J. exp. Biol. 161, 257–271 (1991)
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ACTIVATION OF A PEPTIDERGIC SYNAPSE LOCALLY MODULATES POSTSYNAPTIC CALCIUM INFLUX

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Accepted 13 June 1991

Summary

We examined the synaptic connection between Phe-Met-Arg-Phe-NH_2 (FMRFamide)-immunoreactive neurone VD4 and its target neurone P1, both found in the central nervous system of the pond snail *Helisoma trivolvis*. The major FMRFamide-like peak in neurone VD4 appears to be FMRFamide itself, based on its high performance liquid chromatography (HPLC) elution time and immunoreactivity before and after oxidation, but small peaks are also present at the elution times of Phe-Leu-Arg-Phe-NH_2 (FLRFamide) and Gly-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (GDPFLRFamide).

The modulatory actions of the neuropeptides found in neurone VD4 were tested on the postsynaptic target cell P1. Bath application of both the tetrapeptides FMRFamide and FLRFamide at a concentration of 10^{-5} mol l^{-1} reduced the macroscopic voltage-sensitive calcium current of neurone P1 in culture; FMRFamide by 45% and FLRFamide by 51%. Bath application of the heptapeptide GDPFLRFamide (10^{-5} mol l^{-1}) reduced the calcium current by only 8%.

We reconstructed the synaptic connection between VD4 and P1 in culture. Action-potential-evoked calcium transients in neurites growing from P1 cells in culture were monitored using Fura-2. Addition of FMRFamide, FLRFamide or GDPFLRFamide reduced the magnitude of the calcium transient in P1. Stimulation of VD4 mimicked the effects of peptide application and caused localized reductions in the action-potential-evoked calcium transients in P1 at the points of contact between the neurites of neurones VD4 and P1. These results suggest that neurone VD4 modulates the calcium influx of neurone P1 through the release of endogenous FMRFamide-related peptides and that this modulatory action is restricted to sites of synaptic interaction.

Introduction

Neuropeptides are known to have a wide array of modulatory actions on neuronal targets. One of their effects is to regulate synaptic transmission through

Key words: neuromodulation, FMRFamide, *Helisoma trivolvis*, neuropeptide, synaptic modulation.

the modulation of ion channels (Walker *et al.* 1988; Man-Son-Hing *et al.* 1989). In particular, neuropeptides have been shown to modulate calcium currents (Brezina *et al.* 1987; Walker *et al.* 1988; Man-Son-Hing *et al.* 1989; Bley and Tsien, 1990) in many neurones. Since neuropeptides can act distally as neurohormones or serve paracrine functions, in addition to their direct neurotransmitter roles, it is often difficult to determine the source of peptides underlying this type of modulatory action. In the present study, we examined the connection between a putative peptidergic neurone (neurone VD4) and its target (neurone P1) (N. Syed, personal communication).

Neurone VD4 is a putative homologue of the *Lymnaea stagnalis* visceral white interneurone (VWI) (Benjamin, 1984), which has been shown to be a crucial element of the respiratory central pattern generator (Syed *et al.* 1990). By isolating VD4 and its target neurone P1 in culture, we were able to study the events underlying presynaptic modulation by a peptidergic source.

The peptide FMRFamide was initially identified in the clam *Macrocallista nimbosa* (Price and Greenberg, 1977). It has since been found in several molluscan species (Price, 1986), together with other FMRFamide-related peptides (FaRPs). FaRPs have been shown to have potent modulatory actions on central neurones of these molluscs (Cottrell *et al.* 1984; Colombaioni *et al.* 1985; Belardetti *et al.* 1987; Brezina *et al.* 1987; Kramer *et al.* 1988). In the nervous system of the pond snail *Helisoma trivolvis*, FMRFamide and two other FaRPs have been identified (Bulloch *et al.* 1988) and shown to exert modulatory effects both peripherally and centrally (Coates and Bulloch, 1985; Murphy *et al.* 1985; Bulloch *et al.* 1988).

