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CYCLIC ADENOSINE MONOPHOSPHATE MEDIATION OF PEPTIDE NEUROHORMONE EFFECTS ON THE LOBSTER CARDIAC GANGLION

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SUMMARY

Two cardioexcitors which can be extracted or released from crustacean neurohaemal structures, the pericardial organs, alter the endogenous bursting activity of isolated lobster cardiac ganglia in different ways. A small peptide increases the burst frequency of the ganglion, the duration of each burst, and the number of spikes per burst, while 5-hydroxytryptamine (5-HT) increases burst frequency, but decreases the burst duration and number of spikes per burst. Four lines of evidence are presented that cyclic adenosine monophosphate (cAMP) mediates the effects of peptide on the ganglion, but does not play a significant role in 5-HT action: (1) exogenous cAMP mimics the effects of peptide but not of 5-HT on burst parameters; (2) inhibition of the enzyme phosphodiesterase, which increases endogenous levels of cAMP, mimics the effects of peptide and potentiates peptide action but not that of 5-HT; (3) putative blockers of adenylate cyclase block the action of peptide but not of 5-HT; (4) peptide causes a large increase in cAMP content of the ganglion, while 5-HT application results in a much smaller increase.

INTRODUCTION

Evidence has accumulated over the past decade that some of the modulatory actions of peptide and monoamine neurohormones or neurotransmitters may involve alteration of the intracellular biochemical machinery of target neurones, rather than, or in addition to, a more direct effect on the state of a gated ion channel in the cell membrane. As is the case in non-neuronal systems, peptides and monoamines are thought to interact with neurones primarily via surface receptors. Activation at the external cell surface is often translated into intracellular messages by alteration of the metabolism of cyclic nucleotides, which in turn may modify the electrophysiological properties of the target neurones.

Adenosine 3′,5′ monophosphate (cAMP) has been implicated as a mediator of the action of several peptide hormones and monoamines in both vertebrates and invertebrates. While early studies on the role of cAMP in mammalian nervous systems

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focused on changes in synaptic function (McAfee & Greengard, 1972; Siggins *et al.* 1971) primarily at the level of the postsynaptic cell, it is now clear that a much broader range of neuronal functions can be influenced by changes in cyclic mononucleotide metabolism. Evidence for presynaptic effects of 5-hydroxytryptamine mediated by cAMP have been obtained from molluscan systems (Brunelli, Castellucci & Kandel, 1976), and effects of the mononucleotide on the release of transmitter from vertebrate motor neurones have been reported (Standaert & Dretchen, 1979). Direct effects on non-synaptic membrane properties have been suggested, including alteration of pacemaker conductances (Tsien, 1977) and other slow rhythmically generated potentials that underlie bursting characteristics of some neurones (Levitan, 1979).

Most of the early studies implicating cAMP in the mediation of peptide and monoamine actions analysed neuronal systems in which it was necessary to stimulate impinging pathways electrically in patterns that bore no obvious relation to the normal activities of the neurones in question. It has been difficult, therefore, to evaluate the role of cAMP metabolism in the normal interactions of neurones, or in the mediation of physiologically relevant behaviours. Only recently has evidence been presented for effects of peptides known to occur endogenously on more-or-less normal discrete behaviours in vertebrates (e.g. Moss, 1977) or on well defined neuronal pathways known to subserve physiologically important behaviours (Mayeri, 1979). Both peptides and monoamines, however, are known to alter the rhythmic activity of neuronal oscillators, and such systems therefore present an opportunity to study the role of mononucleotides during normal neuronal behaviour and interaction. Pharmacological experiments altering cAMP metabolism suggest that the effects of native peptides on some invertebrate single-cell oscillators are mediated by cAMP (Levitan & Triestman, 1977; Levitan, 1979), and that endogenous properties of the cell membrane, independent of synaptic activity, are being altered.

The observations on single cell oscillators noted above suggested that it would be worthwhile examining the role of cAMP in the modulation of a simple multicellular network, the crustacean cardiac ganglion, which shows endogenous rhythmicity and which can be modulated by both neurosecretory peptides and monoamines. The anatomy of the ganglion was first described by Alexandrowicz (1932) and the physiology by Welsh & Maynard (1951). One or more of the four 'small cells', with efferent processes confined to the ganglion, typically functions as an intrinsic pacemaker for the entire system; five 'large cells', with axons exiting from the ganglion, are motor neurones innervating the heart musculature (Anderson & Cooke, 1971). The extensive synaptic and electrotonic connections between the nine cells which ensure synchronous rhythmic output to the heart have been analysed (Hartline, 1979), as have some of the endogenous membrane properties which account for pacemaker activity and burst formation of the individual cells (Tazaki & Cooke, 1979*a*, *b*).

The rhythmic activity of the ganglion can be modulated by a variety of inputs. *In situ*, it is controlled directly by cardioexcitor and cardioinhibitory nerves which, respectively speed up and slow down the beat (Maynard, 1953). In addition, neurohormones act as cardioexcitors on isolated ganglia and intact hearts (Maynard & Welsh, 1959). In lobsters, three pairs of neurohaemal structures, the pericardial organs (POs) (Alexandrowicz, 1953; Evans, Kravitz & Talamo, 1976) are found in the openings of the branchial vessels into the pericardial sinus. These organs cons-

of the terminals of neurosecretory axons which, in diverse species, contain and secrete one or more cardioactive peptides (Cooke, 1964; Berlind & Cooke, 1970; Belamarich & Terwilliger, 1966; Sullivan, 1979), 5-hydroxytryptamine (Sullivan, 1978); octopamine (Kravitz *et al.* 1975) and dopamine (Cooke & Goldstone, 1970; Sullivan, Friend & Barker, 1977). The mechanisms of action of these peptides and monoamines on the ganglion (Cooke, 1966; Cooke & Hartline, 1975) and on heart musculature (Battelle & Kravitz, 1978) have been incompletely analysed. It is clear, however, that a peptide cardioexcitor from PO interacts with the isolated ganglion in a different manner than does 5-HT (Cooke, 1966). We therefore attempted to determine, in the work reported here, whether either or both of these neurohumours acts via cAMP, as a preliminary stage of a more extensive analysis of cellular mechanisms of action.

MATERIALS AND METHODS

A cardiac ganglion from *Homarus americanus* was carefully extirpated from the heart, along with small bits of attached myocardium, and secured with glass pins to a layer of clear resin (Sylgard) in the bottom of a small plexiglas chamber. The preparation was perfused at a constant rate with aerated lobster ringers (Cole, 1941) buffered to pH 7·4 with Tris maleate. The perfusion fluid contained 1 mM glucose, and the temperature was maintained at 14–16 °C.

Extracellular records were obtained using a suction electrode applied to the trunk or one of the anterior Y-branches of the ganglion. Suction electrodes were used in order to allow continuous recording of activity and drug application in saline throughout the course of an experiment, and to minimize stretching of the ganglion. Intracellular recordings were obtained from one or more large cell somata, using microelectrodes filled with 3 M-KCl (resistances 12–34 MΩ). Cell three (Hartline, 1967) at the base of the Y-branch was most frequently penetrated. Occasional intracellular recordings were obtained from axonal regions of the cell. Signals were monitored with a high-impedance DC amplifier (WPI-M4) with an internal bridge circuit to allow simultaneous recording and current-passing through the same electrode. Recordings were displayed on a differential oscilloscope, and stored on magnetic tape (Vetter) and/or on a chart recorder (Brush 220).

