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Substrates of Command Ability in a Buccal Neuron of *Pleurobranchaea*

II. Potential Role of Cyclic AMP

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Summary. 1. The bilaterally paired ventral white cells (VWCs) of the buccal ganglion of *Pleurobranchaea* drive rhythmic motor output in the neural network controlling the movements of the buccal mass, which mediates both ingestion and egestion behaviors. The VWCs exert their action during minutes-long episodes of endogenously sustained depolarization and repetitive firing attended by pronounced action potential broadening (Gillette et al. 1980). In isolated preparations the ability to sustain such burst episodes is variable and somewhat labile (Gillette et al. 1980). We have assessed the ability of the intracellular messenger cyclic-3′,5′-adenosine monophosphate (cAMP), its non-hydrolyzable analogs and inhibitors of degradation to stimulate action potential broadening and bursting in the VWC.

2. Intracellular injection of cAMP enhances progressive spike broadening (Figs. 1 and 2C) and stimulates spontaneous spiking activity and endogenous burst episodes in an apparent dose-dependent fashion (Fig. 2). Bath application of cAMP analogs or the phosphodiestrase inhibitor isobutylmethylxanthine also stimulates spike broadening and recurrent burst episodes in the intact neuron (Figs. 3 and 5) and induces the capacity to sustain prolonged bursts triggered by brief current stimulation in the isolated neuron cell body (Fig. 4).

3. Work described in in the preceding paper (Gillette et al. 1982) suggested that regulation of $(Ca^{++})_i$ may affect progressive spike broadening in the VWC via a Ca^{++}-activated K^+ current ($I_{K,Ca}$) and possibly via inward Ca^{++} current. The action of cAMP in enhancing spike broadening resembles that of agents and treatments that reduce $(Ca^{++})_i$ and/or suppress $I_{K,Ca}$. Further, cAMP stimulation reverses a suppression of spike broadening caused by high Ca^{++} saline (Fig. 5) (which may increase $[Ca^{++}]_i$) and, conversely, that intracellular injection of a 'high' Ca^{++} buffer (Ca^{++}-EGTA, 5×10^{-7} M free Ca^{++}) reverses the cAMP stimulation of spike broadening (Fig. 6).

4. Stimulation with cAMP causes an abbreviation of the waveform and amplitude of the undershoots of single, unbroadened action potentials; this action resembles the effect of agents and treatments that reduce $[Ca^{++}]_i$ and/or $I_{K,Ca}$ (Figs. 7, 8). This effect is reversed by intracellular injection of high Ca^{++} buffer (Fig. 9).

5. These results suggest that one possible mechanism of action of cAMP is to reduce the ability or availability of intracellular free Ca^{++} to activate $I_{K,Ca}$. A non-exclusive alternative is that cAMP enhances a Ca^{++} current. The evidence that both of these mechanisms could be regulated by an increase in the internal Ca^{++} buffering ability of the cell is discussed.

6. The potential context of the VWC's behavioral function is one in which their command ability is induced by sensory or other input during arousal of some buccal behavior through neurotransmitter stimulation of endogenous cAMP.

Abbreviations: *cAMP* cyclic-3′,5′-adenosine monophosphate; *IBMX* isobutylmethylxanthine; *CPT-cAMP* 8-p-chlorophenylthio-adenosine-3′,5′-cyclic monophosphoric acid; *VWC* ventral white cell

Introduction

The bilaterally paired ventral white cells (VWCs) of the buccal ganglion of the predatory marine snail *Pleurobranchaea* show intrinsic and notable functional plasticity in their electrical activity. Aspects of this plasticity are related to their capacity to command coordinated motor output in the neural oscillator network of the buccal ganglion (Gillette et al. 1980). This plasticity is markedly displayed in two ways: first, in the ability of these cells to shift between a state of quiescence and one of periodic activity in which they sustain minutes-long depolarized burst

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episodes and secondly, in their ability to develop extremely broadened action potentials during repetitive firing. These qualities confer the capacity of the VWCs to drive motor output (Gillette et al. 1980). In the previous paper (Gillette et al. 1982) we described the parameters and likely mechanisms of progressive spike broadening, and provided evidence that internal Ca^{++} regulation could effectively regulate functional plasticity and thus the command efficacy in these neurons.

It is likely that modulation of plasticity in these potent neurons has considerable significance in the expression of behavior. In searching for potential natural modulators of VWC activity, we have investigated the possible actions of cyclic-3′,5′-adenosine monophosphate (cAMP), which may modulate neuron expression in many invertebrate nervous systems (e.g., Treistman and Levitan 1976; Brunelli et al. 1976; Shimahara and Tauc 1977; Kaczmarek and Strumwasser 1981). We report here that cAMP, its agonists and analogs are capable of inducing prolonged burst episodes and enhancing progressive spike broadening. The results suggest that the actions of cAMP stimulation in this neuron may directly cause a net decrease in $I_{K,Ca}$ and/or an enhancement of the calcium conductance. We consider the possibility that these mechanisms could be affected through modulation of the rate of removal of free internal Ca^{++}, and devote discussion to the potential role of cAMP regulation of VWC activity in behavior.

Materials and Methods

Dissection and Preparation. Procedures were essentially as described in the previous paper (Gillette et al. 1982). Buccal ganglia were dissected out and pinned ventral side-up in saline maintained at 12–14 °C. In most experiments the connective tissue sheath was removed for intracellular recording from the VWC, a procedure sometimes facilitated by a 5 min incubation in 0.2% pronase. Such brief pronase treatments have no obvious effect on the activity of the VWC. Surgical isolation of the VWC soma was performed by undercutting the soma with fine scissors, teasing away adhering somata, and did not involve pronase treatment (Gillette et al. 1980, 1982).