The sources of the FMRFamide responsible for the modulation of these *H. trivolvis* neurones have not yet been established, although there are numerous FMRFamide-like immunoreactive neurones within the central nervous system (CNS), including VD4 (Murphy *et al.* 1985). The goals of this study were to identify the FaRP complement of neurone VD4; to determine the actions of these FaRPs on the calcium currents of the target cell P1, and to examine the regulation of calcium influx in P1 when synaptically connected neurone VD4 was stimulated to release its neuropeptides.

Materials and methods

All experiments were performed on laboratory-raised, adult specimens of the albino (red) pond snail *Helisoma trivolvis*. Animals were maintained in aquaria on a diet of lettuce and Purina Trout Chow.

Cell culture

Neurones P1 and VD4 were isolated from the pedal and visceral ganglia, respectively, using the techniques outlined by Haydon *et al.* (1985). In brief, the central ganglia were removed from the animal and treated with 0.2% trypsin for 25 min. Neurones were plated in either adhesive or non-adhesive culture conditions. Cells plated on 35 mm Falcon (no. 3001) Petri dishes coated with poly-L-

lysine adhered to the substratum, a prerequisite for neurite outgrowth. For voltage-clamp experiments in which spherical neurones were required, cells were plated onto 35 mm Falcon (no. 1008) Petri dishes containing 2 ml of defined medium [DM; 50 % Leibowitz-15 (Gibco) with *H. trivolvis* salts; Wong *et al.* 1981] to which 20 μl of fresh snail haemolymph was added. Haemolymph prevents neuronal adhesion to the culture dish and suppresses neurite extension (Haydon, 1988).

Immunocytochemistry

Indirect immunocytochemical methods were applied to cultured neurones VD4 and P1. Neurones were left overnight at 4 °C in Zamboni's fixative, rinsed in 0.187 mol l^{-1} phosphate-buffered saline (PBS), and exposed for 10 min to 0.3 % Triton-X and 0.02 % sodium azide in PBS containing 3 % goat serum, prior to application of the primary antibody. A rabbit polyclonal antiserum raised against FMRFamide (1:200 dilution) was provided by J. Bishop (Chronwall *et al.* 1984; O'Donohue *et al.* 1984) and was applied to the neurones for 24 h at room temperature (RT, 22–25 °C). Following further washes in PBS, the preparations were incubated with fluorescein-conjugated goat anti-rabbit IgG (Sigma, 1:100 dilution) for 2 h at RT. To test the specificity of the primary antibody, preadsorption controls were conducted using FMRFamide and GDPFLRFamide at concentrations of 5×10^{-4} mol l^{-1}.

Determination of the FaRPs in neurone VD4

Isolated VD4 neurones (10 per sample) were dissolved in acetone (0.5 ml); the acetone was then filtered through a Nylon syringe filter (0.45 μm pore size), dried (Speed-Vac) and the residue resuspended in aqueous high performance liquid chromatography (HPLC) solvent (1 ml of 0.1 % trifluoroacetic acid, TFA). Half of the extract was loaded onto the HPLC directly and the other half was oxidized with hydrogen peroxide before HPLC, as previously described (Price *et al.* 1990). A flow rate of 0.5 ml min^{-1} through an RP-300 column (2.1 mm×220 mm) with a gradient from 16 % acetonitrile (ACN) in 0.1 % TFA to 36 % ACN in TFA over 20 min was used. Fractions (0.5 min) were collected from both runs, dried (Speed-Vac) and resuspended in 0.1 ml of radioimmunoassay (RIA) buffer; 25 μl samples of each fraction were used for RIA with antiserum Q2 (Bewick *et al.* 1990; Price *et al.* 1990). The RIA was standardized with the peptide NDPFLRFamide. Peaks were identified by comparing their elution times with those of synthetic peptides and by their behaviour after oxidation.

Electrophysiology

Conventional electrophysiological techniques were used to record synaptic interactions between neurones P1 and VD4 within the nervous system and in cell culture. To record from neurones in ganglia, microelectrode penetration was facilitated by a 30 s trypsin treatment of the visceral and pedal ganglionic sheaths. The intracellular recording electrodes were filled with 1.5 mol l^{-1} KCl (resistance,

20–30 MΩ) and the preparation was bathed in normal *H. trivolvis* saline containing; 51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Hepes at pH 7.3. Neuronal activity was monitored using Getting preamplifiers (model 5A) and stored on videotape using a Vetter 420F recorder.