Normal ganglia exhibit intermittent bursts of small and large cell spikes, occurring at a frequency of about one per second, each burst being followed by a silent period. The burst frequency, duration of each burst, and number of spikes per burst were analysed quantitatively before and after experimental treatments. In extracellular records, the burst duration was the time between the first and last spike in a train. In intracellular studies the duration of a burst was considered to be the time between the first recorded rapidly depolarizing potential (synaptic potential or action potential), and the nadir of the post-burst hyperpolarization. In most intracellular records from the soma the number of spikes per burst was difficult to measure with confidence because of the severe attenuation of action potentials recorded at a distance from the spike-generating membrane, but when penetrations were made closer to the axon, changes in spike numbers from individual large cells were readily obtained. Spike counts from extracellular records included both small and large cell action potentials.

The pattern of spiking within a burst was qualitatively analysed from extracellular records by use of a discriminator circuit. Discriminator pulses corresponding to individual spikes within a burst were displayed in sequential fashion by a raster (WPI 104A). In an untreated ganglion the pattern of discharge is regular and slight changes in pattern resulting from the application of tested substances are readily apparent (Fig. 1A, and Cooke & Hartline, 1975).

All drugs (Sigma) were applied directly to the chamber (volume = 2 ml), and mixing was complete within 15–20 s. Drug concentrations cited are effective levels in the chamber after complete mixing. Because of substantial differences in the baseline burst rates and durations between preparations, all effects of test substances were normalized as percentage changes compared to an internal baseline value determined for each experimental situation. The percentage change was calculated between a control period of 30 s just before application of the test substance, and the peak 30 s period of the response. Damaged ganglia, or ones that did not maintain a consistant baseline, were not used for analysis.

Cyclic AMP levels in cardiac ganglion and cardiac muscle were determined by radioimmunoassay (Steiner, Parker & Kipnis, 1972). Ganglia were removed from perfusion during control periods or at the peak of response to test substances, placed on a piece of parafilm, and immediately frozen with dry-ice and acetone. Heart muscle was similarly superfused and drug-treated; the muscle was quickly dissected free and frozen. Tissue cAMP was extracted in ice cold absolute ethanol (Farmer, Harrington & Brown, 1975). Following homogenization and centrifugation, the pellet was preserved for protein determination (Lowry *et al.* 1951).

Extracts of cardioexcitor from pericardial organs (XPO) of lobster and two species crabs, *Carcinus maenus* and *Libinia emarginata*, were obtained by the method of Cooke (1966). Homogenates of the neurohaemal areas in distilled water were boiled for 3 min before dilution with lobster ringers. Concentrations of XPO are expressed as the number of POs per ml of perfusion fluid in the chamber. To confirm that the effective factor in XPO is a peptide, extracts were treated with trypsin (20 μg/ml) for 5 h at 37 °C. Protease digestion was terminated by boiling the extract for 4 min. Control extracts were similarly treated except for the addition of trypsin The activity of treated extracts was also tested with an assay highly sensitive to 5-HT, the rate of fluid secretion by isolated Malpighian tubules of the insect *Rhodnius prolixus* (Maddrell, Pilcher & Gardiner, 1971).

RESULTS

Neurohormone effects

The peptide and monoamine neurohormones in pericardial organs have markedly different effects on the electrical activity of the cardiac ganglion, although they are all cardioexcitors in the sense that they generally increase the frequency of bursting. Basic differences in the effects of 5-HT and the extract of the pericardial organs (XPO) were established both by extracellular techniques, which monitored the activity of several large and small cells but did not attempt to distinguish between individual units, and by intracellular recordings from large cell bodies. Our results are in general agreement with those of others (Cooke, 1966; Cooke & Hartline, 1975).

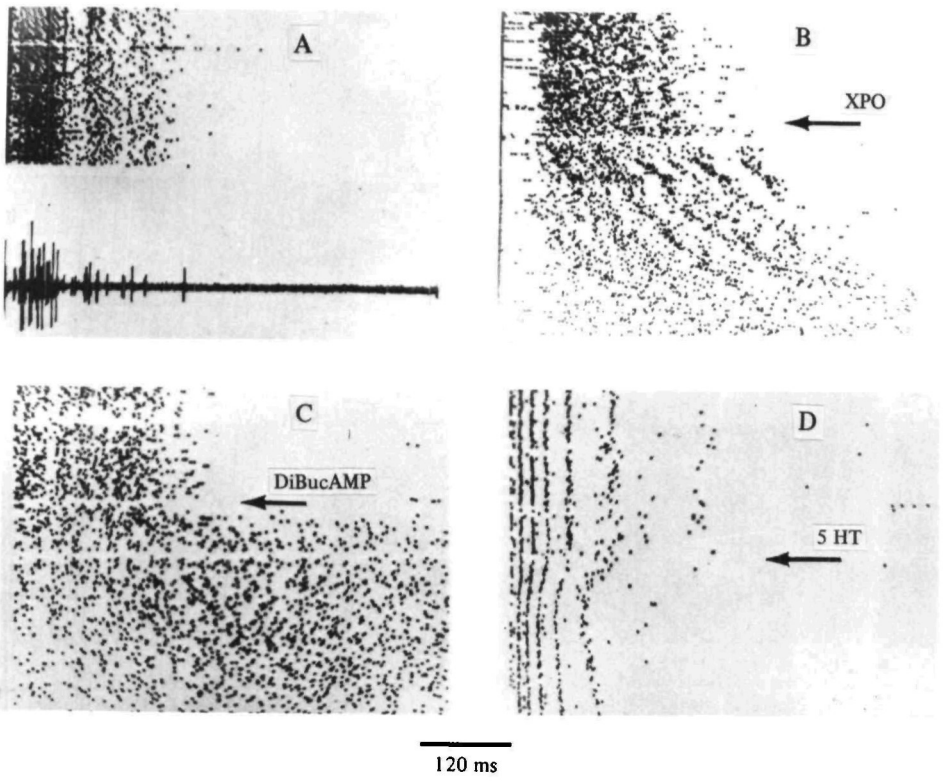

Fig. 1. Comparison of changes in extracellularly recorded burst patterns resulting from exposure to various agents. Each horizontal row of dots corresponds to a single burst, with individual dots representing single action potentials. (Both small and large cell spikes are included in most cases.) About 80 consecutive bursts are illustrated in records B–D, from top to bottom. Arrows indicate the onset of action of applied agents. (A) An untreated ganglion, showing the relation of the dot display to spike pattern. Note regularity in spike number, burst duration and pattern. (B) After the introduction of XPO (0·01 PO/ml) the burst is increased in duration, with a concomitant decrease in intraburst frequency. (C) Dibutyryl-cAMP (10^{-6} M) causes a rapid change in firing pattern, including burst prolongation, and increase in number of spikes per burst. (D) 5-HT (10^{-7} M) decreases burst duration and number of spikes, and increases intraburst spike frequency. (Records from different preparations.)