Bath Solutions. All preparations were initially bathed in artificial saline (mM) (420 NaCl, 25 $MgCl_2$, 25 $MgSO_4$, 10 KCl, 10 $CaCl_2$, 5 Tris-HCl buffer, pH 7.5). High-Ca^{++} salines were made either by equivalent substitution for Mg^{++} or measured volumes of 1 M $CaCl_2$ were added directly to the bath and mixed by pipetting. Cyclic nucleotide analogs and the inhibitor of cyclic nucleotide phosphodiesterase 3-isobutyl-1-methylxanthine (Sigma) were made up as 1 mM stock solutions in saline and stored as frozen aliquots. When used, appropriate volumes were added to the bath and mixed by gentle pipetting to final concentrations of 10^{-5} to 10^{-4} M. The cAMP analogs used were 8-p-chlorophenyl-thio-adenosine-3′,5′-cyclic monophosphoric acid (ICN) and 8-benzylamino-adenosine-3′,5′-cyclic monophosphoric acid (Sigma).

Intracellular Injections. Intracellular injections were performed using air pressure from a hand-held plastic syringe connected to an electrode by tightly fitting polyethylene tubing. A chlorided silver wire entering the tubing near and projecting into the electrode butt allowed intracellular records to be taken from the injection electrode. Intracellular injections were performed as gently as possible to avoid traumatizing the VWC and never caused noticeable swelling of the soma. Somata volumes were variously estimated at $2–4 \times 10^{-9}$ l; while the volumes of solutions injected are not known, they are estimated to lie within 10^{-12} to 10^{-11} l.

A low concentration of adenosine-3′,5′-cyclic monophosphoric acid (cAMP) for injection was made by dissolving the acid in in 0.1 M KCl to a final concentration of 3 mM. A higher concentration (80 mM) of cAMP was prepared by adding the acid to distilled H_2O and adjusting the pH to 7.0 with KOH. A Ca^{++}-EGTA (ethyleneglycol-bis(β-amino-ethyl ether) N,N′-tetra-acetic acid) buffer was prepared as previous (Gillette et al. 1982) and had the final concentration of (mM) 125 EGTA, 250 KCl, 100 histidine hydrochloride with $CaCl_2$ added to give a calculated free Ca^{++} concentration of 5×10^{-7} M at pH 7.3.

Stimulation and Recording. Conventional intracellular recordings were made using KCl-filled microelectrodes with initial resistances of 8–15 megohms. Intracellular current injection was usually performed via a second intracellular electrode, except in the case of the isolated soma where a balancing bridge circuit was used for current injection directly through the recording electrode. Records were either photographed from a storage oscilloscope screen, made directly on a Brush 2400 4-channel chart recorder, or recorded on tape for later transcription. In an effort to control for long lasting effects of membrane voltage on membrane conductance parameters, conditioning hyperpolarization to −65 mV for 1 min immediately preceded stimulation of single or trains of action potentials.

Results

cAMP Stimulation of Spike Broadening and Bursting

Figure 1 shows storage oscilloscope records from one of three experiments, of spike broadening during stimulated trains of action potentials before and after intracellular injection of 3 mM cAMP into a VWC in the isolated buccal ganglion. In the tests of spike broadening performed before (Fig. 1 A) and immediately after a period of repeated cAMP injections

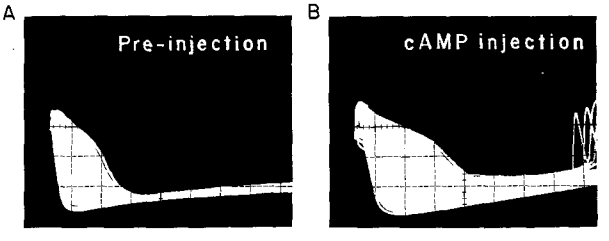

Fig. 1 A, B. Intracellular injection of 3 mM cAMP enhances progressive spike broadening. Records are superimposed storage oscilloscope traces of action potentials stimulated by continuous intrasomatic injection of 5 nA depolarizing current. Each test was preceded by a 60 s conditioning hyperpolarization to −65 mV. **A** Pre-injection test. 198 spikes were stimulated over a 50 s interval. **B** Post-injection test. 163 spikes were stimulated over a 38 s interval. *Calibration:* grid bars 20 mV vertical, 20 ms horizontal

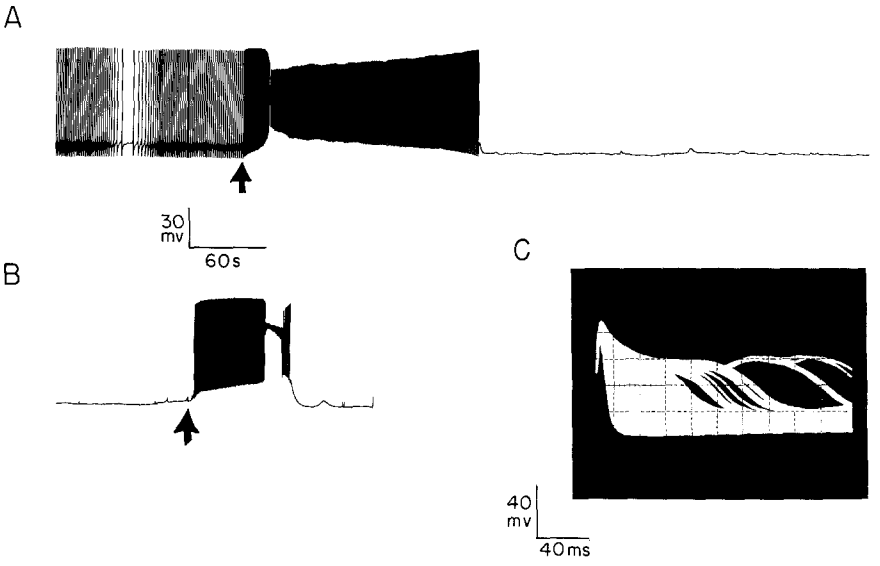

Fig. 2A–C. Induction of prolonged burst episodes and attendant spike broadening by intracellular injection of 80 mM cAMP.
A, B Records from two separate experiments. Prolonged burst episodes are stimulated within 2–3 s of small pressure injections (about 1 s in duration) of 80 mM cAMP (pH 7.0).
C Superimposed oscilloscope sweeps showing the development of extreme spike broadening during a burst episode stimulated by a pressure injection

(Fig. 1B) the extent of the post-injection broadening was increased by nearly 50% over that of the pre-injection test. This result was obtained even though the duration of the pre-injection test was longer (50 s) and the number of spikes stimulated (198) was greater than in the test following cAMP injection (38 s duration and 163 spikes).