Whole-cell voltage-clamp

Neurones were isolated from the nervous system and were cultured in 1% haemolymph in DM for 2–3 days. Immediately prior to the experiment, cells were individually transferred to a recording chamber (a Falcon no. 1008 dish previously coated with poly-L-lysine) containing DM. The spherical neurone adhered to the base of the dish and remained immobilized for the duration of the voltage-clamp experiment. Patch pipettes (with d.c. resistances of 1–2MΩ) were filled with a solution containing 35 mmol l^{-1} CsCl, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} EGTA, 5 mmol l^{-1} ATP, 1 mmol l^{-1} GTP and 5 mmol l^{-1} Hepes (pH adjusted to 7.3 with CsOH). The external solution consisted of 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$, 1.7 mmol l^{-1} KCl, 30 mmol l^{-1} tetraethylammonium bromide, 10 mmol l^{-1} 4-aminopyridine, 30 mmol l^{-1} sucrose and 10 mmol l^{-1} Hepes (pH adjusted to 7.3 with tetraethylammonium hydroxide). Signals were filtered with a corner frequency of 1 kHz. Cells were held at a potential of −60 mV and depolarizing command potentials were delivered at 30 s intervals. Leakage and capacitative transients were digitally subtracted using appropriately scaled hyperpolarizing pulses during data acquisition using pClamp software (Axon Instruments, CA).

Calcium imaging

Membrane-impermeant Fura-2 pentapotassium salt (Molecular Probes Inc., CA) was pressure-injected (using a Picospritzer; General Valve) into neurone P1 following penetration of the cell with a micropipette containing 10 mmol l^{-1} dye in 10 mmol l^{-1} Hepes. The dye was allowed to diffuse through the neurites before data acquisition. We estimated that Fura-2 was at a concentration of no more than 100 μmol l^{-1} after microinjection. Neurone VD4 was penetrated with a microelectrode containing 1.5 mol l^{-1} KCl.

The neurites of neurone P1 were examined with a Nikon Diaphot inverted microscope through a 40× oil-immersion objective. Fura-2 fluorescence was excited by a xenon arc lamp using 340 nm and 380 nm bandpass filters. Neutral density filters were placed in the light path to attenuate the light signal, thereby preventing cell damage and reducing photobleaching of Fura-2. Fura-2 emission (510 DF 40 nm filter) was collected using a SIT camera (Hammamatsu). The raw 340 nm and 380 nm images, along with corresponding background images, were digitized and stored using a Quantex QX-7 image processor. All image processing and analysis were performed using QFM quantitative fluorescence software (Quantex Corp., CA). Ratioed images (340 nm/380 nm), from which the back-

ground image brightness at each wavelength had been subtracted, were used to calculate the internal calcium concentrations using the formula:

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_o/F_s),$$

where K_d is the dissociation constant for Fura-2/Ca^{2+}(214 nm) (Cohan *et al.* 1987), R is the ratio of fluorescence intensities at 340 nm and 380 nm, R_{min} and R_{max} are the ratios at zero calcium and at saturating calcium, respectively, and F_o/F_s is the ratio of the intensities of free and bound Fura-2 at 380 nm (Grynkiewkz *et al.* 1985). The system was calibrated using Fura-2 (100 μmol l^{-1}) in a solution iso-osmotic with *H. trivolvis* haemolymph (130 mosmol l^{-1}), and containing either no added calcium and EGTA (5 mmol l^{-1}) for a zero-calcium medium or a saturating level of calcium (4 mmol l^{-1}). Calibration solutions were viewed in 20 μm thick capillaries (Vitro Dynamics; Rockaway, NJ) to maintain a constant pathlength for obtaining accurate estimates of F_o/F_s.

On completion of calcium imaging, the KCl-filled microelectrode was removed from neurone VD4 and the cell was re-impaled with a Lucifer-Yellow-filled microelectrode. Lucifer Yellow was ionophoresed into neurone VD4, and the fluorescence of the dye was excited using a 485 nm bandpass filter, a 510 nm dichroic mirror and a 515 nm longpass filter. Lucifer Yellow images of presynaptic neurone VD4 were compared to the Fura-2 images of postsynaptic neurone P1 and to the phase-contrast images obtained prior to experimentation to determine sites of contact between the neurites of the two cells. In some preparations successful Lucifer Yellow injections were not achieved because of the fragility of neurone VD4. However, since we found little evidence of neurite fasciculation between P1 and VD4 it was possible to determine discrete regions on P1 neurites that contacted VD4, and other regions that were without apparent contact.