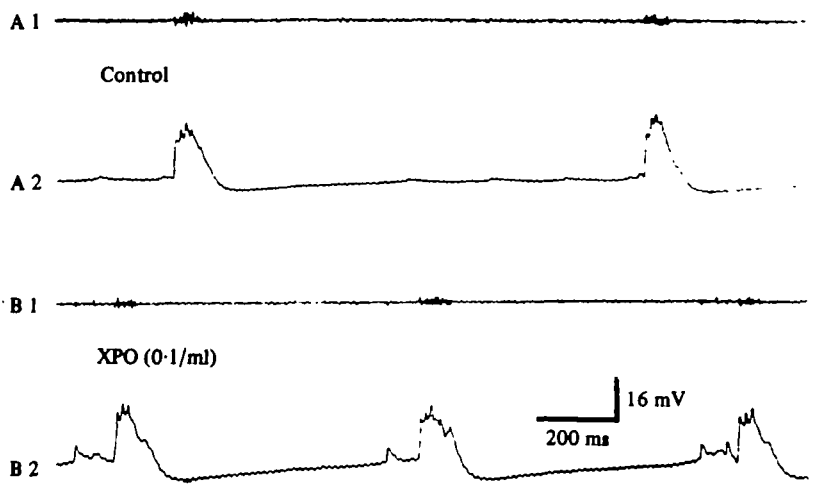

Fig. 2. Bursts recorded simultaneously with an extracellular electrode on the trunk of a ganglion (A_1, B_1) and an intracellular electrode in the soma of a large cell (A_2, B_2) before and after the application of XPO (0·1 PO/ml). Burst frequency, burst duration, and amplitude of the post-burst hyperpolarization are all increased. Voltage calibration refers to intracellular traces in all figures.

XPO, when applied to isolated ganglia, usually increased both the frequency of bursting and the duration of individual bursts (Figs. 1 B, 2). The average percentage increase in burst rate is linearly related to the logarithm of concentration of the extract, as has previously been reported for intact hearts (Maynard & Welsh, 1959). *Libinia* XPO was an order of magnitude more effective than *Carcinus* extract, and *Homarus* XPO was more potent than that of either of the crabs. Almost all XPO applications resulted in a duration increase (sometimes by as much as threefold), but in about 20% of the preparations the burst rate remained unchanged or decreased. In preparations which did not respond with an increased rate, the effects on burst duration were larger than normal.

Use of a raster and window discriminator made it possible to analyse extracellularly recorded bursts in somewhat greater detail. The nine neurones of the ganglion fire in stereotypic patterns which are repeated at each burst (Hartline, 1967; Cooke & Hartline, 1975). XPO applications increased the number of spikes in each burst, apparently affecting both large and small cells. There was usually a concomitant decrease in intraburst spike frequency (Fig. 1 B).

Intracellular recordings were obtained from all five large motor neurones. Penetrations were signalled by a drop in potential of 35–45 mV (mean = 40·5). The burst recorded intracellularly from a cell body is characterized by an underlying depolarizing phase with superimposed rapid deflexions attributable to synaptic input and to electrotonically decremented action potentials (Fig. 2). In almost all of our experiments, the burst was followed by a pronounced hyperpolarization and then by a slow depolarizing (pacemaker) potential. The pacemaker potential was usually terminated abruptly by synaptic potentials, in a manner typical of 'follower' cells in other ganglia (Bullock & Terzuolo, 1957).

Application of XPO (0·1 PO/ml) prolonged (average increase and standard error, ± 18%) the depolarizing phase of the burst (Fig. 2) and increased the number of

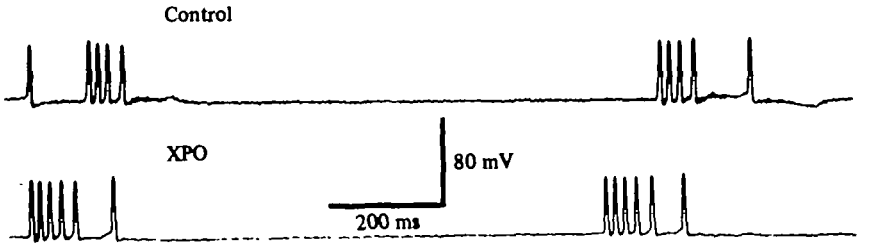

Fig. 3. Bursts recorded intracellularly from a region between the soma and site of spike initiation, showing large action potentials, and little of the underlying slow potentials. XPO increases in the frequency of bursting and number of spikes per burst in single large cells (in this experiment from $4{\cdot}9 \pm 0{\cdot}1$ spikes per burst in the 30 s period preceding XPO to $5{\cdot}8 \pm 0{\cdot}2$ during the peak 30 s of the response). The increase in burst duration recorded from this particular cell (119 ± 9 ms to 133 ± 9 ms) is not statistically significant.

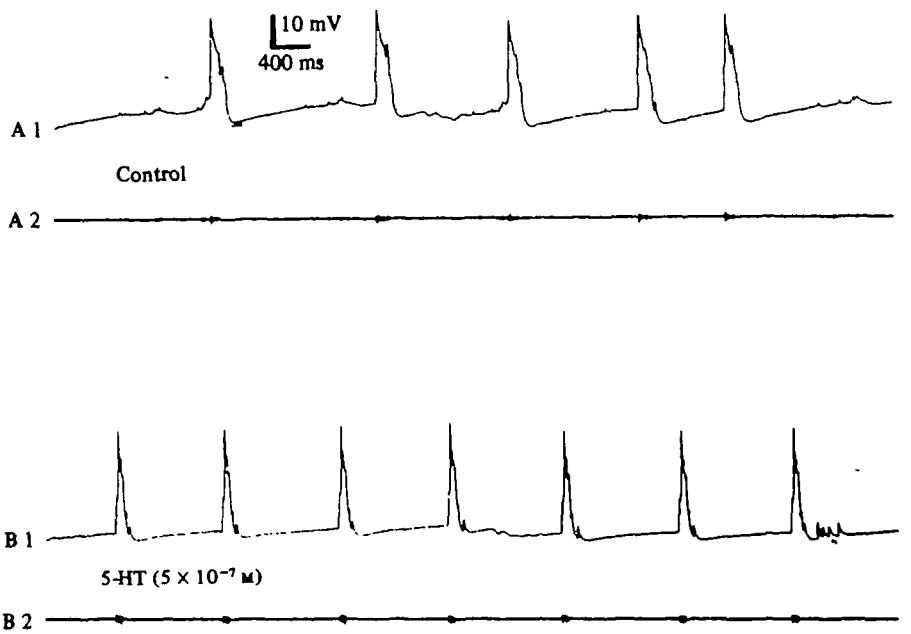

Fig. 4. Intracellular recordings (A_1, B_1) from a large cell and extracellular traces from the ganglionic trunk (A_2, B_2) before and during perfusion of the ganglion with 5-HT (5×10^{-7} M). Burst frequency is increased, burst duration is significantly decreased, and amplitude of the post-burst hyperpolarization is decreased by 5-HT.

spikes in single large cells (Fig. 3) and in the ganglion as a whole (Fig. 1 B) ($+75 \pm 14\%$). The post-burst hyperpolarization became more pronounced($+46 \pm 4\%$) and in most cases the slope of the pacemaker potential greater.

5-HT increased burst frequency at concentrations as low as 10^{-8} M. However, 5-HT, unlike XPO, decreased burst duration (Figs. 1 D, 4) and the number of spikes per burst (5×10^{-5} M 5-HT decreased the average number of spikes per burst by $33 \pm 8\%$) in almost all trials. In soma recordings, the amplitude of the post-burst hyperpolarization decreased during 5-HT treatment, and the slope of the pacemaker potential correspondingly decreased.