Injection of 3 mM cAMP did not induce triggerable burst episodes, although the injections were followed by spontaneous action potential activity at about 2/s in the previously quiescent neurons tested ($n=3$). Injections of cAMP at the higher concentration of 80 mM *did* induce prolonged burst episodes lasting up to 3 min with latencies of less than 1 s in two previously quiescent cells (Fig. 2A,B). These bursts were attended by rapid and extreme prolongation of action potentials, reaching durations over 200 ms (Fig. 2C). Weaker pulses of cAMP, as judged subjectively by the manual force applied to the injection apparatus, induced shorter bursts in an apparent dose-dependent fashion. The weakest and briefest pulses failed to produce true burst episodes but initiated or accelerated spontaneous spiking for periods lasting from 15 s to several min. Prolonged burst episodes in the spontaneously bursting VWC are commonly observed to recur fairly regularly at intervals varying between 5–30 min, depending on the preparation. The cAMP injections did not induce such rhythmicity. The transient effects of native cAMP injections may have in part arisen from rapid degradation of injected cAMP by endogenous phosphodiesterase.

VWC Stimulation by cAMP Analogs and IBMX

Chemically modified analogs of cAMP are widely used to study the intracellular effects of cAMP when the native compound is weakly or transiently effective; much of the efficacy of the analogs probably arises from their ability to partition into the cell, their resistance to degradation by phosphodiesterase, and their ability to activate cAMP dependent protein kinase (Meyer and Miller 1974). We therefore employed cAMP analogs of the 8-substituted class, 8-para-chlorophenylthio-cAMP (CPT-cAMP), 8-benzylthio-cAMP, and 8-benzyl amino-cAMP, to further examine the effects observed by injection of cAMP. In separate experiments isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterase, was used to enhance endogenous cAMP levels by reducing break down rates of the nucleotide.

Addition of the cyclic AMP analogs or IBMX to the bath saline at 10^{-5}–10^{-4} M augmented the frequency of spontaneous spiking within 2–5 min in all experiments ($n=56$), an effect which was correlated with the increase in anomalous rectification normally preceding the occurrence of a spontaneous burst. In previously non-bursting VWCs ($n=18$), addition of analogs or IBMX at the highest concentrations used (10^{-4} M) invariably stimulated the appearance of typical prolonged and recurrent burst episodes within 6–20 min. In those VWCs which showed spontaneous burst activity prior to drug application, the effects of IBMX and the analogs were usually manifested both as an increase in the duration of the bursts and as a decrease in the interburst interval ($n=36$). The typical decrease in interburst interval is shown in Fig. 3, which is an intracellular record of the activity of a spontaneously bursting VWC for nearly 3 h. In this experiment the interburst intervals of the 4 bursts occurring prior to drug addition ranged from 22 to 31 min. Addition to CPT-cAMP at 5×10^{-5} M caused the early appearance of a burst followed by recurrent bursts at shorter intervals. In this case, the