The effects of bath-applied FMRFamide or GDPFLRFamide on calcium transients of neurone P1 were determined in some cell pairs. Calcium transients were acquired either using images as described above or with a photomultiplier tube using UMANS type 5.0 software (C. M. Regan, Urbana, IL).

Results

Comparison of the in situ *and* in vitro *connections between VD4 and P1*

Stimulation of VD4 produced a slow hyperpolarization of neurone P1 *in situ* (Fig. 1A). This synaptic connnection was reconstructed in cell culture. Neurones VD4 and P1 were isolated from the CNS and plated in culture conditions that permitted neurite outgrowth. After 3–8 days in culture, both neurones extended neurites that allowed contact to be established between the cells. Stimulation of neurone VD4 reliably produced a slow inhibitory postsynaptic potential in neurone P1, a response similar to that observed *in situ* (Fig. 1B). This synaptic action is chemically mediated since it is (i) reduced in magnitude by experimental hyperpolarization of neurone P1, (ii) accompanied by an increase in membrane

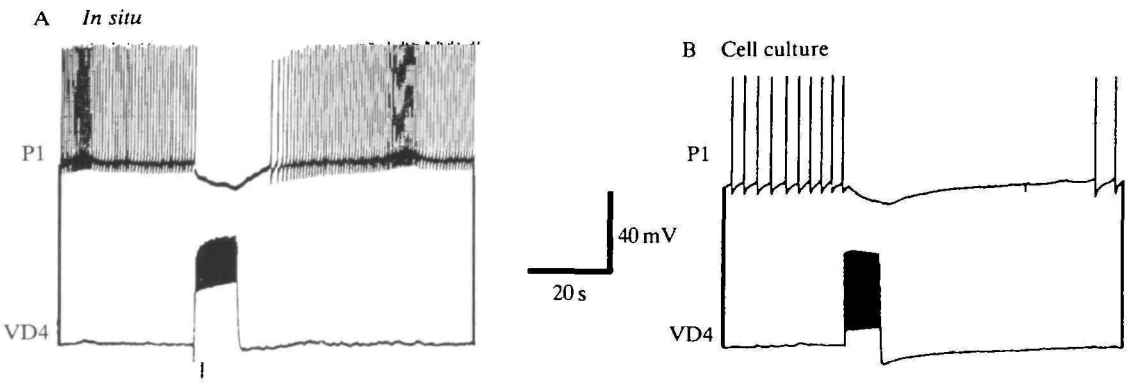

Fig. 1. Synaptic interactions between neurones VD4 and P1 *in situ* and in cell culture. (A) In the isolated central nervous system, depolarization of neurone VD4 produced a hyperpolarization in neurone P1. (B) Stimulation of neurone VD4, plated in cell culture with neurone P1, produced an inhibitory postsynaptic potential which closely resembled the recording *in situ*.

conductance in P1, and (iii) reversibly abolished by high-Mg^{2+}/zero-Ca^{2+} saline (not illustrated).

FMRFamide immunocytochemistry of cultured neurone VD4

Neurite-bearing neurones VD4 and P1, grown for 3–8 days in culture, were fixed, permeabilized, incubated with an FMRFamide antiserum and labelled with a fluorescein-conjugated second antibody (see Materials and methods). The neurites and cell body of neurone VD4 stained positively ($N=7$), while neurone P1 was unlabelled, displaying only somatic autofluorescence (Fig. 2). Preincubation of the FMRFamide antiserum with either FMRFamide ($N=4$) or GDPFLRF-amide ($N=3$) at concentrations of 5×10^{-4} mol l^{-1} successfully preabsorbed the antiserum, preventing positive immunoreactivity of neurone VD4. Thus, neurone VD4, but not neurone P1, exhibits FMRFamide-like immunoreactivity.