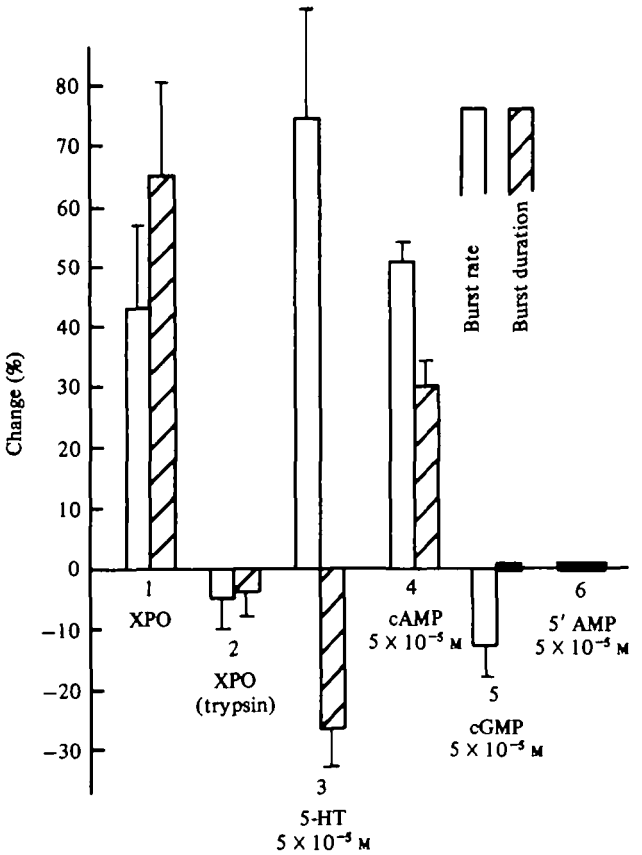

Fig. 5. Summary of effects of neurohumours and mononucleotides on burst frequency (clear columns) and burst duration (hatched columns). XPO and 5-HT both increase burst frequency, but 5-HT decreases duration. Trypsinized PO extracts are without significant effect. cAMP increases burst frequency and duration in a fashion similar to XPO. Control treatment with 5' AMP is without effect on the ganglion, while cGMP slightly decreases burst frequency. Bars indicate standard errors of the mean.

Because 5-HT can be released into haemolymph from electrically stimulated POs (Sullivan, 1978) at levels comparable to effective concentrations for cardioexcitatory effects, it was necessary to eliminate the possibility that the monoamine, rather than a peptide, might be the active factor in PO extract. *Carcinus* XPO was therefore treated with trypsin. Treated extract loses its excitatory effects as compared to untrypsinized controls (Fig. 5). Enzyme-digested extracts retain their ability to promote fluid secretion by insect Malpighian tubules, an effect probably attributable to 5-HT. This sensitive bioassay indicates that each *Carcinus* PO contains about 80 pmol of 5-HT (A. Berlind, unpublished). The effective concentration of 5-HT after dilution of the extracts (about 4nM) would therefore have been below threshold for cardiac ganglion activation.

The effective concentrations of two other cardioexcitors found in PO, octopamine and dopamine, also appear to be too low to affect the ganglion *in vitro* and *in situ*. Octopamine accelerates bursting rate of isolated ganglia only in concentrations of 10

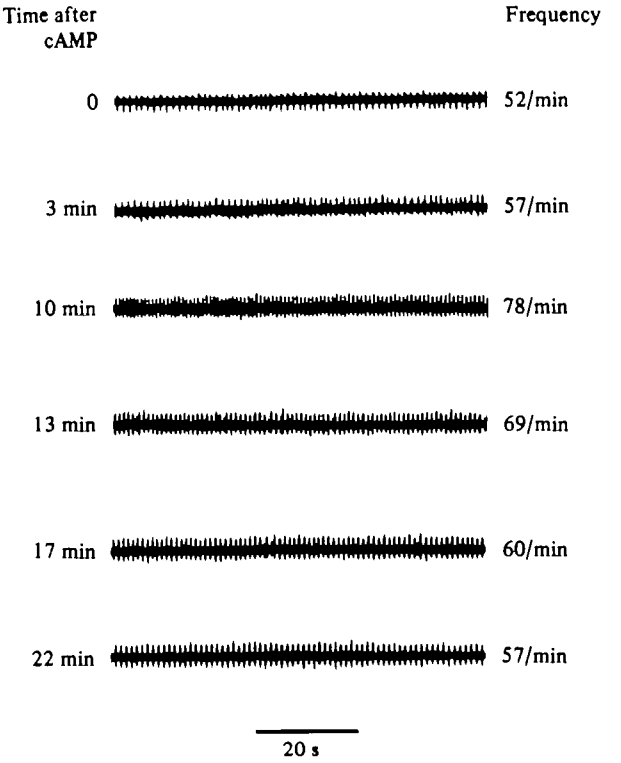

Fig. 6. Extracellular records, at slow chart speed, showing the time course of effects of cAMP (10^{-4} M) on burst frequency. The latency to onset of action varied greatly in different ganglia and for different agents; responses to dibcAMP occurred more rapidly than to cAMP when tested on the same preparation.

μM or higher, a level four to five orders of magnitude higher than what would be expected in our extracts (B. Battelle and E. A. Kravitz, personal communication). Dopamine concentrations in XPO have not yet been determined, but this monoamine is known to be readily oxidised under conditions of extract preparation.

Intracellular messenger for neurohormone

Both monoamines and peptides are believed to exert their effects primarily via interaction with molecular membrane receptors. The peptide (or peptides) and monoamines from pericardial organs apparently act via different receptors (Cooke, 1966) and have distinct effects on the bursting activity of the cardiac ganglion. The mechanism by which the receptors are linked to changes in cellular activity are presumably also different. Because cyclic nucleotides have been implicated as secondary intracellular messengers in many actions of both peptides and monoamines, we attempted to determine whether these substances are involved in the modulation of rhythmic output of the ganglion by either or both of the neurohormones.

(*a*) *Exogenous cAMP.* Applications of adenosine 3′,5′ monophosphate (cAMP) to the intact, isolated cardiac ganglion resulted in an increase in the burst rate (Fig. 6). Cyclic AMP was effective at levels as low as 10^{-6} M. The dose–response curve relating percentage increase in burst frequency to logarithm of the concentration of cAMP is shown in Fig. 7. N^6,O^2-dibutyryl cAMP (dibcAMP) had cardioexcitatory effects a

