J. exp. Biol. (1979), 81, 131–151
With 16 figures
Printed in Great Britain

SYNAPTIC AND HORMONAL MODULATION OF A NEURONAL OSCILLATOR: A SEARCH FOR MOLECULAR MECHANISMS

BY IRWIN B. LEVITAN, ANTHONY J. HARMAR* AND WILLIAM B. ADAMS

Friedrich Miescher-Institut, P.O. Box 273, CH-4002 Basel, Switzerland

SUMMARY

1. The central ganglia of a number of gastropod molluscs (including the marine snail *Aplysia californica* and the terrestrial snail *Helix pomatia*) contain neurones which exhibit endogenous patterns of oscillatory activity.

2. This oscillatory activity can be modulated for long periods of time by synaptic and hormonal stimulation.

3. Stimulation of appropriate pre-synaptic nerves causes long-lasting hyperpolarization in these neurones, with complete abolition of oscillatory activity. This synaptic response is mediated by an increase in K^+ conductance, together with a decrease in inward (Na^+/Ca^{2+}) conductance. The ionic conductances affected by synaptic stimulation are those responsible for producing the rhythmic oscillations.

4. The oscillatory activity can also be modulated by the vertebrate neurohypophyseal peptides, vasopressin and oxytocin, and by an endogenous peptide-containing extract of molluscan ganglia. In contrast to synaptic stimulation, these agents cause an increase in oscillatory activity.

5. The endogenous molluscan factor which produces an increase in oscillatory activity can be purified by affinity chromatography on bovine neurophysin linked to Sepharose. This indicates that the molluscan nervous system may contain a neurohypophyseal-like peptide.

6. Oscillatory activity can be modulated by manipulation of cyclic nucleotide metabolism in these neurones. Increases in cAMP alone are associated with *abolition* of oscillatory activity; this mimics long-lasting synaptic hyperpolarization. Increases in cAMP and cGMP together are associated with an *increase* in oscillatory activity and mimic the effects of the vertebrate and molluscan peptides. Thus, it is possible that cyclic nucleotides play a role in these physiological responses.

INTRODUCTION

In the last two decades the use of gastropod molluscs for neurobiological studies has become widespread. Such animals possess relatively simple central nervous systems, consisting of several ganglia communicating with each other and with the periphery by means of long connective nerves. Within each ganglion there is a complex central neuropile region containing neuronal processes, glial cells, and synaptic contacts,

* Present address: Department of Pharmacology, University of Bristol, Medical School, Bristol BS8 1TD, England.

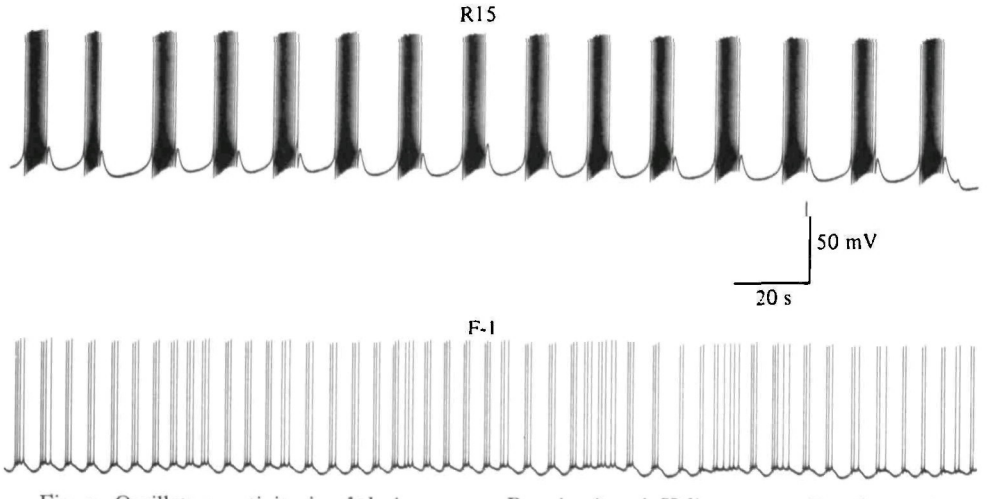

Fig. 1. Oscillatory activity in *Aplysia* neurone R15 (top) and *Helix* neurone F-1 (bottom). *Aplysia* abdominal ganglion and *Helix* circumoesophagel ring were pinned to Sylgard, and the endogenous electrical activity in neurones R15 and F-1, respectively, was recorded with intracellular microelectrodes. In general, as is evident from these tracings, R15 tends to burst more regularly and more vigorously than F-1. However, wide variations in bursting vigor from animal to animal are seen in both species. In some specimens F-1 may exhibit beating rather than bursting, or be completely silent. Such patterns are rarely seen in R15.

whereas the neuronal cell bodies are arranged around the periphery and are easily accessible to the experimenter. These cell bodies are often very large (in some cases as much as 0·5–1 mm in diameter) and many can be identified reliably from one animal to the next on the basis of their specific topographical, morphological, chemical, and electrical characteristics. In such widely studied gastropod species as the ophistobranch marine snail *Aplysia californica*, and the pulmonate terrestrial snails *Helix pomatia*, *Helix aspersa* and *Otala lactea*, the characteristics of many individual neurones and of synaptic connections between them have been thoroughly documented (Frazier *et al.* 1967; Gainer, 1972; Kerkut *et al.* 1975). Thus, the gastropod nervous system is the tissue of choice for many electrophysiological, pharmacological, and biochemical studies of synaptic transmission and other aspects of neuronal function.

The central ganglia of *Helix*, *Otala* and *Aplysia* contain neurones which exhibit similar patterns of endogenous electrical activity, in which a regularly oscillating membrane potential gives rise to alternating periods of action potential bursts and interburst hyperpolarizations (Fig. 1). The depolarizing phase of the oscillation is due to the presence of a voltage and time-dependent inward current (see Meech, p. 93). which in *Aplysia* neurone R15 may be carried largely by Na^+ ions (Smith, Barker & Gainer, 1975), and in *Helix* neurone F-1 largely by Ca^{2+} ions (Meech, p. 93; Eckert & Lux, 1976). The hyperpolarizing phase results from inactivation of the inward current, together with activation of an ion-, time-, and voltage-dependent outward current carried by K^+ ions (Meech, p. 93; Heyer & Lux, 1976). This endogenous oscillatory behaviour can be altered by physiological and pharmacological treatments, including stimulation of appropriate presynaptic nerves, and application of hormones or neutrotransmitters. For example, stimulation of the right connective nerve of *Aplysia* at low stimulus intensities produces a fast excitatory synaptic potential

(EPSP) in R15, leading to a net increase in the activity of the cell (Parnas & Strumwasser, 1974; Schlapfer *et al.* 1974). Stimulation of the branchial nerve, or of the right connective at higher stimulus intensities, produces a complex postsynaptic response in R15, consisting of a fast EPSP followed by one or more inhibitory components (IPSP's). The predominant phase is the inhibitory (hyperpolarizing) one, which eliminates oscillatory activity and can last seconds, minutes or even hours, depending on the stimulus parameters (Parnas & Strumwasser, 1974). A similar long-lasting IPSP can be evoked in *Helix* neurone F-1 by stimulation of the right pallial nerve (Lambert, 1975).

The rhythmic activity of neurone F-1 (and the homologous neurone 11 in *Otala*) can also be modified by application of vertebrate neurohypophyseal peptides such as vasopressin and oxytocin (Barker & Gainer, 1974). At low concentrations these peptides increase the frequency and number of action potentials within each burst, and also cause a marked increase in the depth and duration of the interburst hyperpolarization. The net effect is a long-lasting stimulation of the activity of the cell, and an increase in oscillatory activity, in contrast to the effects of synaptic hyperpolarization. A similar response can be observed in these neurones following application of a crude peptide-containing extract obtained from molluscan ganglia (Ifshin, Gainer & Barker, 1975; Treistman & Levitan, 1976*a*; Levitan & Treistman, 1977*b*). That is, the molluscan nervous system itself contains a factor or factors which can alter the activity of these rhythmically oscillating neurones.