Identification of the FaRPs in neurone VD4

An extract of five cells supplied sufficient material for either HPLC or RIA. Two major peaks are apparent with the unoxidized extract. The largest is at the elution position of FMRFamide and the next largest is at the position of FLRFamide. A small peak is seen near the position expected for GDPFLRFamide (Fig. 3). After oxidation, the FMRFamide peak disappears (oxidation shifts the position of the peptide but, more importantly, makes it almost unreactive with the antiserum used), but the putative FLRFamide and GDPFLRFamide peaks remain, as expected. Since the RIA was standardized with NDPFLRFamide, which is about 10 times more immunoreactive than FMRFamide, the major peak in Fig. 3A actually represents about 10 pmol of peptide or 2 pmol per cell.

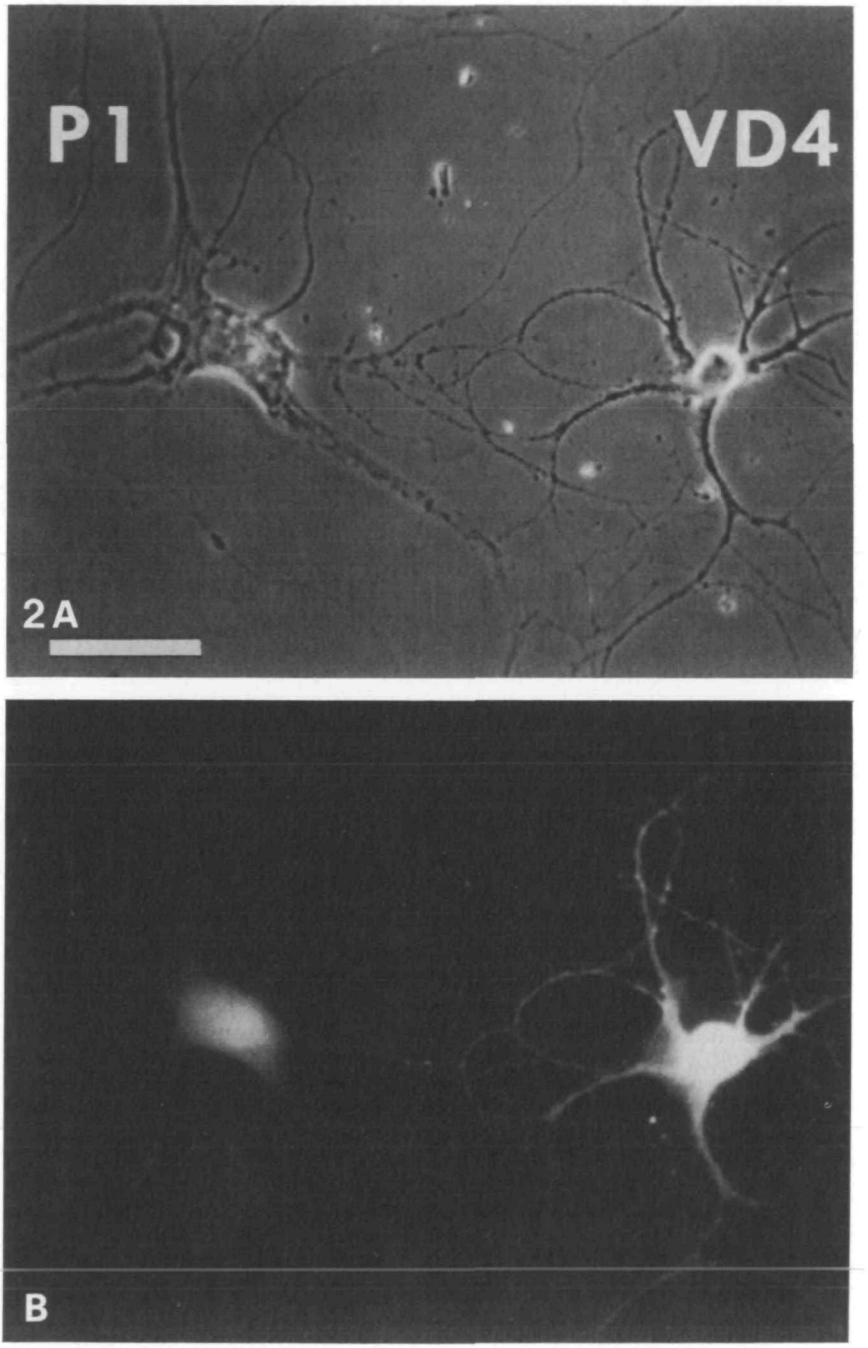

Fig. 2. FMRFamide immunocytochemistry of cultured neurones P1 and VD4. (A) Phase-contrast image showing the neuritic outgrowth of neurones P1 and VD4 in adhesive cell culture conditions. (B) FMRFamide-like immunofluorescence is confined to the neurites and soma of neurone VD4. The soma of neurone P1 exhibits autofluorescence. Scale bar, 100 μm.

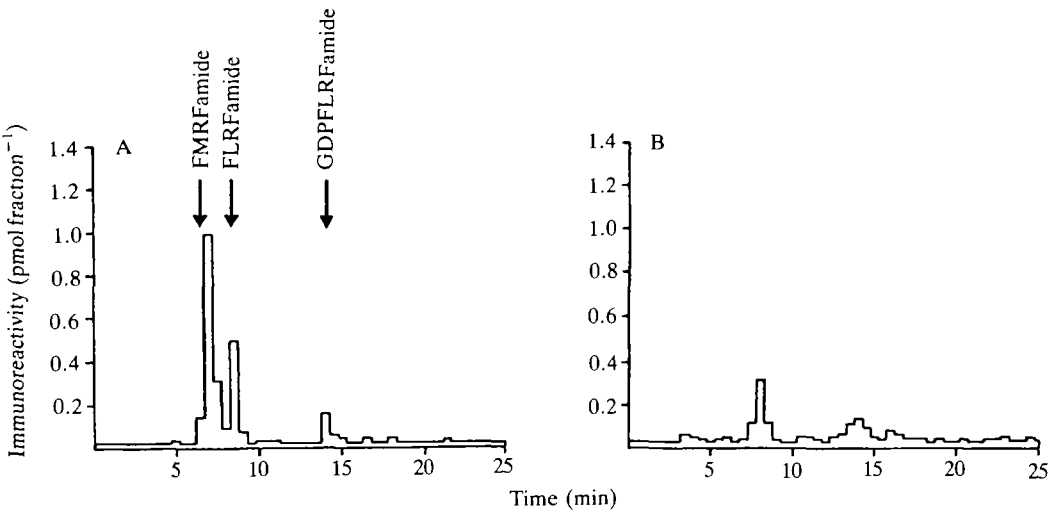

Fig. 3. Immunoreactivity profiles of high performance liquid chromatography (HPLC) runs as determined by radioimmunoassay (RIA). The immunoreactivity in each 0.5-min fraction was calculated from the RIA and plotted against the elution time. The elution times of