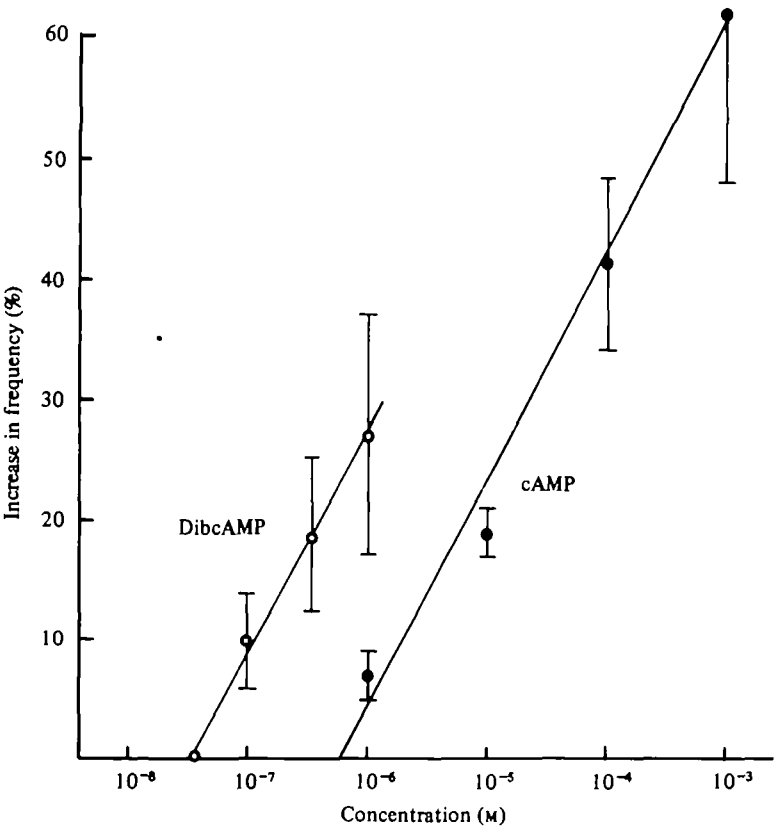

Fig. 7. Dose–response curves showing the effects of externally applied cAMP and dibcAMP on burst frequency. The less polar analogue is about tenfold more potent at low doses. Each point represents the mean response from at least six experiments, on different preparations (bars = $\pm 1 \times$ S.E.M.).

lower concentrations than cAMP. This less polar derivative exhibits a dose–response curve of the similar slope to that of cAMP. Its greater effectiveness was probably due to its superiority in passing through the plasma membrane and its greater resistance to hydrolysis by phosphodiesterases (Posternak, Sutherland & Henion, 1962). At higher doses, dibcAMP had inconsistent effects (not illustrated), probably attributable to the fact that the butyryl moiety seems to have effects of its own on ganglionic activity. At high levels (10–1000 μM) sodium butyrate decreases the frequency of bursting.

The effects of exogenously applied cAMP or its analogue on burst pattern were more similar to those of XPO than to those of 5-HT. DibcAMP increased burst duration and the number of spikes per burst and decreased intraburst spike frequency (Figs. 1 C, 5). Small and large cell spiking activity was altered similarly. In intracellular records, the depolarization of the soma was prolonged, and the post-burst hyperpolarization deeper, effects which are also seen in response to XPO. Guanosine 3′:5′ monophosphate (cGMP) (5×10^{-5} M) in contrast, decreased the burst rate

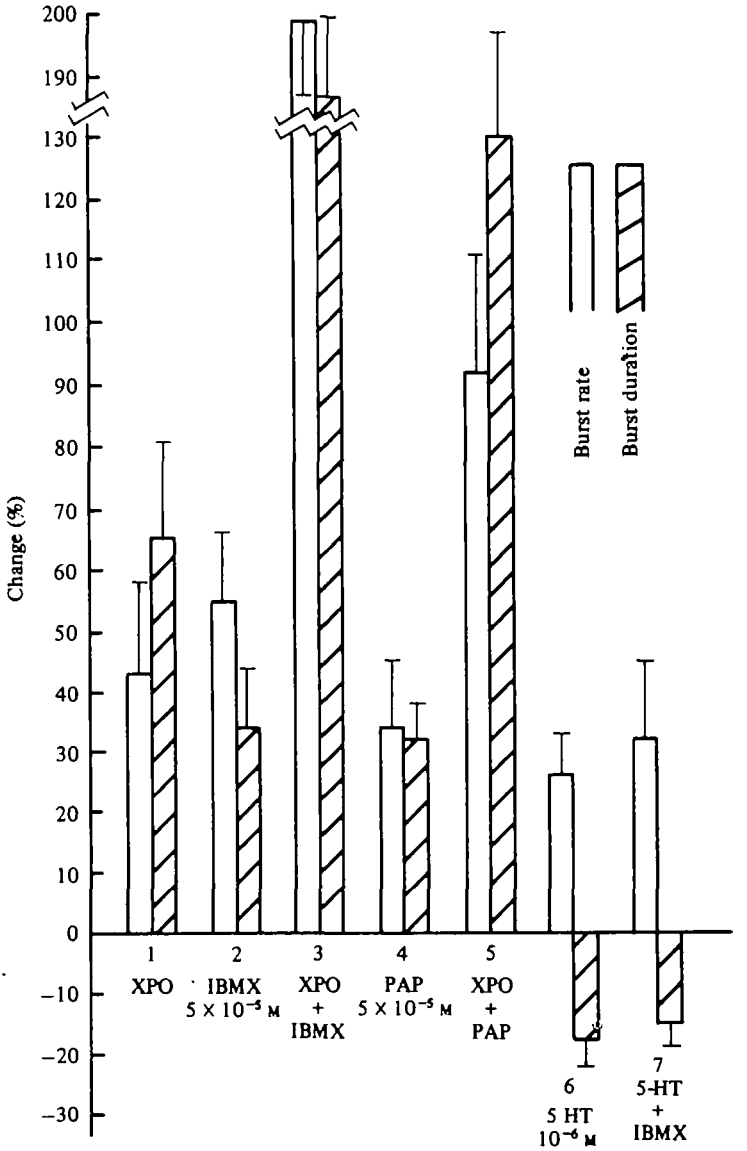

Fig. 8. Summary of effects of phosphodiesterase (PDE) inhibitors on burst frequency and duration. Perfusion of isobutylmethylxanthine (IBMX) (2) or papaverine (4) mimics the effects of XPO or cAMP application, increasing burst frequency and duration. Both PDE inhibitors strongly potentiate the effects of XPO (in columns 3 and 5 the effects of XPO are calculated as a percentage change from the new baseline following PDE blocker effects). The effects of 5-HT are not enhanced by IBMX (6, 7) or papaverine (not shown). Bars represent ±1 × S.E.M.

slightly and had little effect on burst duration (Fig. 5). Control applications of 5′AMP, the product of cAMP hydrolysis by phosphodiesterase, did not alter ganglionic activity at similar levels (Fig. 5).

(*b*) *Phosphodiesterase inhibitors.* Since intracellular phosphodiesterase (PDE) hydrolyses cAMP to a physiologically inactive product, inhibition of this enzyme

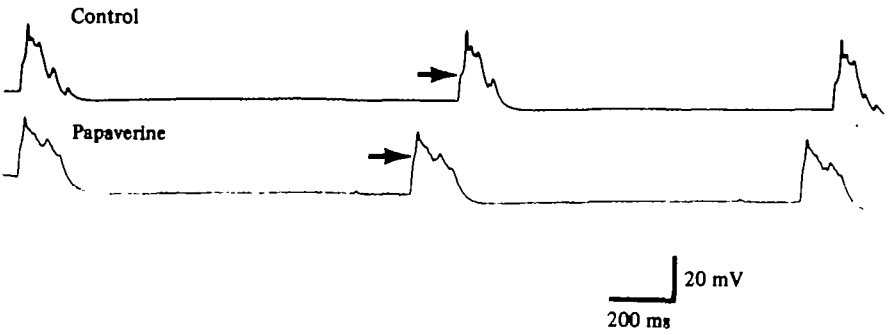

Fig. 9. Bursts recorded intracellularly from a large cell before (top) and after exposure to the phosphodiesterase inhibitor papaverine (5×10^{-5} M). Burst frequency and duration are markedly increased. The amplitude of the earliest component of the complex, depolarizing waveform (arrows) is clearly increased, by a factor of about 1·5 (see Discussion).

should result in an increase in endogenous intracellular levels of cAMP. Different classes of inhibitors were perfused over the ganglion to determine whether the expected resultant changes in cAMP can alter the rhythmic activity of ganglia without exposure to neurohormones and whether the response to XPO or 5-HT is altered.