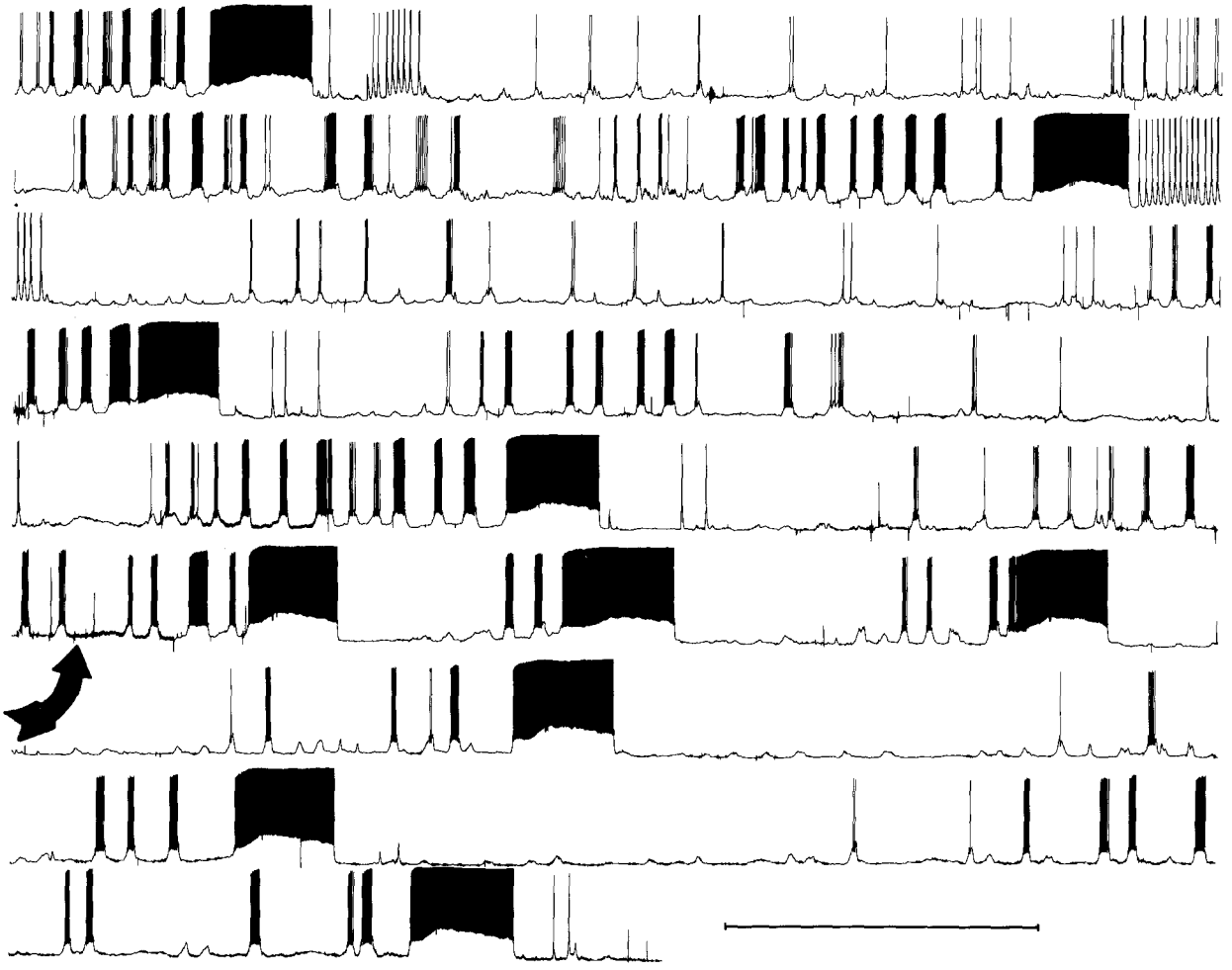

Fig. 3. Cyclic nucleotide stimulation of bursting activity in a spontaneously bursting VWC. Intervals separating the first 4 episodes of prolonged bursting range from 22–31 min. Addition of CPT cAMP at 5×10^{-5} M (*arrow*) stimulated an early burst and shortened the intervals between 6 subsequent bursts. In the continued presence of CPT-cAMP interburst intervals showed progressive lengthening toward pre-drug levels. Rhythmic depolarizations and associated spiking activity of shorter durations occurring between bursts presumably arise from spontaneous synaptic inputs from the buccal network (Gillette et al. 1980); increased spike activity preceding the VWC bursts presumably reflects lower current thresholds for spiking due to enhanced anomalous rectification late in the burst period. *Calibration bar:* 5 min

average burst duration was increased by 12% over the pre-drug average. The decrease in interburst interval caused by cyclic nucleotide stimulation was usually found to decline over 1–2 h. In the experiment shown in Fig. 3, over 6 bursts recorded in the presence of CPT-cAMP the duration of the interburst interval increased successively from 3.67 to a near pre-drug level of 20.33 min. The other cAMP analogs produced results similar to those seen with CPT-cAMP.

Upon drug washout the effects of cAMP analogs and IBMX were reversed within 10 min ($n=16$). In fact, the neuron usually became much less excitable than before drug application, showing drastically increased current thresholds for spiking, reduced spike broadening during driven trains, minimal spontaneous spike activity and absence of spontaneous burst episodes. Recovery of excitability to what seemed pre-drug levels occurred slowly over the course of 1–2 h.

Stimulation of the Isolated VWC Soma

In order to exclude the possible effects of the numerous synaptic inputs to the VWC from the feeding network, the effects of the analog CPT-cAMP were assessed in the isolated soma, cut from the axon close to its origin (less than 50 μm) and entirely removed from the ganglion. Physiologically, the isolated soma differs from the intact neuron in three obvious ways. First, the resting potential may be higher, −60 to −70 mV, possibly due to isolation from depolarizing synaptic input. Second, upon current stimulation

overshooting action potentials develop progressively from oscillations in the depolarized baseline potential (Gillette et al. 1980). Last, current thresholds for spike initiation are relatively low, presumably due to isolation from the axon and dendritic branches.

CPT-cAMP enhancement of progressive spike broadening and endogenously sustained bursting was demonstrated in three experiments on surgically isolated VWC cell bodies. In most intact or isolated VWC somata burst episodes may be triggered by injecting depolarizing current; however, in some preparations such bursts cannot normally be triggered (Gillette et al. 1980). Figure 4A shows an example of an isolated soma in which the depolarization by 1 nA of injected current for a minute and 20 s, while inducing rapid action potential discharge, failed to produce more than three spikes and a decaying depolarizing afterpotential outlasting current stimulation. The progressive emergence of overshooting action potentials from oscillations of the membrane potential previously described for the isolated soma is reflected in the form of the initial portion of the spike train recorded at slow speed in Fig. 4A, while the decay of the K^+ dependent spike undershoots associated with spike broadening is seen in the latter portion of the train. Subsequent addition of CPT-cAMP to the bath was followed within 15 min by the appearance of the ability to produce a sustained burst of spikes. The burst episode shown in Fig. 4B was triggered by a relatively small injected current (0.25 nA) and outlasted the triggering stimulus by more than 4 min. In addition, overshooting action potentials developed more rapidly in the presence of the cAMP analog (compare the initial portions of the trains in A and B).