standards were determined in separate runs. An extract of 10 VD4 neurones was divided in half. Half was directly fractionated by HPLC (A) and half was oxidized before HPLC (B). Thus, the amount of immunoreactivity in each run corresponds to that from five cells.

Peptide modulation of calcium currents in P1

We examined the actions of FMRFamide, FLRFamide and GDPFLRFamide on the calcium currents of neurone P1. Two macroscopic calcium currents were identified in P1; a transient, low-voltage-activated current (LVA) was detected on depolarization to −20 mV and a sustained, high-voltage-activated current (HVA) was detected at command potentials of −10 mV or greater (see Haydon and Man-Son-Hing, 1988). The tetrapeptides FMRFamide and FLRFamide were equipotent in reducing the HVA calcium current of P1 (Fig. 4A,C,D). However, the heptapeptide GDPFLRFamide, exerted only a small modulatory effect on the HVA calcium current and only at high concentrations (Fig. 4C,D). The threshold concentrations of both tetrapeptides was 3×10^{-8} mol l^{-1}, whereas for the heptapeptide it was 3×10^{-6} mol l^{-1}. At a concentration of 10^{-5} mol l^{-1}, the highest dose tested, the tetrapeptides FMRFamide and FLRFamide reduced the current by 45.4±12.6% (mean±S.D., N=6) and 51.0±9.4% (N=5), respectively. The heptapeptide GDPFLRFamide only reduced the current by 7.7±6.4% (N=9) (Fig. 4C). The actions of these FaRPs were fully reversible upon washout. The LVA calcium current was unaffected by the FaRPs.