The methylxanthines aminophylline, theophylline and isobutylmethyxanthine (IBMX) were all effective in increasing burst rate, duration, and post-burst hyperpolarization (Fig. 8), thus mimicking the effects of XPO. IBMX was especially potent, even at micromolar concentrations. Because the methylxanthines are known to have many physiological actions on other tissues unrelated to PDE inhibition (for example, a calcium-releasing effect from intracellular storage sites (Katz, Taka & Kirschberger, 1975)), a chemically unrelated PDE blocker, papaverine, was also tested. It had effects on cardiac ganglion similar to those of the methylxanthines (Figs. 8, 9).

PDE inhibitors also appear to be effective in potentiating the excitatory effects exerted by XPO on ganglion output. The excitatory effects of XPO in the presence of IBMX, aminophylline or papaverine were greater than can be accounted for by a simple additive effect of the separate actions of neurohormone and PDE inhibitor (Fig. 8). In contrast, the effects of 5-HT were not appreciably altered by exposure to PDE inhibitors.

(*c*) *Adenylate cyclase inhibitors.* The results described above implicate cAMP as the probable secondary messenger mediating peptide cardioexcitor effects on the ganglion. If the machinery which leads to a rise in intracellular cAMP were blocked, any effects which could be exerted only by this machinery should also be blocked. To test the hypothesis that cAMP formation is a necessary step in XPO action, we applied a number of purported adenylate cyclase blockers to the ganglion. Since the blockers we have used might individually have direct effects on cellular functions other than cyclase activity, we have employed several agents thought to interact with the cyclase by different mechanisms.

Adenosine, which blocks the synaptically induced elevation of cAMP levels in vertebrate brain (Fain, Pointer & Ward, 1972) slightly decreased burst rate in the cardiac ganglion, but only at very high (10 mM) concentrations. Alloxan, an adenylate cyclase inhibitor in the vertebrate nervous system (Cohen & Bitensky, 1969), also

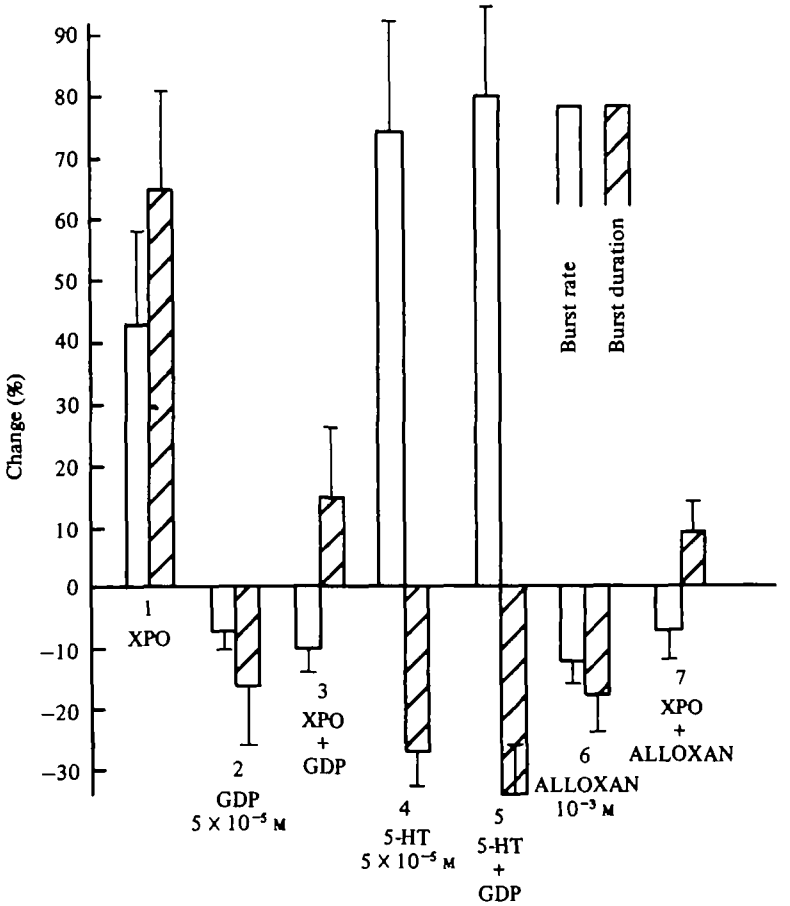

Fig. 10. Effects of putative adenylate cyclase blockers. GDP (2) and alloxan (6) both decrease burst rate and duration slightly. Applications of XPO are much reduced in effectiveness in the presence of either blocker (3, 7) while the effects of 5-HT are unaltered (5). Bars represent ±1 × S.E.M.

decreased the burst rate at lower concentrations (0·1 mM) but over long durations gave inconsistent results. M. W. Bitensky (personal communication) found similar inconsistencies over long durations. Both alloxan (Fig. 10) and adenosine effectively blocked the excitatory effects of XPO on electrical activity of the ganglion.

Guanosine triphosphate (GTP) is necessary for the interaction between hormone receptor and adenylate cyclase in other systems (Rodbell *et al.* 1974). Guanosine diphosphate (GDP) can compete for the GTP binding site in some systems, but does not stabilize the receptor-cyclase interaction and can therefore block activation of the enzyme (Rodbell *et al.* 1974; Eckstein *et al.* 1979). GDP applied extracellularly was effective in lowering the endogenous rate of bursting of the ganglion at relatively low concentrations (5×10^{-6} M). More importantly, GDP was able to block the increase of frequency normally seen in response to XPO, and to dampen the effects on burst duration (Fig. 10). 5-HT effects, in contrast, were only slightly altered by GDP.

Table 1. *Levels of cAMP in cardiac ganglion and heart muscle*

(Radioimmunoassay)

Tissue	Treatment	cAMP 10^{-13} M/mg protein‡	Control (%)
Cardiac ganglion (5)†	Control	$8{\cdot}3 \pm 1{\cdot}1$	100
Cardiac ganglion (3)	XPO (0·1/ml)	$44{\cdot}4 \pm 6{\cdot}1$**	535
Cardiac ganglion (4)	5-HT (10^{-6} M)	$17{\cdot}8 \pm 4{\cdot}5$*	214
Cardiac muscle (14)	Control	$3{\cdot}7 \pm 0{\cdot}2$	100
Cardiac muscle (2)	5-HT (10^{-6} M)	$16{\cdot}4 \pm 7{\cdot}0$**	440

* Differs significantly from untreated control ganglia ($P < 0{\cdot}05$).
** Differs significantly from untreated control ganglia or muscle ($P < 0{\cdot}005$).
† Number of measurements in parentheses.
‡ Mean ± standard error.