Because their endogenous oscillatory activity can be enhanced or inhibited for long periods (many seconds or minutes) by environmental manipulations, it seemed that these neurones might be particularly appropriate for studying molecular events involved in long-term modulation of oscillatory behaviour. Relatively long-lasting changes in neuronal function may be mediated by different intracellular events than the much shorter changes often associated with neurotransmitter action. In particular, rapidly reversible fluctuations in ionic conductances may reflect rapidly reversible conformational changes in membrane components which control ionic permeability. On the other hand, long-lasting conductance changes may require more stable metabolic modification of the appropriate membrane components.

One possible metabolic modification that could play such a role is cyclic nucleotide-mediated phosphorylation of membrane proteins which, directly or indirectly, control membrane permeability to various ions. It has been proposed that cyclic nucleotides may be involved in oscillatory behaviour in many systems (Rapp & Berridge, 1977), and Greengard (1976) has put forward a detailed scheme for cyclic nucleotide involvement in the nervous system in particular. Much of the early evidence in favour of this scheme is based on work with the mammalian superior cervical ganglion, in which McAfee & Greengard (1972) showed that dibutyryl cAMP could produce a long-lasting hyperpolarization similar to the IPSP resulting from pre-ganglionic stimulation. However, these results and their interpretation have been challenged by several authors (Gallagher & Schinnick-Gallagher, 1977; Dun & Karczmar, 1977), and the controversy remains unresolved. The other major evidence for a cyclic nucleotide involvement in mammalian nervous system is that of Bloom and his colleagues, who have concluded that cAMP may mediate the nor-adrenergic synaptic input from the locus coeruleus onto cerebellar Purkinje cells (for review see Bloom,

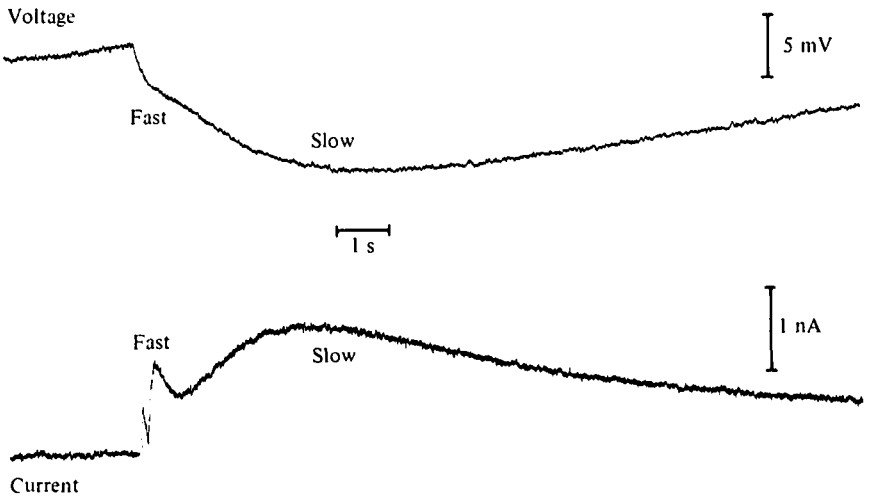

Fig. 2. Synaptic response recorded in neurone F-1 following a single pulse delivered to the right pallial nerve. The hyperpolarizing voltage response is shown at the top, and the outward current flow which gives rise to the hyperpolarization is shown at the bottom. For the current measurement, the neurone was voltage-clamped to the same potential used for measurement of the voltage response. Outward currents are taken as positive, inward currents as negative. Note that there are two distinct components to the synaptic response, one fast and one slow. Only the slow response is considered here. Taken from Adams & Levitan (submitted).

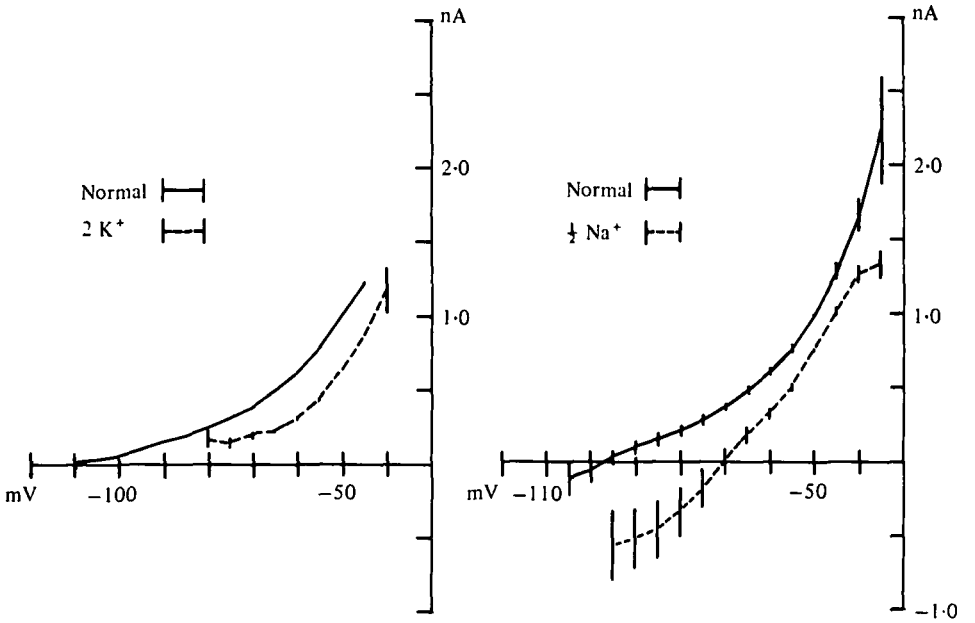

Fig. 3. Effect of ion changes on the amplitude of the slow synaptic current component in neurone F-1 at different membrane voltages. Left: doubling the K^+ concentration in the bathing medium causes a shift in voltage dependence or a decrease in the amplitude of the current. Right: halving the Na^+ concentration (lithium replacement) allows the current to reverse at approximately −70 mV. Current axes are normalized to allow results of several experiments to be combined. Vertical bars on curves represent ± S.E.M. Not shown: changing from (1/2 Na^+) medium (in which the current clearly reverses), to (1/2 Na^+ + 2 K^+) medium, shifts the current–voltage curve to the right, indicating that the changes in the response in 2K^+ medium (left) do indeed result from a shift in voltage dependence of the response. Thus, both Na^+ and K^+ ions play a role in this synaptic response. Taken from Adams & Levitan (submitted).

1975). In *Aplysia*, there are recent indications that cAMP may be involved in pre-synaptic facilitation of neurotransmitter release (Brunelli, Castellucci & Kandel, 1976; Shimahara & Tauc, 1977; Klein & Kandel, 1978). The present report describes some attempts to characterize both synaptic and hormonal modulation of the oscillatory activity in *Aplysia* neurone R15 and *Helix* neurone F-1, with respect to the ionic basis of the responses and the possible involvement of cyclic nucleotides. The methodology used for the biochemical measurements, and for intracellular recording of electrical activity, is standard and has been described in detail elsewhere (Levitan, Madsen & Barondes, 1974; Treistman & Levitan, 1976*a*, *b*; Levitan & Treistman, 1977*a*, *b*; Adams & Levitan, in preparation).

The slow conductances that mediate oscillatory behaviour are modulated by synaptic stimulation

The slow *membrane* conductances that mediate the oscillatory activity observed in some molluscan neurones are described in detail by Meech (p. 93). We will review them briefly here since we wish to compare them with the *synaptic* conductances mediating the long-lasting inhibition of bursting, elicited in R15 and F-1 by stimulation of appropriate pre-synaptic nerves. Molluscan neurones appear to have *slow* conductance pathways for sodium (g_{Na}), potassium (g_K) and calcium (g_{Ca}). These pathways can be distinguished from the *fast* conductances that produce action potentials (1) by having kinetics that are two to three orders of magnitude slower, and (2) by pharmacological means (the slow sodium channel is not blocked by tetrodotoxin; lithium can substitute for sodium in the fast channel but not the slow). During the depolarizing phase of oscillatory activity sodium and/or calcium conductance is high and potassium conductance is low, resulting in a net inward flow of current. During the hyperpolarizing phase the situation is reversed and current flows outward.