To determine whether the FaRPs might act through G-protein-coupled receptors, GTPγS (100 μmol l^{-1}) replaced the GTP in the patch pipette to promote irreversible receptor-activation of G proteins. In large neurones, such as P1, the

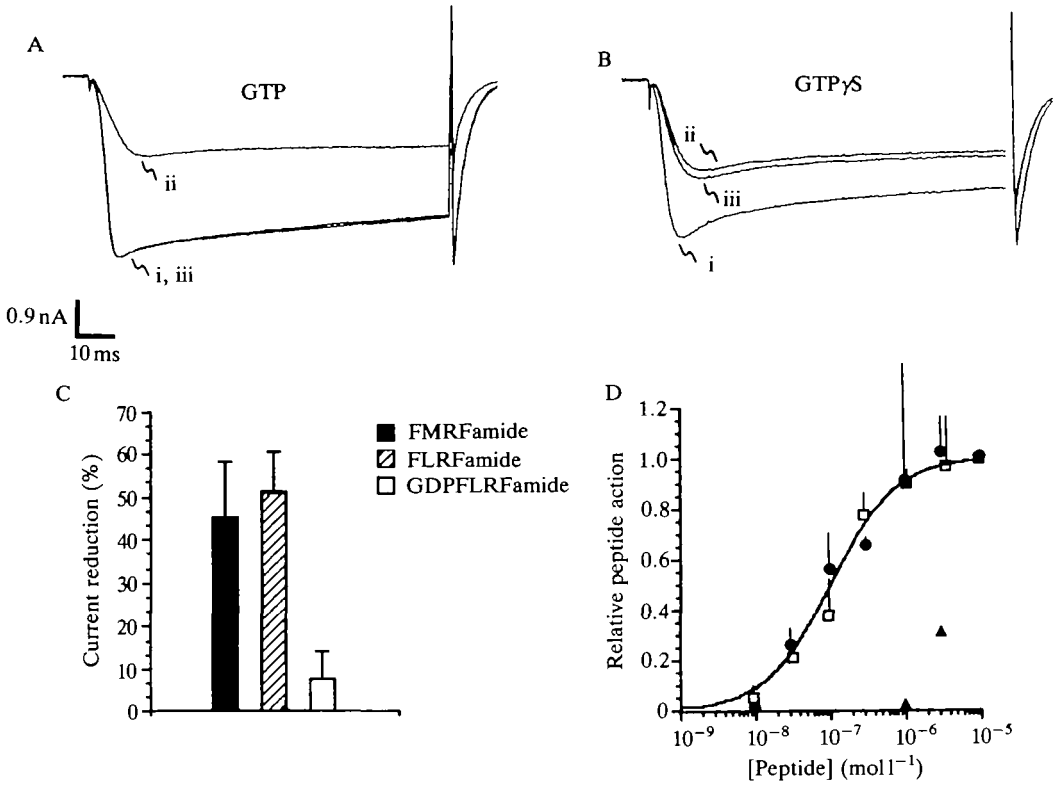

Fig. 4. Modulation of the macroscopic calcium current in neurone P1 by FMRFamide, FLRFamide and GDPFLRFamide. (A) An inward calcium current is shown (downward direction) in response to a 100 ms depolarizing step to +10 mV from a holding potential of −60 mV. (i) Control; (ii) bath application of 10^{-6} mol l^{-1} FMRFamide; (iii) washout. When GTP is included in the patch pipette the reduction in calcium current due to FMRFamide is fully reversible. (B) When GTPγS (100 μmol l^{-1}), the nonhydrolyzable analogue of GTP, is present in the patch pipette, the FMRFamide-induced reduction in calcium current is irreversible. Traces i–iii are equivalent to those in A. (C) Comparison of the effects of 10^{-5} mol l^{-1} FMRFamide (N=6), FLRFamide (N=5) and GDPFLRFamide (N=9) on the peak calcium current of neurone P1. Data are presented as the percentage decrease in the peak calcium current of neurone P1 (mean and S.D.). (D) Dose–response curves for the effects of FMRFamide-related peptides on the calcium current of neurone P1. The line of best fit for both the FMRFamide and FLRFamide data correlates with an apparent K_d of 10^{-7} mol l^{-1}. Data were normalized for individual cells by expressing the reduction in calcium current as a fraction of the reduction upon application of 10^{-5} mol l^{-1} peptide. FMRFamide (●) (N=6), FLRFamide (□) (N=4), GDPFLRFamide (▲) (N=4).