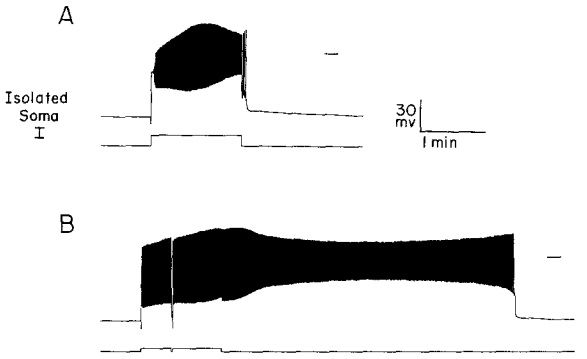

Fig. 4A, B. Stimulation of the ability to endogenously sustain prolonged bursting in the isolated soma by a cAMP analog. **A** Normal saline. Stimulation by 1 nA of depolarizing current for 82 s failed to elicit prolonged activity outlasting the stimulus. **B** 20 min after addition of CPT-cAMP to the saline at 10^{-4} M. Two tests are shown, separated by a 3 s observation period. Spike activity and prolonged depolarization outlasted current stimulation of the 2nd test by 4.4 min. In addition to the enhanced triggerability of burst episodes in the presence of the analog, peak spike amplitude was attained much faster (compare the initial portions of the stimulated trains), and the applied current necessary to stimulate spiking was markedly reduced (0.25 nA)

Specificity of cAMP Stimulation

We examined the specificity of cAMP action by comparing the actions of the analogs CPT-cAMP and CPT-cGMP, which differ structurally only as do native cAMP and cGMP ($n=3$). At levels of 10^{-4}–3×10^{-4} M, CPT-cGMP either failed to augment sponaneous bursting activity ($n=2$) or produced a low degree of stimulation (a single 2 min burst in 2 h of observation). CPT-cAMP, however, was highly potent at 10^{-4} M in stimulating recurrent and prolonged burst episodes in these preparations. The 8-substituted cAMP derivatives, such as CPT-cAMP, are inactive as activators of cGMP-dependent protein kinase from lobster tail, while stimulating cAMP-dependent protein kinase better than cAMP itself (Meyer and Miller 1974). Conversely, CPT-cGMP is as poor an activator of cAMP-dependent protein kinase as cGMP, and strongly activates the cGMP-dependent protein kinase. The much greater efficacy of the cAMP analog on the VWC suggests that the stimulation of activity is specifically mediated via the cAMP pathway and, in view of the resistance of the cAMP analogs to enzymatic hydrolysis, it is unlikely that the stimulation is a non-specific consequence of hydrolysis.

Cyclic Nucleotide Stimulation of Spike Broadening Antagonized by $Ca^{++}{}_i$

In the companion paper we showed that treatments aimed at altering free internal Ca^{++} alter the rate and extent of progressive spike broadening, apparently acting through a Ca^{++}-activated K^+ conductance and possibly through opposite but agonistic effects on inward Ca^{++} current. For instance, intracellular injection of the Ca^{++} chelator EGTA causes the enhancement of spike broadening in a stimulated train in much the same way as does cyclic nucleotide stimulation. Conversely, augmenting $[Ca^{++}]_i$ by intracellular injection of a high Ca^{++} buffer or by raising saline Ca^{++} suppresses spike broadening.

We find that cyclic nucleotide stimulation can reverse the effects of augmenting $[Ca^{++}]_i$, and that augmenting $[Ca^{++}]_i$ antagonizes the effects of cyclic nucleotide stimulation. The results of one such experiment ($n=3$) using high external Ca^{++} to increase $I_{K,Ca}$, and the reversal of its effects by IBMX, are illustrated

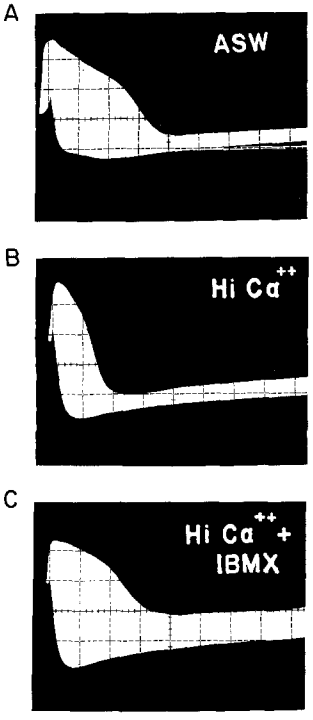

Fig. 5A–C. High external Ca^{++} suppresses progressive spike broadening in the VWC; this effect is partially reversed by the inhibitor of cyclic nucleotide phosphodiesterase IBMX. **A** A record of spike broadening in normal saline (ASW). Stimulating current 3 nA; 439 spikes were fired during a 3 min test period. **B** Suppression of spike broadening 20 min after the bath Ca^{++} was raised to 40 mM. Stimulating current was raised to 5 nA to compensate for an increased current threshold. 453 spikes were fired during 3 min. **C** Enhancement of spike broadening in the presence of high Ca^{++} after addition of IBMX to 10^{-4} M. 5 na stimulating current; 450 spikes fired during 3 min. Spike broadening is suppressed by high external Ca^{++}, presumably through augmentation of gK_{Ca}. The partial reversal of high Ca^{++} suppression by IBMX resembles the effects of treatments which reduce gK_{Ca}. *Calibration:* grid bars 20 mV vertical, 20 ms horizontal

in Fig. 5. High external Ca^{++} may increase $[Ca^{++}]_i$ and intensify inward current by steepening the transmembrane gradient. This results in intensification of $I_{K,Ca}$ (Eckert and Tillotson 1978; Gorman and Thomas 1980); the increase $[Ca^{++}]_i$ could also contribute to the inactivation of I_{Ca} (Kostyuk and Krishtal 1977; Tillotson 1979). The records of Fig. 5 show tests of spike broadening before (Fig. 5A) and 30 min after addition of 30 mM extra Ca^{++} to the bath (4× normal $[Ca^{++}]$) (Fig. 5B); the high external Ca^{++} condition suppresses progressive spike broadening. Subsequent addition of IBMX to the bath to a concentration of 10^{-4} M had the effect of restoring progressive spike broadening toward the pre-high Ca^{++} level (Fig. 5C). This experiment indicates that cyclic nucleotide stimulation can reverse the effects of increased $[Ca^{++}]_i$.