Long-lasting synaptic inhibition of oscillatory activity can be elicited in these cells by stimulation of appropriate pre-synaptic nerve trunks with single electrical pulses (Fig. 2). It appears that these responses are not monosynaptic, but the interneurones cannot be readily located (Frazier *et al.* 1967). The inhibitory response in *Helix* neurone F-1 consists of a fast (< 1 s) and a slow (1–10 s) phase (Fig. 2). The outward currents which give rise to the voltage response can be readily studied under voltage clamp (Fig. 2). Ion replacement experiments indicate that the slow inhibitory phase in F-1 is mediated in part by an *increase* in g_K and, in part, by a *decrease* in g_{Na} (Fig. 3). Since Na^+ tends to flow down its electrochemical gradient into the cell, reducing g_{Na} leads to a reduction of inward current and hyperpolarization. In *Helix* it appears that the decrease in g_{Na} predominates: reversal potentials for the slow synaptic response are rarely found in normal medium (Fig. 3), but reduction of Na^+ decreases the Na^+ component and gives reversal potentials at or near K^+ equilibrium (Fig. 3).

Stimulation of the pre-synaptic nerve with a train of pulses produces an inhibition in R15 that can last for periods of up to several hours (Parnas & Strumwasser, 1974). Ionic mechanisms similar to those in F-1 appear to mediate this response, but in R15 it is possible to separate the Na^+/Ca^{2+} and K^+ components temporally (Fig. 4*a*). Shortly after the stimulus train a reversal potential close to the presumed potassium equilibrium potential is found (Fig. 4*b*) indicating that an increase in g_K predominates. With increasing time the reversal potential shifts in the negative direction (Fig. 4*c*)

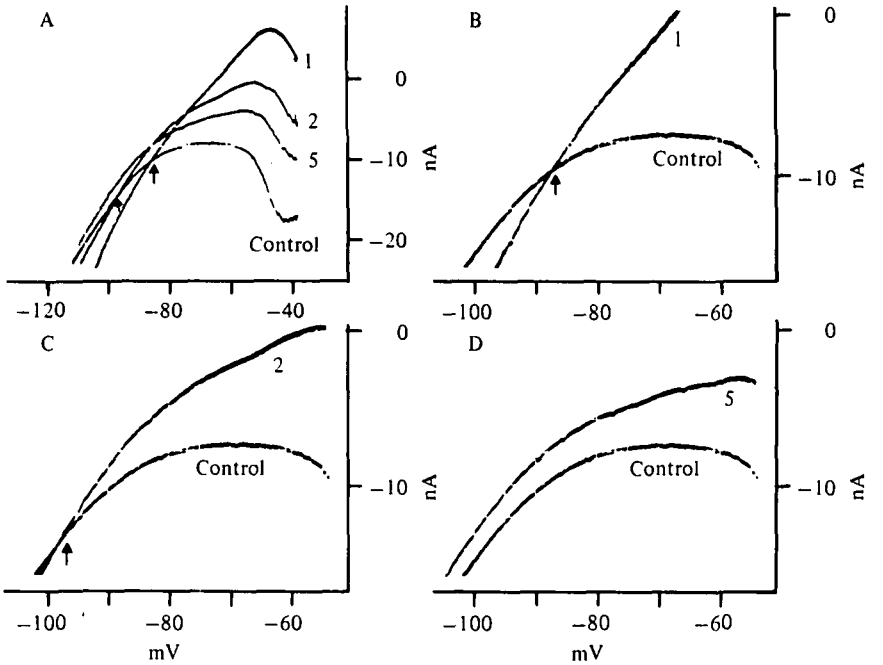

Fig. 4. Long-term changes in membrane current–voltage relationship in neurone R15 elicited by multiple stimuli delivered to the branchial nerve. The neurone was voltage-clamped and the voltage was continually swept between −120 mV and −40 mV. Immediately following the control curve, a train of supra-maximal stimuli was delivered to the branchial nerve. In an unclamped cell, such stimulation produces hyperpolarization and elimination of firing for more than 20 min. Shown in (A) are the control sweep, and the first, second and fifth sweeps after synaptic stimulation; (B) compares the control and first sweeps at higher gain. Note that the curves intersect at approximately the K^+ equilibrium potential (arrow), indicating that synaptic stimulation causes an increase in K^+ conductance. During the second sweep (C) the intersection point has moved to a more negative voltage, indicating less involvement of a K^+ component. By the fifth and subsequent sweeps (200 s or more later), no intersection is found (D), showing that this long-lasting phase of synaptic hyperpolarization is mediated entirely by a decrease in inward current. Taken from Adams, Levitan & Parnas (submitted).

until finally no reversal potential is seen (Fig. 4*d*). At these later times g_K appears to be of minimal importance and the synaptic response is due to a long-lasting decrease in inward (Na^+/Ca^{2+}) current. We have not yet carried out ion replacement experiments to determine whether Ca^{2+} or Na^+ ions carry the inward current in R15 which is turned off by synaptic stimulation.

The similarities between the conductances that mediate the inhibitory synaptic response and those that mediate the hyperpolarizing phase of oscillatory activity, namely the slow kinetics and the concomitant increase in g_K and decrease in g_{Na} (and/or g_{Ca}), led us to suspect that they might be related and might in fact be the same conductances. To test this idea we measured the synaptically induced current in R15 immediately preceding and immediately following a burst. The rationale was as follows: application of a voltage clamp just before the beginning of a burst detects net *inward* current due to a high $g_{Na/Ca}$ and low g_K. Application of a clamp immediately following a burst, at the same potential as before, detects net *outward* current (Adams & Levitan, in preparation). If the individual conductances that are modulated by the

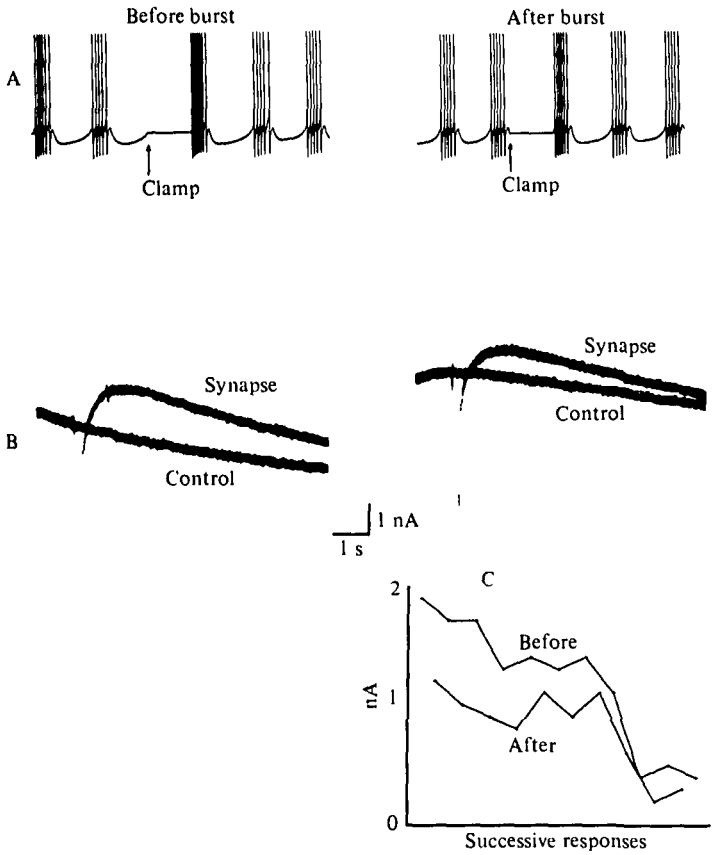

Fig. 5. Interaction between synaptic and burst conductances. (A) R15 was allowed to burst normally and then voltage-clamped abruptly to −36 mV immediately before (left arrow) or after (right arrow) a burst. One second later the branchial nerve was stimulated with a single pulse just slightly above threshold for eliciting a synaptic response. (B) The currents recorded with (synapse) and without (control) stimulation, before (left) and after (right) the burst, are shown. (C) Amplitude of net current flow elicited by the synapse (difference between 'control' and 'synapse' in (B) before and after the burst. Measurements were made alternately over a period of approximately 1 h. Note that, although the responsiveness of the neurone decreases considerably during that time (possibly due to an increase in threshold), the synaptic response is always larger before the burst.

synapse are the same as those which change during the oscillatory cycle, then activation of the synapse when $g_{Na/Ca}$ is high and g_K is low (immediately before a burst) should elicit a larger net current flow than when the reverse is true (immediately after a burst). Fig. 5 shows the results of such an experiment. The net current elicited by synaptic stimuli delivered before the burst are indeed larger than those elicited after the burst and remain larger through a series of measurements (Fig. 5).

