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SHORT COMMUNICATIONS

STIMULATION OF CHEMOSENSORY PATHWAYS AND INTRACELLULAR ALKALINIZATION MIMIC cAMP ACTIVATION OF ENDOGENOUS BURSTING IN FEEDING COMMAND NEURONES

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Cyclic nucleotides modulate the activity of diverse types of excitable cells (Twarog & Muneoka, 1973; Berridge, 1975; Triestman & Levitan, 1976; Greengard, 1980; Bernier, Castellucci, Kandel & Schwartz, 1982; Gillette, Gillette & Davis, 1982*b*; Evans, 1984). In the carnivorous gastropod *Pleurobranchaea*, it would appear that cAMP modulation of a single pair of neurones effects the expression of a complex coordinated rhythmic behaviour. These neurones, the ventral white cells (VWCs), command vigorous motor output from the neuronal feeding oscillator through an intrinsic capacity to generate minutes-long high intensity bursts of action potentials (Gillette, Gillette & Davis, 1980). This high frequency firing causes the action potentials to increase in duration (broaden). The increase in action potential duration is a specific antecedent to enhanced motor output through a Ca^{2+}-dependent mechanism (Gillette, Gillette & Davis, 1982*a*). Elevated intraneuronal cAMP is a specific activator of prolonged VWC bursting in both the isolated nervous system and the isolated VWC soma (Gillette *et al.* 1982*b*). The relationship between this endogenous effector (cAMP) and extrinsic activators of the motor programme has not been established.

I report here that the electrical changes in VWCs activated by food stimulation of the chemosensory sites involved in food detection and ingestion, as well as the changes activated by alkalinizing intracellular pH-shifts, both resemble in detail the changes induced by augmentation of intracellular cAMP. All three treatments stimulate recurrent burst episodes and cause attenuation of the after-hyperpolarizations of single stimulated action potentials. Decay of the activation in the continued presence of food shows kinetics strikingly similar to the decay of cAMP or NH_4^+ stimulation. This is consistent with a model wherein ingestion is modulated at this strategic neuronal site by a pH-sensitive mechanism which controls intracellular levels of cAMP.

Pleurobranchaea californica were pre-tested for their response to food stimuli (squid homogenate, SH) and egestive stimuli (100 % EtOH or 1 % Haemosol in sea water). Immediately after behavioural testing, the animal was dissected to leave the anterior half (hemi-animal) intact. The buccal ganglion was immobilized on a

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Fig. 1. Prolonged burst episodes stimulated by food (A) and intracellular alkalinization with NH_4^+ (B) are similar to those stimulated by 8-*para*-chlorophenylthio-adenosine-3′,5′-cyclic monophosphoric acid (CPT-cAMP) treatment which elevates cAMP within the ventral white cells (VWCs) (C). Stimulation (arrow) of the oral veil of the hemi-animal with squid homogenate produced bursts (A) similar in spike frequency, undershoot decrement, general form and recurrent nature to prolonged VWC bursts recorded in the isolated buccal ganglion and stimulated by adding 20 mM-NH_4^+ 6 min earlier (B) or the non-hydrolysable analogue CPT-cAMP (5×10^{-5} M) 3 min earlier (C) to the bath. Burst stimulation was reversed upon washing the ganglion with normal saline. Bar = 1 min.

platform to allow intracellular recording of the response of the VWC during chemosensory stimulation of the oral veil or buccal cavity. This preparation responded to the two classes of stimuli with behaviour appropriate to the nature of the stimulus. Experiments using chemical agents to stimulate bursting were performed on isolated buccal ganglia pinned out in normal saline (NS, see Gillette *et al.* 1980), pH 7·5, to which test substances were added. All effects were reversed upon washing.