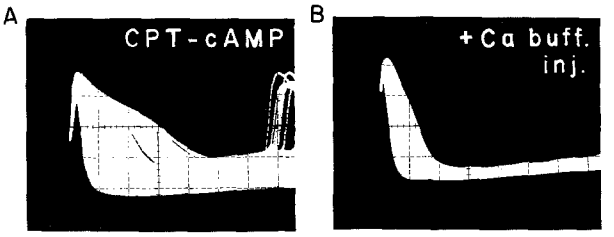

Fig. 6A, B. Enhancing effects of a cAMP analog on progressive spike broadening are suppressed by intracellular injection of 'high' Ca^{++} buffer. **A** Spike broadening in the presence of CPT-cAMP. Action potentials were stimulated by 2 nA of depolarizing current during a 50 s test. **B** Post-injection of a Ca^{++}-EGTA solution, 5×10^{-7} M free Ca^{++}. Test stimulus 2.75 nA, test duration 3 min. *Calibration:* grid bars 20 mV vertical, 20 ms horizontal

The reversibility of cyclic nucleotide enhancement of progressive spike broadening by intracellular injection of a 'high' Ca^{++} buffer (Ca^{++}- EGTA, 5×10^{-7} M free Ca^{++}) is shown in Fig. 6. The test of spike broadening in Fig. 6A was made in the presence of $\times0^{-4}$ M CPT-cAMP. The test shown in Fig. 6B was made shortly after intracellular injection of the "high" Ca^{++} buffer solution and shows significant reduction in the extent of broadening, although the stimulating current was increased by 37%, and the test duration was increased by 360%.

Cyclic Nucleotides Attenuate Spike Undershoot

The results of the experiments described above and our previous findings that alterations in gK_{Ca} were capable of modulating progressive spike broadening (Gillette et al. 1982) led us to examine specifically the effects of cyclic nucleotides on the undershoots of single, unbroadened action potentials. In our accompanying report we established that a manifestation of the $I_{K,Ca}$ could be observed in the undershoots, or afterhyperpolarizations, of single unbroadened action potentials. Treatments which reduce $I_{K,Ca}$, such as lowering external Ca^{++}, intracellular injection of EGTA, addition of the calcium blocker Co^{++}, and substitution of Ba^{++} for Ca^{++} all cause a typical abbreviation of the amplitude and waveform of the undershoot trough. Conversely, those treatments which enhance $I_{K,Ca}$, high external Ca^{++} and intracellular injection of 'high' Ca^{++} buffer, characteristically accentuate the amplitude and waveform of the undershoot. We find that cyclic nucleotide stimulation abbreviates the undershoot in a fashion identical to the treatments which reduce $I_{K,Ca}$ (Fig. 7). This feature was an invariant result of all 56 experiments using cAMP agonists. The records of Fig. 7 show action potentials stimulated prior (Fig. 7A) and sub-

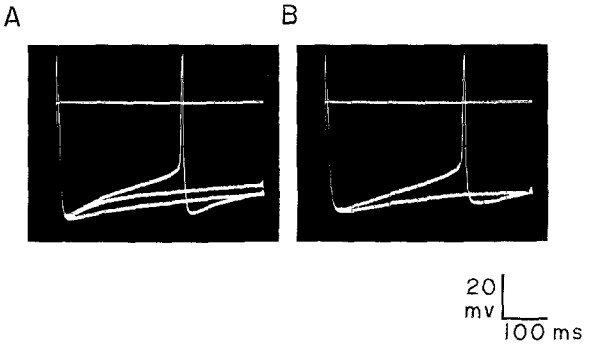

Fig. 7A, B. Abbreviation of the amplitude and waveform of the action potential undershoot by IBMX. Records are superimposed oscilloscope sweeps triggered by action potentials stimulated by varying levels of depolarizing current. Current was injected via a second intracellular electrode; conditioning hyperpolarization to −65 mV for 1 min preceded each stimulus. **A** Normal saline records showing the characteristic accentuation of the undershoot troughs. Stimulus currents 1.0, 1.5, and 2 nA. **B** Records taken 15 min after adding IBMX to the bath at a final concentration of 10^{-4} M. Amplitudes of undershoots are reduced by 3–4 mV and accentuation of the undershoot is largely abbreviated. Stimulus currents 0 and 0.5 nA. Reference traces are 0 mV. Spike heights clipped

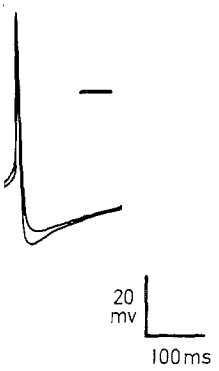

Fig. 8. Superimposed VWC spikes stimulated in normal saline (*lower trace*) and in the presence of 10^{-4} M IBMX (*upper trace*). Attenuation of the undershoot amplitude (4 mV) and waveform is typical of a reduction in the Ca^{++}-activated K^+conductance (gK_{Ca}) (see text). Stimulation currents: normal saline, 2 nA; IBMX, 1 nA. Reduction of current threshold for spikes is also typical of reduced gK_{Ca} (Gillette et al. 1982). Reference line is 0 mV potential

sequent (Fig. 7B) to the addition of the phosphodiesterase inhibitor IBMX, a treatment which should augment native cyclic nucleotides. IBMX caused both a loss of acuteness of the undershoot waveform and a loss of undershoot amplitude by 3–4 mV. A direct demonstration of the changes in undershoot waveform and amplitude is shown in Fig. 8, in which a spike stimulated in IBMX is superimposed on one stimulated prior to drug addition. Associated with the attentuation of the undershoot in IBMX shown in Fig. 8 was a 1.5 mV decrease in the amplitude of the spike overshoot. The small decrease in overshoot amplitude could possibly result in the 4 mV decrement in the amplitude of the undershoot shown through voltage-dependent decrease in the activation of outward current. However, spike records more perfectly matched with respect to the overshoots also show similar attenuation of the undershoot induced by CPT-cAMP treatment. Therefore, the attentuation of spike undershoot is most likely due to an effect of cAMP on the spike currents whose tails form the undershoot.