To enhance the differences in current flow before and after the burst, we augmented the bursts by injecting current and then applied synaptic stimuli before and after the augmented bursts (Fig. 6). The difference in the current flowing across the membrane pre-burst and post-burst is approximately 8 nA, almost exactly the same as the difference between the currents elicited by synaptic stimulation pre-burst and post-burst (Fig. 6). That is, the difference in the synaptic response pre-burst and post-

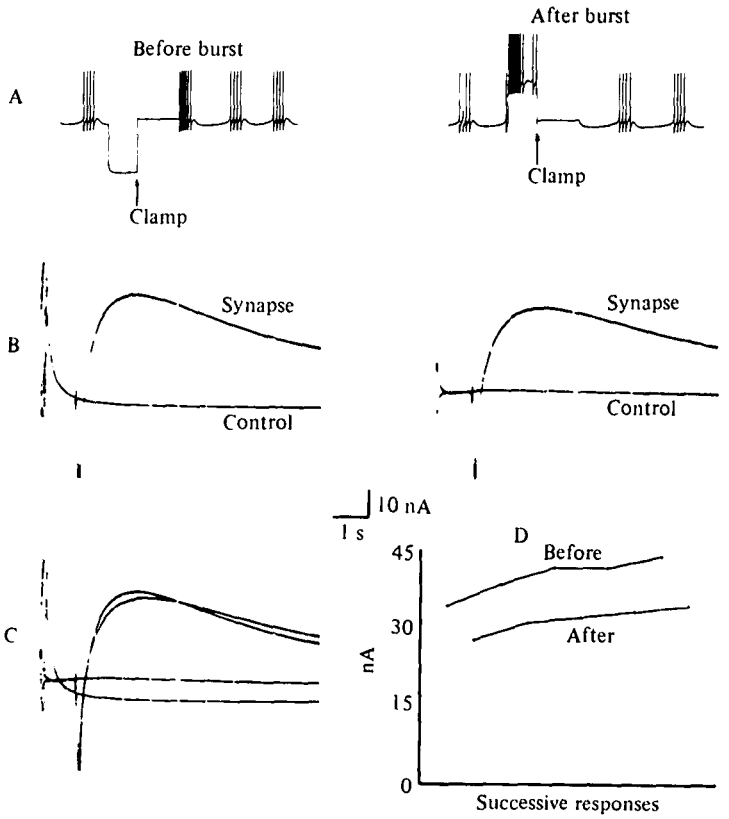

Fig. 6. Interaction between synaptic and burst conductances using artificially enhanced bursts. (A) Same as Fig. 5, except that current was injected during the *interburst* preceding the pre-burst clamp (left arrow), and during the *burst* preceding the post-burst clamp (right arrow), to enhance the burst currents. The cell was clamped to −30 mV. In addition, stimulation of the branchial nerve was supramaximal. (B) Currents recorded with (synapse) and without (control) stimulation, before (left) and after (right) the burst. (C) Superposition of the two sets of curves in (B). Note that the currents at the peak of synaptic activation reach very nearly the same value before and after the burst, even though the baselines are separated by approximately 8 nA. (D) Amplitude of net current flow elicited by the synapse before and after the burst. The difference is approximately 8 nA, and can be attributed entirely to the difference in baseline current flow. The experiments shown in Figs. 5 and 6 were done using the same cell.

burst can be attributed entirely to the changing currents responsible for the oscillations. Furthermore, the currents at the peak of synaptic activation reach approximately the same value before and after the burst, suggesting that activation of the synapse eliminates the conductance changes that occur during the oscillatory cycle. These data suggest that oscillatory activity in these cells, and long-lasting inhibition elicited by synaptic stimulation, are indeed mediated by the same ionic conductances.

Hormonal modulation of oscillatory behaviour

In the molluscan nervous system, physiological responses to conventional neurotransmitters, such as acetylcholine, dopamine and 5-hydroxytryptamine, are rarely observed at concentrations below about 10^{-6} M. In contrast, vasopressin and related neurohypophyseal hormones are remarkably potent in stimulating oscillatory activity

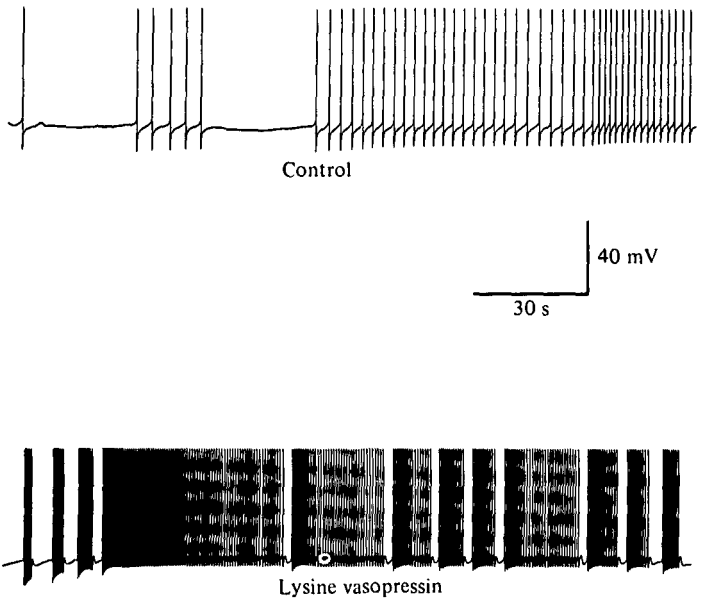

Fig. 7. Effect of lysine vasopressin on endogenous oscillatory activity in neurone F-1. Data shown are control activity in F-1 during perfusion with normal medium, and pattern 10 min after the start of perfusion with lysine vasopressin (10^{-6} M).

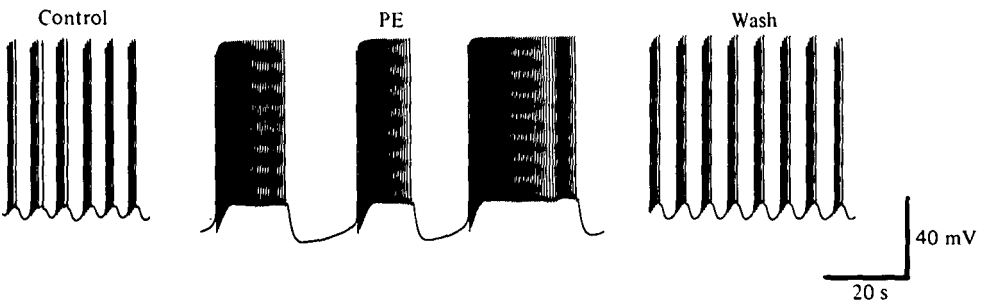

Fig. 8. Effect of crude peptide-containing extract (PE: see Table 1) on endogenous oscillatory activity in neurone F-1. Data shown are control activity in F-1, pattern 40 min after addition of medium containing PE from 2 *Helix* ganglia per ml, and pattern after 70 min wash with normal medium. Taken from Levitan & Treistman (1977*b*).

in *Helix* neurone F-1 (Fig. 7), and in neurone 11 in *Otala* (Barker & Gainer, 1974), threshold responses being observed at hormone concentrations as low as 10^{-9} M (Barker, Ifshin & Gainer, 1975). The high sensitivity of these neurones to vasopressin and related hormones suggested the presence in molluscan ganglia of receptors specifically sensitive to these peptides. This, together with the observation that crude acetic acid extracts of molluscan brain contain a factor that has effects qualitatively similar to those of vasopressin (Fig. 8, Ifshin, Gainer & Barker, 1975), led us to search for a vasopressin-like peptide in such extracts. At present, no conclusive proof for the existence in invertebrates of peptides resembling the neurohypophyseal hormones is available (Sawyer, 1977). Such proof would not only be of considerable evolutionary

Table 1. *Procedure for purification of a vasopressin-like factor from molluscan ganglia*

1. Ganglia boiled and homogenized in 5% acetic acid
2. Extract with H_2O – keep H_2O-soluble fraction
3. Sephadex G-10 – keep excluded fraction (> 700 daltons) = PE
4. Sephadex G-75 – keep included fraction (< 20000 daltons)
5. Neurophysin-sepharose affinity column