Food stimuli, but not egestive stimuli, applied to either the oral veil or buccal cavity of the hemi-animal produce gradual synaptic excitation of the VWC which culminates in prolonged burst episodes with attendant spike broadening in the VWC (Gillette & Gillette, 1983); it is only during these prolonged bursts that the VWCs drive the vigorous buccal motor programme (Gillette *et al.* 1980) characteristic of feeding behaviour (Croll & Davis, 1981; Gillette & Gillette, 1983). The bursts stimulated by food (Fig. 1A) and NH_4^+ treatment (Fig. 1B) resemble in detail those stimulated in the isolated buccal ganglion by a number of treatments which elevate intracellular cAMP (Fig. 1C): injection of native cAMP into the VWC, bath application of non-hydrolysable cAMP analogues (e.g. 8-*para*-chlorophenylthio-adenosine-3′,5′-cyclic monophosphoric acid, CPT-cAMP), or bath application of isobutylmethylxanthine (IBMX), which is an inhibitor of the cAMP degradative enzyme, phosphodiesterase (Gillette *et al.* 1982*b*). Bursts stimulated by this array of agents are similar in spike frequency and progressive spike broadening as well as their general form and recurrent nature (Fig. 1).

A second similarity in the effects of these three treatments is an attenuation of the dip or trough in the undershoot normally seen in individual stimulated spikes in normal saline (NS, Fig. 2A, C, E). It was previously shown (Gillette *et al.* 1982*b*) that a concomitant of VWC burst stimulation in the isolated ganglion by treatments which augment intracellular cAMP is an attenuation of the form and amplitude of th

ion potential. A 4 mV attenuation of the undershoot caused by elevated intracellular cAMP is clearly seen in the superimposition of the spike recorded in IBMX with that of NS (Fig. 2B). Similarly, in isolated ganglia bathed with NH_4^+, the undershoot of single VWC spikes is attenuated in amplitude (5 mV) and has a flattened form (Fig. 2E, F). NH_4^+ lowers the current threshold, as does IBMX (Fig. 2B, F).

The undershoots of spikes stimulated in normal saline in the hemi-animal (Fig. 2C) reveal the dip or trough seen in the VWCs of the isolated ganglion (Fig. 2A, E). Activation of the VWC by squid homogenate (SH) in the buccal cavity produced attenuation in both the amplitude (3 mV) and form of the trough (Fig. 2D), like that