Cyclic Nucleotide Attenuation of Spike Undershoot Reversed by High $Ca^{++}{}_i$

Cyclic nucleotide attenuation of the undershoot was found to be reversible by intracellular injection of 'high' Ca^{++} buffer (5×10^{-7} M free Ca^{++}) (Fig. 9). The undershoot, shown in its abbreviated mode in the presence of 10^{-4} M CPT-cAMP (Fig. 9A) was

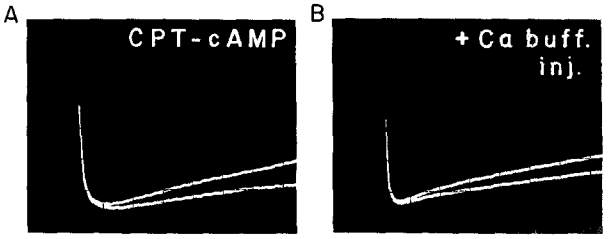

Fig. 9A, B. Reversal of cyclic nucleotide attenuation of the spike undershoot by Ca^{++} injection. Records are superimposed oscilloscope sweeps triggered on the downstroke of current-stimulated action potentials. **A** Characteristically attenuated undershoots of spikes in the presence of 10^{-4} M CPT-cAMP. Stimulus currents: 0.6 and 1.0 nA. **B** Accentuation of the undershoot waveform recorded 3 min after pressure injection of Ca^{++}-EGTA solution (5×10^{-7} M free Ca^{++}). Due to adjustment of the oscilloscope trace, a post-injection increase of 2–3 mV in undershoot amplitude is not apparent. Stimulus currents: 1.3 and 1.8 nA

restored to a more accentuated waveform by the Ca^{++} injection (Fig. 9B).

Thus, the effects of cyclic nucleotide stimulation both in reducing the undershoot amplitude and in de-emphasizing the accentuated character of the undershoot match the effects of a variety of conditions which reduce $[Ca^{++}]_i$. These effects are readily interpretable in terms of a reduction in $I_{K,Ca}$, a current whose blockade causes identical effects on the undershoot. However, the possibility that reduced $[Ca^{++}]_i$ causes an abbreviation of the undershoot through an effect in the tails of the inward spike Ca^{++} current (c.f. Gillette et al. 1982 for discussion) cannot be excluded by conventional intracellular recording methods.

Discussion

Modulation of VWC Command Ability in the VWC

The VWCs are behaviorally potent neurons which may play a significant command role in the feeding behavior of the animal. While several populations of neurons have been shown able to drive cyclic motor output in the feeding oscillator of *Pleurobranchaea* (Gillette and Davis 1977; Gillette et al. 1978), the discharges of these neurons are locked to distinct phases of the motor rhythm by alternating inhibitory and excitatory feedback from the network. The VWCs are unique among previously identified neurons in that they drive the oscillator through endogenously sustained tonic discharge. Such bursts are prolonged through many cycles of the motor rhythm and are relatively independent of network feedback. Spontaneous burst episodes and attendant spike broadening are observed in VWCs of most isolated nervous systems; however, variability among preparations, observations of lability in the VWCs' capacity to support burst episodes, and the fact that spontaneous activation in the intact organism would result in adaptively inappropriate behavior suggest that their capacity to burst is normally closely regulated.

The results of the experiments reported here indicate that cAMP could modulate the command role of the VWC through regulating the capacity for prolonged burst generation and attendant spike broadening. That cAMP can regulate these functions was shown in the enhancement of progressive spike broadening (Figs. 1 and 2) and stimulation of prolonged bursts (Fig. 2) by intracellular injection of native cAMP, and by the action of cAMP analogs (Fig. 3). The specificity of the effects to cAMP pathways in the cell is supported by the virtual lack of effect of a cGMP analog. The presence of endogenous cAMP is implied by the similarity of the effects of the degradative enzyme inhibitor, IBMX, to those of cAMP and its analogs.

Possible Mechanism of Action of cAMP

The results of the experiments presented here may be explained by either a cAMP stimulated decrease in $I_{K,Ca}$, a direct enhancement of I_{Ca}, or both. The effects of cAMP, its analogs and the stimulant of cyclic nucleotide accumulation. IBMX, in enhancing the rate and extent of progressive spike broadening are accompanied by abbreviation of the component of the spike undershoot waveform attributed in part to the calcium-activated potassium conductance, $I_{K,Ca}$ (Figs. 7 and 8). It was previously shown that experimentally altering the gK_{Ca} modulates the rate and extent of spike broadening (Gillette et al. 1982). The parameters of progressive spike broadening are consistent with a basic mechanism wherein a progressive weakening of a delayed K^+ current permits inward Ca^{++} current to prolong the depolarized phase of the spikes (Aldrich et al. 1979). Thus, as that K^+ conductance wanes, the $I_{K,Ca}$ assumes greater relative importance in spike repolarization and is a significant contributing factor to the rate and extent of progressive broadening (Gillette et al. 1978). The effects of cyclic nucleotide enhancement of progressive spike broadening can therefore be sufficiently explained on the basis of a reduction in the $I_{K,Ca}$; however, there is an untested possibility that an increase in I_{Ca} intensity could also contribute to enhanced spike broadening. Reduction of $I_{K,Ca}$ and increase in I_{Ca} would be expected to act agonistically.