NH_4 acetate → Non-retained fraction

Formic acid → Neurophysin-retained fraction

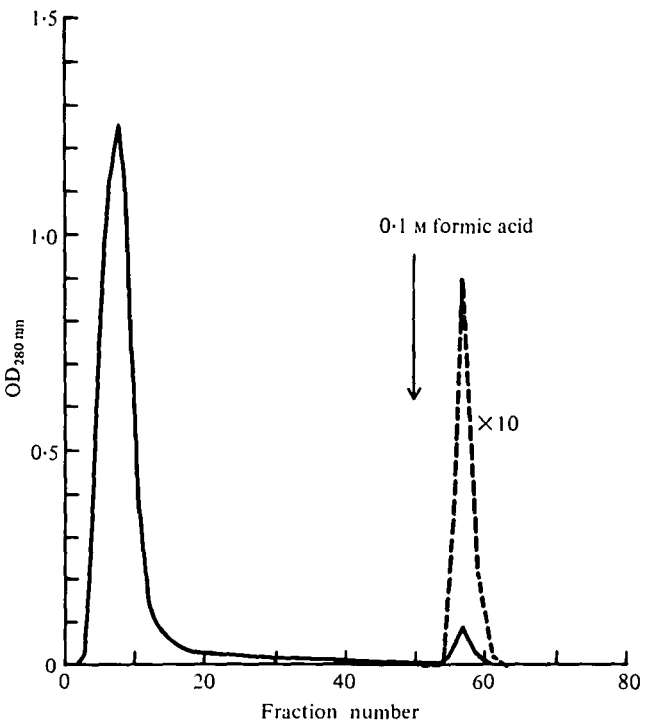

Fig. 9. Purification of a vasopressin-like factor from *Helix* ganglia by affinity chromatography on neurophysin-Sepharose. An acetic acid extract of 167 *Helix* ganglia (after gel filtration on Sephadex G-10 and Sephadex G-75; see Table 1) was lyophilized and redissolved in 5 ml 0·1 M ammonium acetate, pH 5·7. The sample was applied to a 3 × 0·9 cm column of neurophysin-Sepharose and the column was washed with a further 50 ml of ammonium acetate to remove unbound material. At the arrow the column was eluted with 0·1 M formic acid to remove neurophysin-retained material. Fractions of 1·1 ml were eluted and their optical density at 280 nm determined. The peak of optical density eluted by formic acid is shown on a 10× expanded scale.

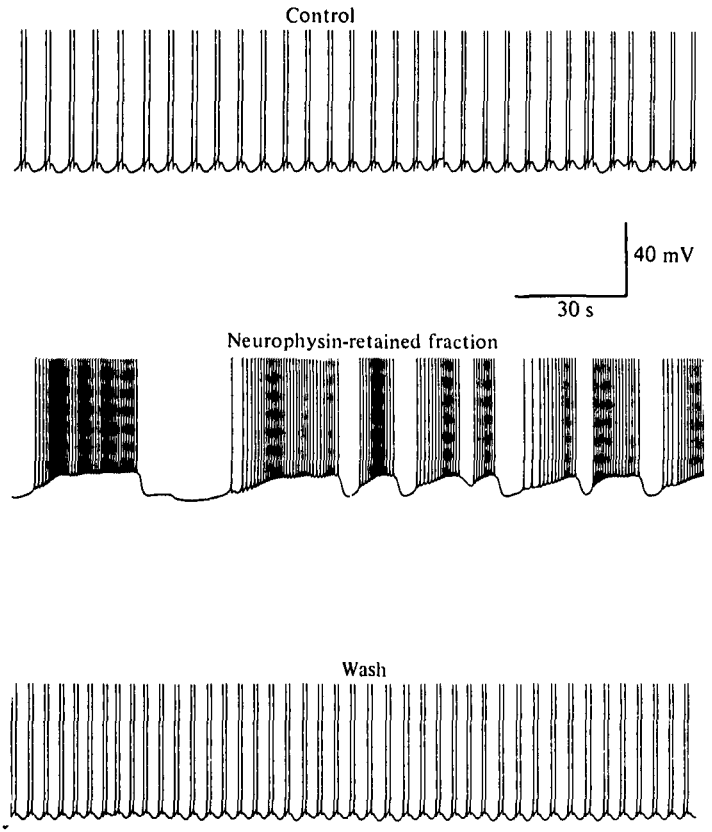

Fig. 10. Effect of purified vasopressin-like factor on endogenous oscillatory activity in neurone F-1. Data shown are control activity in F-1, pattern 15 min after addition of medium containing neurophysin-retained fraction from 7·5 *Helix* ganglia per ml, and pattern after 15 min wash with normal medium.

significance, but would allow us to study in detail the mechanism by which an endogenous molluscan hormone modulates oscillatory behaviour in individual neurones.

All of the known posterior pituitary peptides are stored in the pituitary in association with specific binding proteins (neurophysins) and may be isolated by precipitation as a complex with neurophysin of endogenous or exogenous origin (Acher, Light & Du Vigneaud, 1958). We have been able to purify a vasopressin-like factor from the brain of *Helix pomatia*, using the technique of affinity chromatography. The affinity support used was Sepharose, to which we linked bovine neurophysin. The neurophysin was isolated from bovine posterior pituitary powder (Hollenberg & Hope, 1968), and coupled to cyanogen bromide-activated Sepharose 4B (Flouret *et al.* 1977). A summary of the purification procedure is given in Table 1. Prior to application to a neurophysin-Sepharose affinity column, acetic acid extracts of snail brain were passed through two gel filtration columns – the first (Sephadex G-10) to remove amino acids and other small molecules (material smaller than 700 daltons was discarded), the second (Sephadex G-75) to remove material of higher molecular weight (greater than 10000 daltons). After gel filtration, the extract was applied to a 2 ml column of neurophysin-Sepharose under conditions optimal for the binding of the neuro-

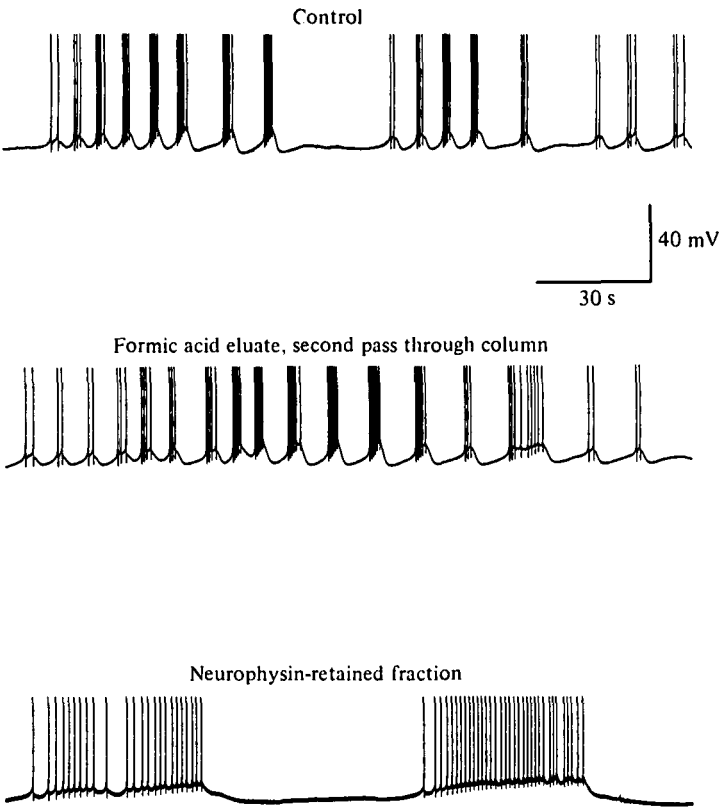

Fig. 11. Depletion of vasopressin-like activity from snail brain extracts by chromatography on neurophysin-Sepharose. A *Helix* ganglion extract partially purified by gel filtration on Sephadex G-10 and Sephadex G-75 (see Table 1) was applied to a neurophysin-Sepharose column in 0·1 M ammonium acetate, pH 5·7; after extensive washing of the column the neurophysin-retained material was eluted with 0·1 M formic acid (as in Fig. 9). Material which was *not* retained at pH 5·7 was *reapplied* to the column; after extensive washing with ammonium acetate the column was eluted with formic acid for a second time. Material eluted from neurophysin-Sepharose after this second pass through the column had essentially no effect on endogenous oscillatory activity in neurone F-1 (middle trace); essentially all the vasopressin-like activity had been absorbed during the first pass through the column (lower trace). Control activity is shown in the upper trace.

hypophyseal hormones (0·1 M ammonium acetate, pH 5·7). The column was extensively washed with the same buffer, which removed over 99% of the applied material (estimated by optical density at 280 nm, see Fig. 9). The column was then eluted with 0·1 M formic acid; a small peak of material which was fluorescamine-reactive and which possessed detectable optical density at 280 nm was eluted (Fig. 9).