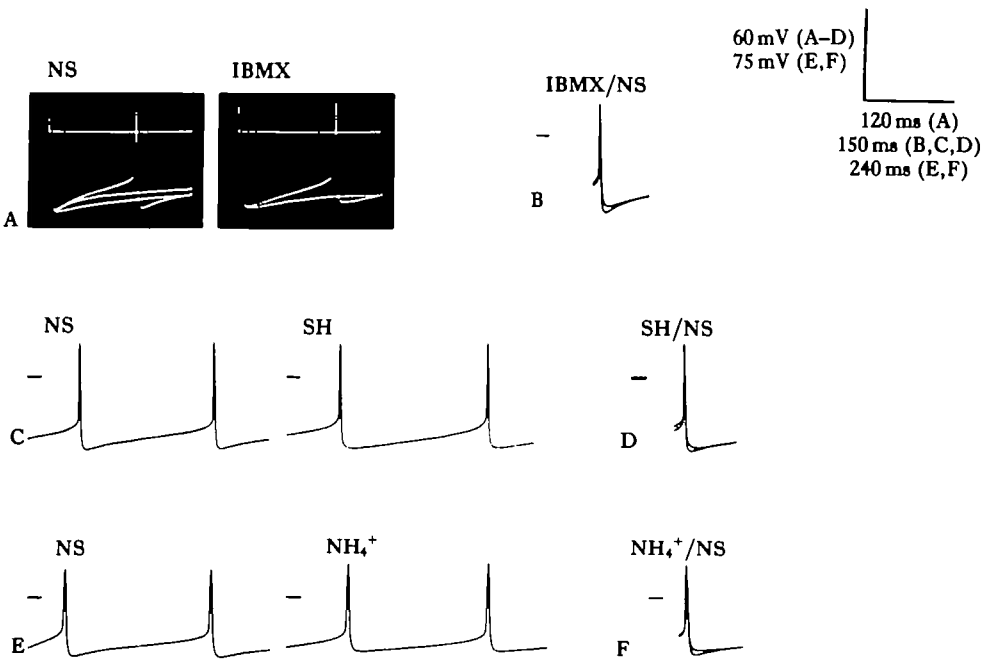

Fig. 2. Abbreviation of the amplitude and waveform of the action potential undershoot by isobutylmethylxanthine (IBMX), appetitive stimuli and NH_4^+. (A) These intracellular recordings were made from dual electrode penetration of a single VWC in an isolated buccal ganglion. Records are superimposed oscilloscope sweeps triggered by action potentials stimulated by varying levels of depolarizing currents. Normal saline (NS) records show the characteristic attenuation of the undershoot trough. Stimulus currents are 1·0, 1·5 and 2·0 nA. Records taken 15 min after adding isobutylmethylxanthine (IBMX) to the bath (10^{-4} M final concentration) show attenuation of the amplitude and form of the trough. Stimulus currents are 0 and 0·5 nA. (B) Superimposed VWC spikes stimulated in NS (lower trace) and in the presence of 10^{-4} M-IBMX (upper trace) are directly comparable in attenuation of undershoot amplitude (4 mV) and form. (C) These intracellular recordings were made with a single microelectrode penetration of one VWC in a hemi-animal preparation. Chart records of action potentials stimulated in NS before squid homogenate (SH) treatment show the characteristic trough in the undershoot, similar in form to that in NS in the isolated ganglion. Spikes stimulated after SH applied to the buccal cavity had activated the VWC (37 s before a prolonged burst) show attenuation of undershoot amplitude and form. (D) Superimposition of the spikes from (C) to demonstrate directly the attenuation of undershoot amplitude (3 mV) and form. (E) Intracellular records (1 min pre-burst) of stimulated spikes from a VWC in the isolated buccal ganglion in NS and after adjusting the bath saline to 20 mM-NH_4^+. (F) Superimposition of the spikes from (E) demonstrates attenuation of the undershoot amplitude (5 mV) and form. In all trials (A–F) each depolarizing stimulus was preceded by a conditioning hyperpolarization of −60 mV for 30 s. Records within experimental paradigms were matched for spike frequency. Reference traces in (A) and bars (B–F) are zero potential.

seen with IBMX treatment (Fig. 2B). Reduction in the current threshold by S placed on the hemi-animal's oral veil was also observed with IBMX treatment of the VWC (Fig. 2B, D). Under conditions where the VWC is activated by food, the attenuation of the undershoot does not occur until late in the activation period during which VWC activity is synaptically driven by the feeding network. It is not seen during the short bursts occurring 1 min before a prolonged burst but becomes clearly apparent shortly (37 s in Fig. 2D) before the prolonged burst. This suggests that undershoot attenuation is a manifestation of a change in an endogenous property regulating this neurone's excitability, since the VWC shifts from following the network to driving the network during the prolonged burst episodes (Gillette & Gillette, 1983).

A third and striking similarity is the recurrence of the burst episode and the decay in the frequency of bursts occurring after the initial activation. This decay in burst frequency attended by increasing inter-burst intervals is a common feature of food