The cAMP analogs and IBMX also stimulate prolonged and recurrent burst episodes in the VWC (Figs. 3 and 4). It is possible that burst stimulation and the abbreviation of the spike undershoot both arise from a cAMP-induced reduction in $[Ca^{++}]_i$. In endogenously bursting neurons of the mollusc *Aplysia*, the termination of bursts and the duration of the interburst interval is accompanied by a slow rise and fall of K^+ conductance (Junge and Stevens 1973). Use of the dye Arsenazo III to monitor fluxes of internal free Ca^{++} in a bursting neuron has provided compelling evidence that the regulation of $I_{K,Ca}$ by intracellular Ca^{++} is sufficient to account for burst termination and the duration of interburst interval (Gorman and Thomas 1978). In this model the rate of accumulation of intracellular Ca^{++} towards a level at which $I_{K,Ca}$ may terminate the burst through hyperpolarization is a major factor determining the duration of the burst, and the rate of removal of free internal Ca^{++} determines via $I_{K,Ca}$ the rate of depolarization to next burst threshold (i.e., the interburst interval). The VWCs resemble the endogenous bursters of *Aplysia* in the regular production of endogenously sustained bursts, but the durations of the VWC burst and interburst intervals are an order of magnitude longer and the $[Ca^{++}]_i$ fluxes with which the cell has to deal may be proportionately greater as well. Since there is evidence that appreciable inactivation of I_{Ca} may result from internal Ca^{++} accumulation during prolonged depolarization (Kostyuk and Krishtal 1977; Tillotson 1979), there may be a role for a $[Ca^{++}]_i$ regulation of I_{Ca} agonistic to that of $I_{K,Ca}$ in the determination of the VWCs' burst durations and the interburst interval. Accordingly, the effects of cyclic nucleotides on prolonging the burst duration in the VWC and on shortening the interburst interval are accountable in economical terms if they

are assumed to act to increase the rate of removal of free internal Ca^{++}, either by extrusion of Ca^{++} from the cell or intracellular sequestration of free Ca^{++}. A cAMP-increased rate of removal of internal Ca^{++} would slow the accumulation of the ion during the burst and thus result in burst prolongation; the increased rate of removal would also shorten the less excitable period between bursts. If this is so, the slow waning of cAMP analog and IBMX-stimulated burst activity may be due to a slow saturation of the augmented Ca^{++} buffering ability of the cell. An alternative explanation where cAMP stimulation becomes refractory is contrary to the observation that VWCs, which have seemingly returned to control levels of excitability after prolonged periods in the presence of the drugs, become very hypoexcitable upon drug washout. The overshooting loss of excitability and burst capacity could result from sudden increase in $[Ca^{++}]_i$, as might occur by release from sequestered stores or reduction in extrusion rate.

Twarog and her co-workers (Twarog 1966; Hidaka et al. 1967; Twarog and Muneoka 1973) earlier proposed that serotonin causes a reduction in intracellular free Ca^{++} in the anterior byssus retractor muscle of the mussel, *Mytilus*, thereby explaining how the neurotransmitter caused relaxation, enhanced electrical excitability and increased both the rate of upstroke and the amplitude of Ca^{++} action potentials. The action of serotonin in this system may be mediated by cAMP (Twarog 1973).

The Potential Context of cAMP Action on the Ventral White Cells

Previously it was shown that spike broadening in the VWC is invariably correlated with initiation or acceleration of rhythmic motor output of the buccal ganglion (Gillette et al. 1980). That spike broadening is necessary to the cell's command ability was shown by manipulations preventing broadening of stimulated action potentials which blocked the cell's action on the motor network, and by the demonstration of rather precise thresholds of spike duration at which the VWC is effective. The evidence that prolongation of Ca^{++} current sustains the broadened spike (Gillette et al. 1982) suggests that broadened spikes may enhance Ca^{++} dependent transmitter release, as has been shown for neurons of other molluscs (Klein and Kandel 1978).

This last observation is relevant to the likely function of the VWCs in the dual roles of motorneuron and command neuron. Each VWC of the bilateral pair acts as an esophageal motorneuron whose peripheral axon ramifies over the superficial muscle sheet of the contralateral side of esophagus; single and short trains of action potentials cause graded longitudinal contraction. The VWCs are active in two patterned firing modes: a phasic one in which short bursts of spikes at 1–2/s are phase-locked to the retraction phase of the motor output of the ganglion by synaptic activity, and that which is the prolonged and endogenously sustained burst episode (Gillette et al. 1980). Thus, during phasic activity the cells can cause rhythmic esophageal shortening with virtually no central effects, since spikes do not appreciably broaden during phasic activity. When the VWC is triggered into its second mode of activity, that in which it drives motor neurons of the feeding network, tonic esophageal shortening is maintained throughout the period in which the VWC drives vigorous and multiple cycles of motor network activity. The VWCs thus show a capacity to act in two distinct behavioral roles in feeding activity in which their motor neuron and command neuron functions are both distinct and coordinated.

The activation of neuronal adenyl cyclase by certain neurotransmitters is an increasingly well documented neuromodulatory event; in particular, both serotonin and dopamine have been shown to stimulate adenyl cyclase in molluscan neurons (Cedar and Schwartz 1972; Levitan 1978). Both of these transmitters are present in the buccal ganglion of *Pleurobranchaea* (Gillette et al. 1980; R. McCaman, personal communication). We expect that sensory or other inputs converge on the VWCs to specifically induce the bursting capacity that drives some aspect of vigorous and cyclic buccal mass behavior. Such a mechanism of neuromodulation is in keeping with the likely role of these neurons as activators of a stereotyped motor program involving the buccal apparatus, the buccal mass. Whether the motor program results behaviorally in egestion or ingestion is presently under investigation. We are currently concerned with exploring these questions.

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