This formic acid eluate has effects upon the oscillatory activity of *Helix* neuron F-1 (Fig. 10), which are qualitatively similar to those of vasopressin or of crude snail brain extract (Figs. 7, 8). A number of controls indicate that the absorption of biologically active material on to neurophysin-Sepharose is specific. Firstly, no material which affects oscillatory activity is retained by a column of Sepharose substituted with glycine in place of neurophysin. Furthermore, the neurophysin affinity column appears to extract the biologically active material almost quantitatively from the crude extract, since, if material which was not retained by neurophysin-Sepharose is

applied to the column a second time, no further biological activity is retained (Fig. 11). It could be argued that the biological activity eluted from neurophysin-Sepharose is due to leakage of vasopressin, oxytocin, or other material present as contaminants in the initial neurophysin preparation. However, no such activity is eluted from columns subjected to a 'dummy' elution cycle in which no sample is applied to the column (not shown).

Although the approach described above has provided strong evidence for the presence of a vasopressin-like peptide in the molluscan nervous system, conclusive proof will require the purification to homogeneity and chemical characterization of the active principle. The extremely small quantities of material available to us may render such characterization difficult, if not impossible. Estimates of the concentration of active material likely to be present in snail nervous tissue (obtained by paper electrophoresis of the purified material followed by staining with fluorescamine) indicate that less than 10 pmol are present in each animal. Several hundred micrograms of material (tens of thousands of snail ganglia) are likely to be required for protein sequence analysis by conventional techniques. However, amino acid analysis is now possible in the picomole range, and methods employing reversed-phase high-performance liquid chromatography have been reported to be capable of detecting as little as 15 pmol of vasopressin and oxytocin (Gruber *et al.* 1976). The growing availability of such techniques may render purification and sequence analysis feasible within the near future.

At any rate, the quantities of material available to us should be sufficient to establish whether vasopressin and the endogenous *Helix* peptide have a common mechanism of action in modulating oscillatory behaviour. It will be particularly interesting to ask which ionic conductances are affected during the peptide-induced stimulation of oscillatory activity and how this relates to synaptic modulation (see previous section). With respect to the biochemical mechanism of action of vasopressin and of the endogenous peptide, two possible modes of action might be envisaged by analogy with the known effects of neurohypophyseal hormones upon vertebrate tissues. The first would involve stimulation of neuronal adenylate cyclase in a manner analogous to that observed in many ion-transporting epithelia in vertebrates (Dousa, 1977; see below). A second possible mechanism of action would involve the gating of calcium influx by vasopressin, as appears to be the case in contractile tissues such as smooth muscle (Altura & Altura, 1977). Experiments to compare the effects of vasopressin and of the endogenous peptide on neuronal oscillatory activity are in progress.

Significance of modulation of oscillatory activity

It is interesting to speculate on the role that neuronal oscillations, and their modulation, may play in behaviour of the organism. The function of R15 in *Aplysia* and its homologues in the land snail is not clear, but it is known that these neurones share several other homologies in addition to oscillatory activity. Neurosecretory-like granules are present in their cytoplasm (Coggeshall, 1967), and the synthesis and processing of low-molecular-weight peptides, which may be neurosecretory products, has been thoroughly documented (Berry, 1975; Loh, Barker & Gainer, 1976; Strumwasser & Wilson, 1976). In an investigation of the possible function of these neuro-

secretory peptides, Kupfermann & Weiss (1976) injected crude extracts of R15 into *Aplysia* and found a small but rapid weight gain apparently due to water retention. Thus, R15 may synthesize and release an anti-diuretic peptide, and long-lasting synaptic hyperpolarization may represent feed-back inhibition of release of this peptide, from the target organ on which it acts. It is particularly significant in this regard that the branchial nerve, which contains axons of pre-synaptic neurones responsible for long-lasting hyperpolarization, also contains processes of sensory neurones from the osphradium, the organ which controls water balance in *Aplysia* (Jahan-Parwar, Smith & von Baumgarten, 1969). Furthermore, the oscillatory activity in R15 can be completely inhibited for long periods of time by appropriate chemical, osmotic or mechanical stimulation of the osphradium (Jahan-Parwar *et al.* 1969; Stinnakre & Tauc, 1966).

In view of our isolation from molluscan ganglia of a vasopressin-like peptide which alters neuronal oscillatory behaviour, it is tempting to compare this system with the hypothalamo-neurohypophyseal system in mammalian brain. The supra-optic nucleus area of the hypothalamus contains neurones which exhibit regular oscillatory activity (Gähwiler, Sandoz & Dreifuss, 1978), and which synthesize and release vasopressin, the antidiuretic hormone (Dreifuss, Harris & Tribollet, 1976). Furthermore, vasopressin itself can modulate the oscillatory behaviour of these neurones (Gähwiler, Sandoz & Dreifuss, 1978). Thus, although the osmotic stresses to which gastropods and mammals are subject clearly must differ in many respects, one may speculate that *some* aspects of control of water balance may be similar, and that the molluscan system may be a suitable model for studying control of release of neurohypophyseal-like peptides.

Effects of manipulating intraneuronal cyclic nucleotide levels

(A) *Phosphodiesterase inhibitors and cAMP derivatives*

As a first approach to investigating the possibility that cAMP and/or cGMP might play a role in modulation of the endogenous oscillatory activity of neurones R15 and F-1, we examined the effects of phosphodiesterase (PDE) inhibitors on this activity. The PDE inhibitor isobutylmethylxanthine (IBMX), added to the bathing medium at concentrations sufficient to produce increases in both cAMP and cGMP (Treistman & Levitan, 1976*a*), markedly alters the activity of R15 (Fig. 12A) and F-1 (not shown). Both the burst and interburst phases of the oscillatory cycle are affected, with the net effect being stimulation of the oscillatory activity. IBMX is also effective in the presence of tetrodotoxin (Levitan, 1979), which eliminates Na^+-dependent action potentials in R15 (and other cells in the ganglion) but leaves the oscillations in membrane potential (Strumwasser, 1971). This indicates that the change in activity produced by IBMX may not require interneuronal communication, which is minimized in tetrodotoxin. The non-methylxanthine PDE inhibitor, papaverine, affects R15 and F-1 in the same way as does IBMX (Treistman & Levitan, unpublished observations).

We examined the effect of cAMP and cGMP applied separately or together, extracellularly or intraneuronally, on the activity of R15 and F-1. Although in several experiments small transient changes were seen, cAMP and cGMP had no dramat[illegible]

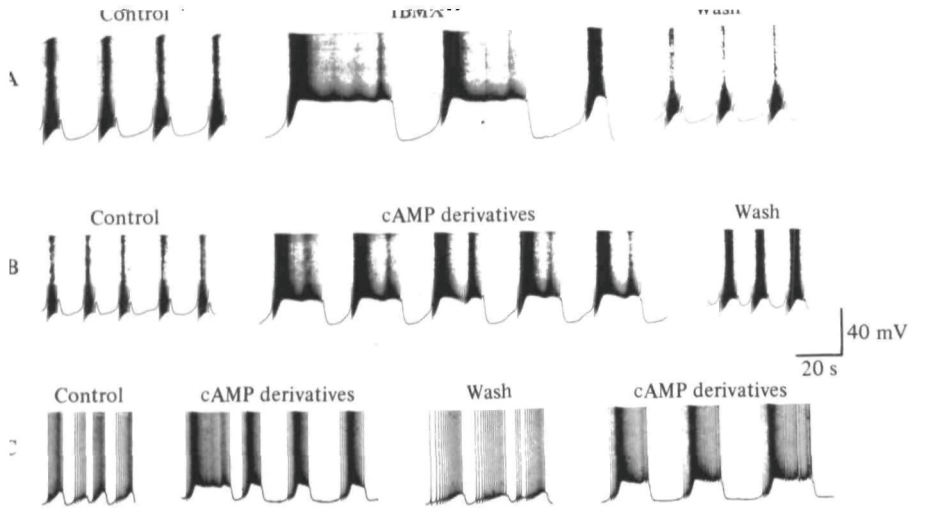

Fig. 12. Effects of IBMX and cAMP derivatives on endogenous oscillatory activity in neurones R15 and F-1. (A) Electrical activity in R15 during perfusion with normal medium (control) 40 min after introduction of 10^{-4} M IBMX, and 70 min after return to normal medium (wash). (B) Same as (A), but a mixture of 8-benzylthio cAMP and 8-parachlorophenylthio cAMP (10^{-3} M each) was used in place of IBMX. (C) Same as (B), but using F-1 instead of R15. The mixture of cAMP derivatives was reintroduced following the wash. Taken from Treistman & Levitan (1976*a*).