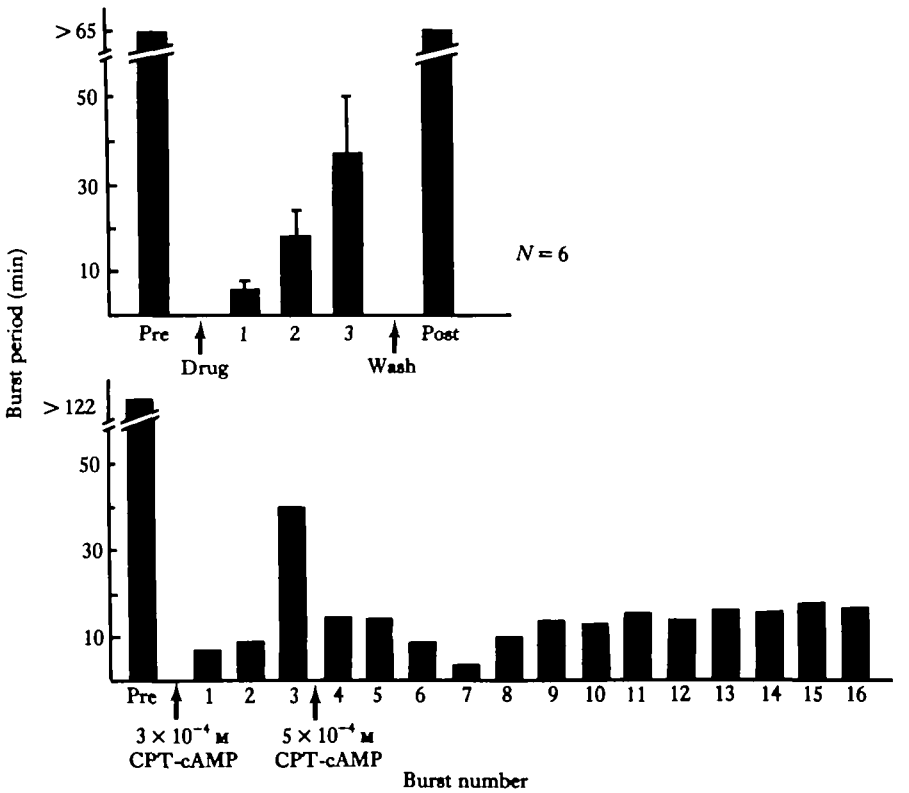

Fig. 3. Relationship between stimulus level of 8-*para*-chlorophenylthio-adenosine-3',5'-cyclic monophosphoric acid (CTP-cAMP) and the rate of decay of recurrent burst episodes. The upper bar graph shows the mean and S.D. of the period between initiation of prolonged burst episodes stimulated in quiescent VWCs ($N = 6$) by bath application of $1–2\times10^{-4}$ M-CPT-cAMP. Note the similar rate of decay for this stimulus level for VWCs from six different experimental animals. The lower bar graph depicts a continuous sequence from one VWC which did not burst prior to addition of CPT-cAMP to the bath. When the bath concentration was adjusted to 3×10^{-4} M the cell started bursting within 15 min in a sequence of recurrent prolonged burst episodes which decayed over 56 min. Ten minutes after the third burst, the bath level was elevated to 5×10^{-4} M-CTP-cAMP. This was followed within 10 min by an oscillatory sequence of prolonged bursts with a short, relatively constant burst period (which includes the burst + interburst interval); no decrement was observed over the next 4 h at which time the experiment was terminated.

mulation, NH_4^+ and cAMP elevation (Fig. 1A, B, C). I have found that for direct tonic cAMP stimulation the rate of decay depends on the level of activation. It is similar for bursts stimulated in a non-bursting VWC by $1–2\times10^{-4}$ M-CPT-cAMP in six different experiments (Fig. 3, upper), however, when the stimulus level is raised to 5×10^{-4} M-CPT-cAMP, the cell oscillates between bursting and quiescence indefinitely (Fig. 3, lower). This type of non-decremental hyperstimulation, under conditions which elevate cAMP for prolonged periods, was always observed at higher CPT-cAMP and IBMX concentrations ($N = 12$). Saline NH_4^+ levels of 20 mM may produce similar long-lasting recurrence of bursting ($>3\,h^{-1}$ in 2/5 neurones). At double these concentrations of either CPT-cAMP or NH_4^+ the burst does not terminate at all. These results suggest that the decay mechanism has been overridden, possibly by overloading the driving mechanism, at the higher cAMP and NH_4^+ levels.