(*d*) *Direct measurement of cAMP levels.* If cAMP is an intracellular messenger which mediates the effects of a PO neurohormone on ganglion activity, levels of the cyclic nucleotide should be increased by application of the primary messenger. Radioimmunoassay (RIA) results from individually extracted ganglia provided evidence for a significant rise in cAMP following exposure to XPO.

Cyclic AMP levels in untreated ganglia were about twice as high as in cardiac muscle (Table 1). Treatment with XPO elevated cAMP content more than fivefold in the ganglion. In contrast, 10^{-6} 5-HT, which increased the cAMP of innervated heart muscle by a factor of 4·4, only induced a twofold increase in ganglionic content. (Our values for untreated heart muscle, and muscle exposed to 5-HT are comparable to figures published previously by Battelle & Kravitz (1978).)

Results from several additional experiments involving alteration of cAMP levels are not included in the table, either because they consisted of only single data points, or because they were performed with a protein binding assay, rather than RIA. The results tend to support the conclusion that cAMP mediates XPO effects, and are noted briefly here, pending further confirmation by RIA:

(1) A single ganglion was frozen and assayed after exposure to trypsin-digested XPO, which had no effect on bursting; cAMP content, as measured by RIA, was 124% of the average value of untreated controls, a very much lower level than measured in any ganglion which responded to undigested XPO.

(2) Treatment with alloxan (10^{-3} M) which, as noted above, blocked the physiological response to XPO, also blocked the rise in cAMP normally observed in ganglia following XPO treatment (single experiment, protein binding assay, 95% of average value of untreated controls). Effects of GDP and adenosine on cAMP levels have not yet been measured and our use of and conclusions with regard to the actions of these agents are therefore based on their proposed effects on other systems.

(3) The methylxanthine inhibitors of phosphodiesterase, aminophylline and IBMX, which alter physiological function in a manner similar to XPO, raised cAMP levels in both ganglion and muscle to levels between 230% and 270% of untreated control (one experiment with each inhibitor).

(4) Treatment of heart with XPO increased cAMP levels in muscle to 310% of controls (three experiments, using protein binding assay). Protein binding assays of AMP in the ganglion showed a rise after XPO treatment almost identical to that detected by RIA in the same tissue.

Conductance changes in large cell membrane. Short hyperpolarizing current pulses were passed across the membrane of a large cell to monitor changes in membrane resistance during the course of the burst cycle, and following exposure of the ganglion to PO neurohormones or agents which alter cAMP metabolism. The brief currents used were short relative to the time constant of the membrane, and the geometry of the cells was undefined, so no absolute values can be given for membrane resistance. Changes in effectiveness of the current pulses in altering membrane voltage were, however, noted, and provided preliminary evidence for opposite effects of 5-HT and XPO on membrane conductance.

The voltage change due to constant current pulses is larger late in the interburst period than at the peak of the post-burst hyperpolarization, indicating that the pacemaker potential is accompanied by, and possibly caused by, a declining membrane conductance. This finding agrees with the work of others, who have attributed the pacemaker potential to a slowly decreasing K^+ conductance (Connor, 1969; Tazaki & Cooke, 1979*b*). Treatment of the ganglion with XPO, PDE inhibitors and cAMP has similar effects on conductance of the large cell membrane: in almost all experiments in which these agents induced an increase in burst frequency (five of six with XPO, and all eight experiments with papaverine or IBMX), the membrane conductance appeared to be lower at all stages of the interburst than before treatment, and increased again as the burst rate declined after washout of the effector. In contrast, 5-HT treatment, which accelerated and shortened the bursts, and caused a decrease in post-burst hyperpolarization, in three of five cases was associated with an increase of membrane conductance at all phases of the interburst.

DISCUSSION

We derive two major conclusions from our results with regard to the role of cAMP in the cardiac ganglion.

(1) The cyclic mononucleotide seems to play a critical role in the mediation of peptide effects on the ganglion, but is involved to a much smaller degree, or not at all, in 5-HT action.

(2) The maintenance of normal bursting characteristics in ganglia not exposed to neurohumours is to some degree influenced by endogenous levels of the cyclic nucleotide.

Four lines of evidence support the conclusion that cAMP mediates the actions of XPO.

(1) Extracellular application of low concentrations of cAMP or its analogues alters burst discharge characteristics in a manner similar to XPO.

(2) Raising intracellular levels of cAMP by applying phosphodiesterase inhibitors alters discharge characteristics in a manner similar to XPO, and potentiates the effects of externally applied XPO.

(3) Application of agents which reportedly prevent the activation of adenylate cyclase alter the ongoing discharge of the ganglion and block the excitatory effects of XPO.

(4) Application of XPO causes a significant rise in cAMP in the ganglion.

We have thus provided evidence fulfilling several, but not all, of the criteria fo

establishing cAMP as a mediator of physiological actions (Greengard, 1976). We have not, for example, attempted to find or characterize biochemically a peptide-sensitive adenylate cyclase or cAMP phosphodiesterase in cardiac ganglion, nor have we succeeded conclusively in localizing the site of cAMP formation within the ganglion. Preliminary attempts using immunofluorescense localization of cAMP suggests that the rise occurs in neuronal elements of the ganglion, rather than in associated tissues, but is probably not localized to a significant degree in the neuronal somata (J. R. Lemos, unpublished).

Our tentative conclusion that 5-HT affects the ganglion primarily by mechanisms not involving cAMP is based on results showing that the electrophysiological effects of the monoamine differ substantially from those of cAMP, XPO or PDE inhibitors, and that 5-HT action is not potentiated by PDE inhibitors or blocked by putative inhibitors of adenylate cyclase. 5-HT application does, however, significantly elevate levels of cAMP in our extracts, although to a much smaller degree than does XPO. It is possible that this increase is in part due to the response not of nervous tissue but of small bits of adhering muscle which were impossible to eliminate completely from our preparation; cAMP levels in muscle tissue show a much greater relative response to 5-HT than does the ganglion. We cannot, however, from our experiments conclusively rule out the possibility that 5-HT exerts some of its effects via cAMP. It is possible, for instance, that distinct pools of cAMP are present within discrete compartments (Menon & Azhar, 1978; Podesta *et al.* 1978) in ganglionic neurones. The pools might be differently affected by our pharmacological agents, or two different cAMP-dependent protein kinases in discrete compartments might be altered by different levels of cyclic nucleotide. In view of the numerous systems in which 5-HT is known to interact with cAMP, our tentative negative conclusions should therefore be regarded with caution.

Our conclusion that peptide cardioexcitor and 5-HT act via different cellular mechanisms is consistent with previous work which differentiates the effects of the two neurohumours on isolated ganglia or isolated, internally perfused hearts. It is known that different receptor mechanisms are involved in the ganglion, since a response to peptide persists after pharmacological blockade of 5-HT receptors (Cooke, 1966; Berlind, Cooke & Goldstone, 1970). When tested on isolated hearts, XPO has a steeper dose-response curve than does 5-HT (Maynard & Welsh, 1959). Finally, localized application of XPO and 5-HT to different regions of isolated ganglia indicates that the details of whole ganglionic and individual cell responses are distinctly different depending on the site of application and nature of the stimulating agent (Cooke & Hartline, 1975). 5-HT, for example, alters burst rate and duration in the whole ganglion when applied either to small cell or large cell regions, while XPO is effective in altering burst rate only if small cell firing is changed. From our experiments with cAMP that did not involve localized applications, it appears likely that both small and large cells are being affected directly (see below), but we are unable to conclude that the same properties of the two classes of cells are being altered.