effects on oscillatory activity resembling those produced by IBMX (Treistman & Levitan, unpublished results). One possible explanation is that the high phosphodiesterase activity in *Aplysia* and *Helix* ganglia (Levitan & Bergström, in preparation) destroys the cAMP and cGMP before they can reach their site of action. Accordingly, we tested the effects of several 8-position substituted derivatives of cAMP that are not broken down by phosphodiesterase (Meyer & Miller, 1974). A mixture of 8-parachlorophenylthio cAMP and 8-benzylthio cAMP, applied to the bathing medium, alters the activity of R15 (Fig. 12B) and F-1 (Fig. 12C) in the same way as does IBMX (Fig. 12A). These derivatives, like cAMP itself, are potent activators of mammalian (Meyer & Miller, 1974) and molluscan (Levitan & Bergström, in preparation) protein kinases. In addition, they can inhibit cAMP and cGMP phosphodiesterases, and at the concentrations used in these experiments they cause intraneuronal accumulation of both cAMP and cGMP (Levitan & Bergström, in preparation).

To determine whether the cAMP derivatives were acting directly on R15 and F-1, rather than on some pre-synaptic or even non-neuronal element, we injected them intraneuronally. Immediately following their injection into R15, the cell hyperpolarized and oscillatory activity was abolished for a number of minutes (Fig. 13). When oscillations resumed, the interburst hyperpolarizing phase was strongly enhanced in both amplitude and duration and the bursts themselves were similar to those observed following extracellular application of the derivatives or IBMX (Fig. 12). Certainly, then, 8-substituted cAMP derivatives can act *directly* on R15 to modulate its oscillatory behaviour. However, it is not clear whether the apparent difference seen following extracellular and intracellular application of the derivatives is simply a quantitative one, or whether the response is indeed qualitatively different

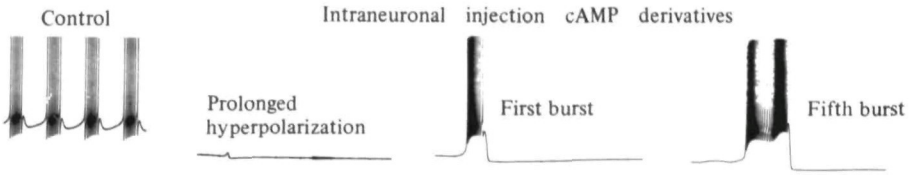

Fig. 13. Effects of intraneuronal injection of cAMP derivatives on endogenous oscillatory activity in neurone R15. A mixture of cAMP derivatives was injected under pressure into R15, to give an estimated intraneuronal concentration between 10^{-5} and 10^{-3} M. Within several minutes the cell hyperpolarized and remained silent for 30 min. When bursting resumed the bursts resembled those observed following extracellular application of cAMP derivatives (Fig. 12). Shown are the first burst and fifth burst (18 min after the first) after the end of the prolonged hyperpolarization. Taken from Treistman & Levitan (1976*a*).

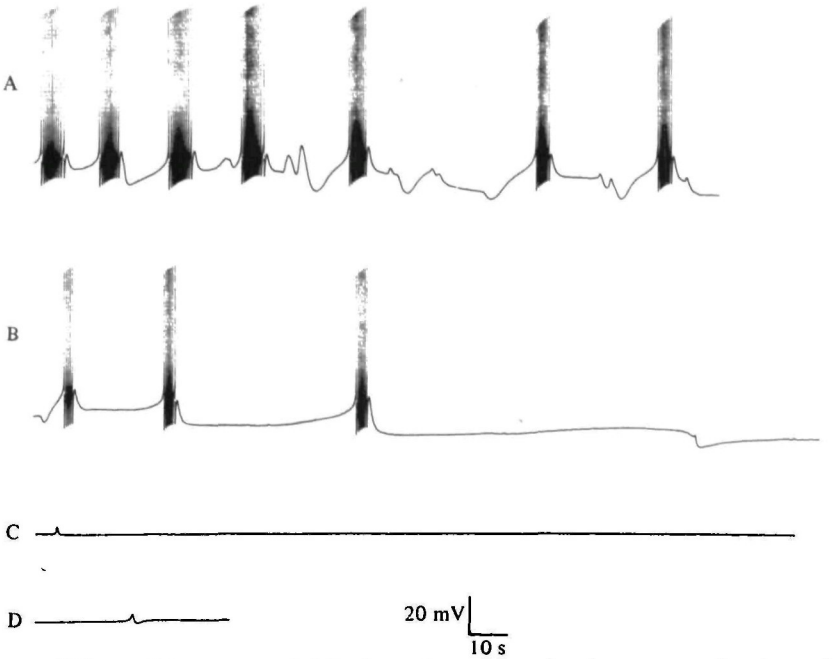

Fig. 14. Effects of intraneuronal injection of guanylylimidodiphosphate [Gpp(NH)p] on endogenous oscillatory activity in neurone R15. Gpp(NH)p was injected under pressure into R15 (A), to give an estimated intraneuronal concentration between 10^{-5} and 10^{-4} M, sufficient to fully activate adenylate cyclase (Treistman & Levitan, 1976*b*). Within several minutes the cell hyperpolarized (trace (B) begins 1·5 min after the end of trace (A)), and it was still hyperpolarized 2 h (C) and 3·5 h (D) later. Action potentials could be elicited by injection of depolarizing current through the recording microelectrode, indicating that the cell was not damaged. Taken from Treistman & Levitan (1976*b*).

following intraneuronal injection. The prolonged hyperpolarizations produced in the latter case (Fig. 13) were also observed in some extracellular application experiments (Levitan & Norman, in preparation).

(B) *Adenylate cyclase activation*

As another approach to manipulation of intraneuronal cAMP levels we utilized the GTP derivative guanylylimidodiphosphate [Gpp(NH)p], which is not hydrolyse by GTPase. As in many other systems, Gpp(NH)p is a potent activator of *Aplysia*

and *Helix* adenylate cyclase (Treistman & Levitan, 1976*b*; Levitan, Bergström & Simonet, 1978). However, in contrast to IBMX and the cAMP derivatives, it does not affect cAMP or cGMP phosphodiesterase (Treistman & Levitan, 1976*b*). We injected Gpp(NH)p into R15, at a concentration sufficient to fully activate adenylate cyclase. The cell rapidly hyperpolarized, and no further oscillatory activity was seen for the duration of the experiment (approximately 4 h) (Fig. 14). The cell was not damaged, since action potentials could be elicited by injection of depolarizing current, rather the neurone appeared to be 'chemically clamped' at about −75 mV (Treistman & Levitan, 1976*b*), which is close to its K^+ equilibrium potential.

This complete and long-lasting abolition of oscillatory activity resembles that produced during long-lasting synaptic hyperpolarization (Fig. 2; Parnas & Strumwasser, 1974). Furthermore, a number of putative amine neurotransmitters can activate adenylate cyclase (Levitan & Drummond, in preparation) and increase cAMP levels (Cedar & Schwartz, 1972; Levitan & Barondes, 1974; Levitan *et al.* 1974) in *Helix* and *Aplysia*. Unfortunately, however, the synaptic neurotransmitter mediating long-lasting hyperpolarization in R15 has not yet been identified, so it remains unclear whether these cAMP measurements are relevant to this particular synaptic response. We are currently attempting to determine whether Gpp(NH)p affects the same ionic conductances which give rise to oscillatory activity and which are altered during long-lasting synaptic hyperpolarization.