Taken together, these experiments suggest that the presence of food at the oral veil or buccal cavity activates a neuromodulatory pathway which stimulates cAMP accumulation within the VWCs. The identity of pathways which naturally activate the VWCs is unknown beyond their appetitive nature. The pathways could comprise chemosensory afferents or collaterals from the feeding oscillator. Aminergic neurotransmitters and neuropeptides have been shown to stimulate cAMP synthesis in molluscan neurones (Cedar & Schwartz, 1972; Barker & Gainer, 1974; Levitan, 1978) and are likely candidates in this system.

Presumably, accumulation of cAMP above a threshold level induces the burst mode. The observation made in this study that attenuation of the VWC undershoot induced by food occurs shortly before the onset of bursting supports this notion. By this interpretation, once bursting is initiated by neuromodulatory synaptic excitation, the cell shifts to a different state of activity, which is self-sustained. This interpretation is strengthened by the finding that prolonged burst episodes can be triggered in the isolated VWC soma (Gillette *et al.* 1980). Voltage and patch clamp analysis of the cAMP-activated state has demonstrated a sustained enhancement of a slow inward current underlying the enhancement of bursting (Gillette, 1981; Green & Gillette, 1983). This current may be turned on by cAMP-mediated phosphorylation, a ubiquitous mechanism of cAMP regulation of cell function (Greengard, 1980).

Decay of the activated state may be caused by the burst activity itself. Intracellular accumulation of Ca^{2+} during the burst would activate hyperpolarizing Ca^{2+}-dependent K^+ conductances (Gillette *et al.* 1982*a*) as well as Ca^{2+}-activated phosphodiesterase, which degrades cAMP (Calhoon & Gillette, 1983). The involvement of Ca^{2+} influx is supported by observations that in 0-Ca^{2+} saline, cAMP-stimulated bursts show no auto-termination (Gillette *et al.* 1982*b*). Thus, very high levels of cAMP stimulation (Fig. 3) may overcome this control mechanism by biasing the cell against these Ca^{2+}-mediated termination and decay mechanisms in favour of the cAMP-mediated 'on' processes.

The pH-sensitivity of VWC activation, which is reflected in the NH_4^+-mediated enhancement of bursting, attenuation of the spike undershoot, and the gradual lengthening of the interburst interval, is remarkable in its detailed similarity to the effects on the VWC of food stimulation of the animal as well as cAMP stimulation of the neurone. A further similarity to cAMP effects is the recent finding that alkalinization of intracellular pH potentiates the slow inward current underlying the burst

(Gillette, 1983). These similarities suggest that alkalinization of the cell (by NH_3 diffusing in through the membrane, acquiring a proton to form NH_4^+ and thus raising the pH) also enhances intracellular cAMP. Recent work supports this interpretation: the degradative enzyme for cAMP in the *Pleurobranchaea* nervous system, a Ca^{2+}-activated phosphodiesterase, shows marked pH sensitivity (Calhoon & Gillette, 1983). Alkalinization of the cell by 0·1–0·2 pH units during NH_4^+ treatment could effectively inhibit phosphodiesterase, allowing cAMP to accumulate (Calhoon & Gillette, 1983). Thus, NH_4^+ should and does mimic treatments which elevate cAMP in the VWC.

These data are consistent with a model wherein consummatory feeding behaviour in this predatory mollusc may be modulated at this strategic central neurone by a complex, pH-sensitive regulation of intracellular cAMP (Calhoon & Gillette, 1983). In this model, appetitive stimulation of buccal chemoreceptors must lead to synthesis and accumulation of cAMP in the VWC. Then cAMP-mediated phosphorylation would activate a slow inward membrane current carried by Ca^{2+}. This electrical change would manifest itself in the burst. Accumulation of Ca^{2+}, and concomitant H^+, would then stimulate phosphodiesterase leading to cAMP degradation, the cessation of bursting, and the end of a feeding bout. Decline of intracellular free Ca^{2+} and H^+ during the post-burst period should prime the cell for reinitiation of the burst in the continued presence of an excitatory stimulus. Thus, in this model the interaction of Ca^{2+}, cyclic nucleotides and pH determines the temporal parameters of the burst cycle.

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