The observed changes in response to XPO (burst rate increase, duration increase) and 5-HT (rate increase, duration decrease) confirm previous observations (Cooke, 1966) and are similar to those observed in single neurones following local application of the agents to the ganglion (Cooke & Hartline, 1975). The physiological significance

of these differential effects is not easy to assess from experiments on isolated ganglia. While the decrease in burst duration and lowered number of spikes per burst in large cells (i.e. motor neurones) responding to 5-HT might be thought to correspond to a decreased stroke-volume in intact hearts, isolated internally perfused hearts normally respond to both XPO and 5-HT with increases of both frequency and amplitude of contraction. The difference between isolated ganglia and intact isolated hearts might in part be due to direct effects of 5-HT on muscle (Battelle & Kravitz, 1978) and to mechanosensory feedback to the ganglion. The details of effects of 5-HT and peptide on the hearts in intact animals are totally unknown; it is likely that responses to circulating hormones may be extensively modified by sensory information or other controlling factors. It is noteworthy, however, that under some conditions, physiological increases in heart rate (excitatory pathway undetermined) are thought to be accompanied by a decrease in stroke volume (Florey & Kriebel, 1974).

The variability in details of response of different ganglia to XPO has been noted previously. Part of the variability might be due to differences in the content of extracts. Since it is likely that more than one cardioactive peptide is present in extracts of a single organism (Belamarich & Terwilliger, 1966; Sullivan, 1979), proportions of the various factors, with different detailed effects, might differ from preparation to preparation. Regular differences were observed in the ganglionic responses to lobster and crab XPO, the latter generally inducing much more pronounced changes in burst duration than in frequency. It is likely, however, that differences in ganglionic responsiveness in part account for the variability of effects, since the same preparation of XPO may affect two ganglia in different ways, as can cAMP and pharmacological agents which alter cAMP metabolism. The availability of purified peptides should help to solve this problem.

Experiments in progress are designed to analyse in greater detail the nature of membrane level changes which are most directly induced by XPO, 5-HT and cAMP. Cooke & Hartline (1975) have suggested that a peptide may exert its most important effects on the generation of 'driver potentials' (slow depolarizing potentials based on voltage-dependent Ca^{2+} influx (Tazaki & Cooke, 1979*a*, *b*) which are triggered by synaptic input and underlie the spike train), while 5-HT may induce a general depolarization at, or near, the spike-generating site. Our observations are certainly consistent with these ideas, but do not rule out numerous other possible modes of actions, including direct effects on the efficiency of synaptic transmission within the ganglion or on conductances related to pacemaker activity.

XPO and cAMP regularly increase the duration of the intracellularly recorded slow depolarization underlying each burst in large cells, perhaps indicating an enhanced and prolonged driver-potential Ca^{2+}-influx. More detailed analysis will be required to determine whether the change in waveform represents primarily an alteration of endogenous characteristics as opposed to enhanced synaptic drive (e.g. Hartline & Cooke, 1969; Freisen, 1975). A direct effect on driver potential mechanisms might also be indicated by the ability of XPO to promote bursting in ganglia where it has been suppressed by ionic alteration or other means, or where it has failed during the course of an experiment (Cooke & Hartline, 1975). We have recently shown, for example, that bursting can be initiated by the application of XPO to totally quiescent ganglia in which neurones have been depolarized by treatment with

ouabain, an active transport inhibitor (K. O. Jelly and A. Berlind, unpublished). It is of interest with regard to this hypothesis that slow voltage-dependent conductances in molluscs, similar in some aspects to crustacean driver potentials, appear to be the direct target of endogenous and exogenous peptide neurohormones and of cAMP (Barker, 1977; Levitan, 1979). The calcium dependency of the crustacean driver potentials also makes this question an attractive one for further investigation, in view of the extensive interactions between cyclic mononucleotide and Ca^{2+} metabolism in a variety of neuronal and non-neuronal systems (Berridge, 1975; Rasmussen & Goodman, 1977).

The changes induced by XPO and cAMP typically included an increased amplitude of the post-burst hyperpolarization recorded from large cells. This phase of cellular activity is reportedly dependent more on the amplitude and nature of the preceding slow depolarizing wave than on spike activity of the neurone (Matsui & Shibuya, 1958) and is possibly caused by an increase in Ca^{2+}-controlled potassium conductance (Tazaki & Cooke, 1979*b*). Our results would therefore be consistent with an indirect effect due to enhanced Ca^{2+} influx during the driver potential. Such an effect would not necessarily explain the increased slope of the pacemaker potential during the interburst period that was observed in most of our experiments with XPO and cAMP. Our data also suggest that the input resistance of large cells is higher at all phases of the interburst period following exposure to XPO or treatments that raise cAMP levels than at the corresponding phases of untreated ganglia. The sites and nature of this alteration are unknown, but are indicative of a more extensive membrane effect than would be expected from a direct action restricted to the driver potential mechanism. We should emphasize that our intracellular observations are confined to the large cells, which are in most cases not serving as the overall pacemakers for the ganglion. If similar changes in input resistance, pacemaker slope, and endogenous slow potentials are occurring in small cells, the effects of XPO and cAMP might be largely explained, without the necessity of postulating synaptic effects (see below).

The effects of 5-HT on burst rate and duration are mimicked by several treatments which are known or expected to depolarize the membranes of the constituent neurones. Burst rate increases are accompanied by a decrease in duration and spike number when ganglia are stimulated via the cardioexcitatory neurones from the central nervous system (Terzuolo & Bullock, 1958) and when isolated ganglia are slightly depolarized by direct current passing into single neurones, by raising the external potassium concentration (Matsui, Kuwasawa & Kuramato, 1977), or by treatment with ouabain (Livengood & Kusano, 1972). In our experiments with 5-HT, we have not consistently observed baseline depolarization (although the potential at the peak of the post-burst hyperpolarization was consistently less negative). It is likely, however, that if slight depolarization occurs at critical sites distant from the soma, where 5-HT is known to be most effective (Cooke & Hartline, 1975), the change might be undiscernible at our recording site due to attenuation of the signal. If the primary effect of 5-HT is in fact to cause a general depolarizing current (perhaps resulting from a conductance increase), there are likely to be important secondary consequences of this change on burst pattern. For instance, sustained depolarization of crab large cells depresses the amplitude and duration of repeatedly evoked driver

potentials (Tazaki & Cooke, 1979*a*), which might be expected, in turn, to depress the duration and spike number in evoked burst trains.

We cannot, on the basis of our data, evaluate the possibility that XPO, cAMP or other agents might have direct influence on the efficacy of synaptic transmission. In some experiments XPO, cAMP, and PDE inhibitors clearly increased the amplitude of the earliest discrete component of the complex depolarization in large cells (e.g. Fig. 9). It is likely, although not certain, that this component represents a synaptic potential rather than a decremented spike. Even if it does represent a synaptic potential, we cannot be certain whether the observed change represents enhanced neurotransmitter output, greater receptor sensitivity of the post synaptic membrane, an intracellular biochemical change which alters the sequelae of transmitter action, a change in the identity of the specific small cell giving rise to the potential, or whether the change merely reflects in increased input resistance of the postsynaptic membrane. Experiments to discriminate rigorously between these possibilities are currently in progress.

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