(C) *Peptide effects on cyclic nucleotides*

The similarity in the changes in oscillatory activity in R15 and F-1 produced on the one hand by peptides (Figs. 7, 8), and on the other hand by PDE inhibitors and cAMP derivatives (Figs. 12, 13), suggested to us that cAMP and/or cGMP might play a role in the neuronal response to peptides. This led us to examine the effect of vasopressin and oxytocin, as well as of the crude peptide-containing extract (PE) from molluscan ganglia, on ganglionic cyclic nucleotide levels. As shown in Fig. 15, the PE does indeed cause cAMP and cGMP concentrations to increase; the amounts of PE required are similar to those which modulate neuronal oscillatory activity (Fig. 8). Vasopressin and oxytocin have similar effects, although the duration and amplitude of the cAMP and cGMP increases are smaller than those produced by PE (Levitan, 1978*a*; Levitan & Treistman, 1977*b*). These cyclic nucleotide changes are not observed in neuronal cell bodies isolated following PE treatment, but rather appear to be restricted to the ganglionic neuropile (Treistman & Levitan, 1976*a*).

The PE also stimulates adenylate cyclase activity in a crude membrane fraction prepared from *Helix* and *Aplysia* ganglia (Fig. 16). Note that the stimulation is maintained for only a short time in this *in vitro* system, as in many others. Stimulation is also seen in membranes prepared from isolated *Helix* and *Aplysia* neuronal cell bodies (Table 2), indicating that these cell bodies contain receptors for the active component of the PE (Levitan, 1978*b*). We have not found conditions under which vasopressin (Levitan, Bergström & Simonet, 1978) or the neurophysin-purified component of the PE (Harmer & Levitan, unpublished results) stimulate *Helix* or *Aplysia* adenylate cyclase *in vitro*. Thus, the factor in the PE which stimulates adenylate cyclase is distinct from the vasopressin-like factor which we purify on neurophysin-Sepharose.

Table 2. *Effect of crude peptide-containing extract (PE) on adenylate cyclase activity in membranes from isolated* Helix *and* Aplysia *neuronal cell bodies*

(Adenylate cyclase activity was measured in the presence and absence of *Helix* or *Aplysia* PE (PE concentrations were the same as those required to alter neuronal oscillatory activity). Taken from Levitan (1978*b*).)

Source of membranes	Stimulation (%) Addition: *Helix* PE	Addition: *Aplysia* PE
Aplysia		
Metacerebral neurone	57	40
Left pleural giant neurone	69	42
R2	50	32
R15	93	66
L7	80	37
Helix		
Pooled unidentified neurones	72	55

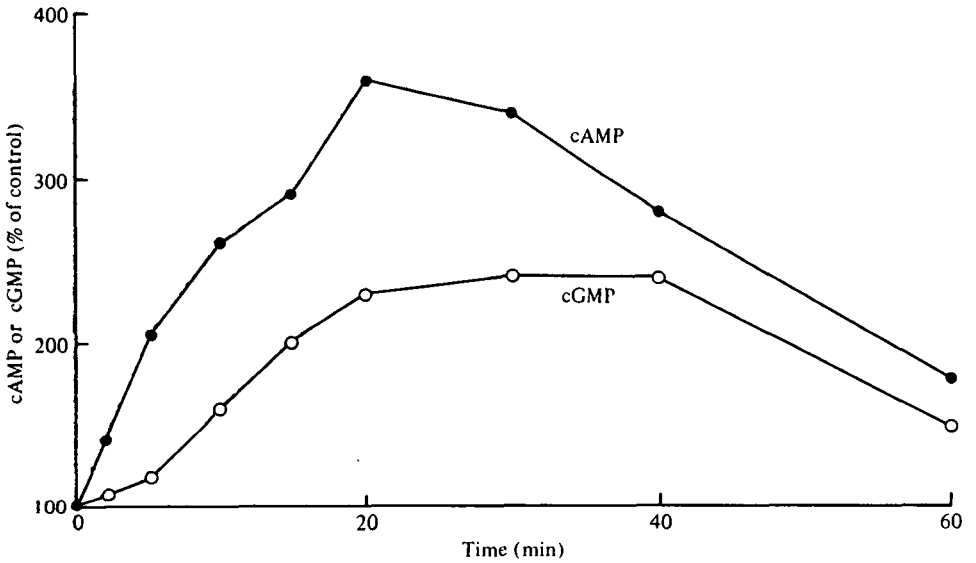

Fig. 15. Effect of crude peptide-containing extract (PE) on cyclic nucleotide concentrations in *Helix* nervous system. *Helix* circumoesophageal rings were incubated for varying periods of time in medium containing PE, prior to measurement of cAMP (●—●) and cGMP (○—○) levels in the tissue. The concentration of PE was the same as that used to alter oscillatory activity in *Helix* neurone F-1 (Fig. 8). Taken from Levitan (1978*a*).

(D) *Role of cyclic nucleotides*

One of the implications of our findings is that cyclic nucleotides are involved in both enhancement and inhibition of oscillatory activity, and it is appropriate to ask how two apparently opposing phenomena may both be mediated by cAMP. Our data suggest that the ionic conductances which are affected by synaptic stimulation are those directly involved in generation of oscillatory activity. This indicates that vasopressin (Barker & Smith, 1977) and the molluscan vasopressin-like peptide on the one hand, and the synaptic neurotransmitter on the other hand, both may modulate

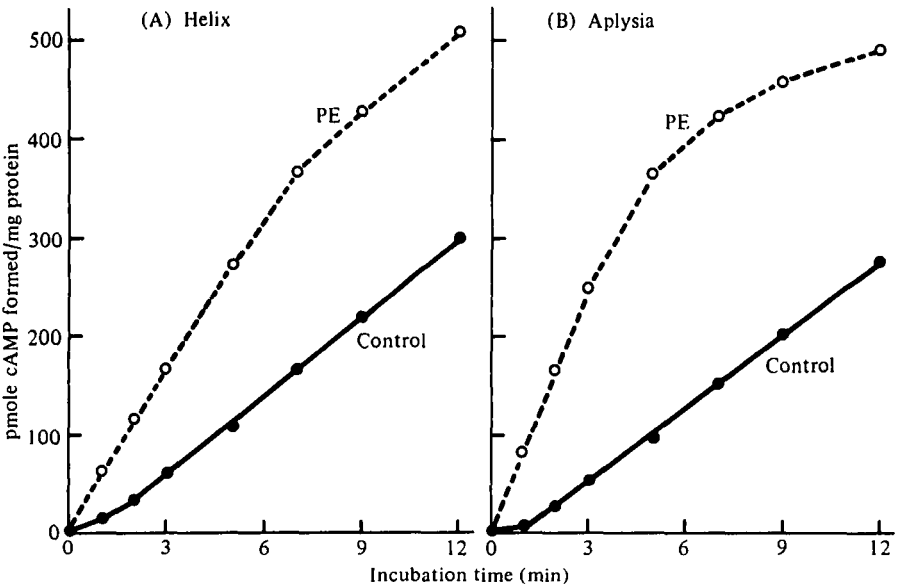

Fig. 16. Effect of crude peptide-containing extract (PE) on adenylate cyclase activity in membranes from *Helix* and *Aplysia* nervous system. Membranes from (A) *Helix* circumoesophageal ring or (B) pooled *Aplysia* ganglia were incubated for varying periods of time, in the absence (●—●) or presence (○- - -○) of *Helix* PE (PE concentration was the same as in Figs. 8 and 15). Taken from Levitan *et al.* (1978).

the same conductances. It then seems possible that cAMP *alone* may act on one or several of the relevant ionic conductances to cause long-lasting hyperpolarization, whereas it may act *together* with some other factor on the same or different conductances in such a way as to produce a net increase in oscillatory activity. Gpp(NH)p treatment, which appears to give a relatively 'pure' increase in cAMP, produces long-lasting hyperpolarization; in contrast, those agents which produce an increase in oscillatory activity (vertebrate peptides, crude PE, IBMX, cAMP derivatives) all cause an increase in *both* cAMP and cGMP. Although it is possible that cAMP and cGMP may act together, it must be emphasized that such a scheme is highly speculative at present. We cannot exclude the possibility that other factors, for example Ca^{2+}, in addition to or in place of cyclic nucleotides, may play a role in long-term modulation of oscillatory behaviour. It is certainly clear that manipulation of cyclic nucleotide concentrations within individual neuronal oscillators can cause profound changes in their oscillatory activity. Definitive proof that such changes are relevant to physiological phenomena must await further investigation.

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