mechanism, it is also possible that in these cells, all the modulators share a common cAMP-dependent mechanism. This is seen in the molluscs *Helix aspersa* and *A. californica* where several neurotransmitters decrease the same potassium conductance in a single cell by a common cAMP-dependent mechanism (1, 6, 9, 46, 52).

Like forskolin, the amines dopamine, octopamine, and serotonin can all induce BPPs in a quiescent AB cell. Although only octo-
pamine significantly increased cAMP levels in the ganglion the other amines induced modest increases in cAMP, and we cannot discount the possibility that they may act via cAMP in a limited number of cells, including the AB. However, the shapes of the bursts are different for each of the amines and forskolin, and the contribution of different ionic currents that generate the bursts are different for each amine (21, 22). Forskolin generates a complex series of events in the AB cell, which have characteristics not only of octopamine-induced BPPs but of dopamine- and serotonin-induced bursts as well. The large-amplitude bursting generated after 5 min of forskolin application (Fig. 3A, 5 min) resemble dopamine- and serotonin-induced BPPs (16, 21); burst amplitudes are \( \sim 20 \) mV and the trough phase of the membrane potential oscillations is hyperpolarized from the control membrane potential. Continued application of forskolin generates octopamine-like BPPs with an amplitude of \( \sim 10 \) mV and which are completely depolarized from the control membrane potentials. Like serotonin and octopamine, forskolin cannot induce BPPs in the presence of \( 10^{-7} \) M TTX. Amine-induced BPPs are very stable and do not exhibit the irregular BPPs that forskolin generates during the first few minutes of its application (Fig. 3A, 2 min) or at low concentrations (Fig. 5). Since forskolin only partially mimics the actions of dopamine, octopamine, or serotonin, cAMP-dependent processes could only account for part of each amine-induced BPP. Any amine acting on the AB cell by a cAMP-dependent mechanism probably activates additional cAMP-independent processes as well.

In summary, cAMP is likely to be a second messenger mediating diverse excitatory effects in all pyloric neurons. Although no neuromodulator has yet been definitively linked to cAMP in this system, octopamine is one likely candidate. There are probably other, as yet unidentified, modulators that act by elevating intracellular cAMP levels in at least a subset of pyloric neurons.

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