concentration of dialyzed GTPγS is small, resulting in little G protein activation; thus, the ligand (FMRFamide or FLRFamide) was applied to promote exchange of GDP for GTPγS. FMRFamide (N=10) and FLRFamide (N=4) caused irreversible reductions in the HVA current of P1 in the presence of GTPγS (Fig. 4B). After peptide washout, subsequent application of peptide was without

effect on the calcium current magnitude. These observations are consistent with the hypothesis that FaRPs act through receptor-coupled G proteins to modulate the HVA calcium current.

Modulation of the calcium transient in P1 cell neurites

Since synaptic interactions frequently occur on neuronal processes, we investigated whether the modulatory actions of the peptides present in VD4, could be detected in neurites of P1. Neurone P1 was plated alone, or in the presence of VD4, on an adhesive culture substratum that supports neurite extension. After 3–8 days of culture, the action-potential-evoked calcium transients of neurite-bearing cells were examined. To monitor internal calcium levels, Fura-2 was pressure-injected into the cell body of neurone P1 from a recording microelectrode. A constant train of action potentials (characteristically consisting of 10 action potentials at 2 Hz) was evoked in P1 at intervals of 1–2 min to promote calcium loading of neurites. The fluorescent emission of Fura-2 within a selected region of the neurites from P1 was detected using a photomultiplier tube or, in some experiments, a field of neurites was imaged using a SIT camera. Calcium levels were estimated using ratiometric techniques.

Bath application of FMRFamide at 5×10^{-7} mol l^{-1} (a concentration found to affect HVA calcium current without preventing spike initiation) caused a 78.3±7.7% (mean±S.E.M, N=4) reduction in the calcium transient of P1 cell neurites evoked in response to a constant train of action potentials. Similar results were obtained with FLRFamide (N=2). Since the threshold concentration for GDPFLRFamide on the HVA calcium current was 10^{-5} mol l^{-1}, we used this

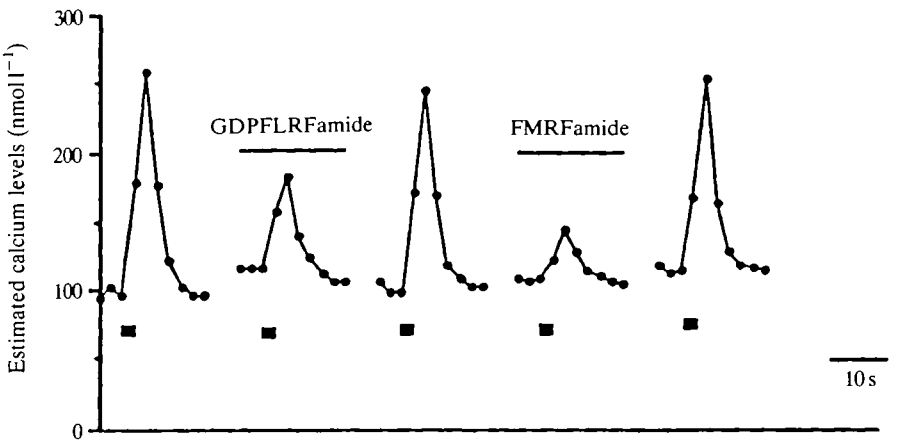

Fig. 5. FMRFamide-related peptide (FaRP) modulation of the calcium transients in neurites of P1. Calcium transients evoked by intracellular stimulation of neurone P1 (denoted by squares) were imaged at 3-s intervals at a selected region on the neurites of a P1 cell. Bath application of FMRFamide (5×10^{-7} mol l^{-1}) and GDPFLRFamide (10^{-5} mol l^{-1}) reduced the magnitude of the calcium transient. The duration of FaRP application is indicated by bars. Calcium transients recovered following peptide washout.

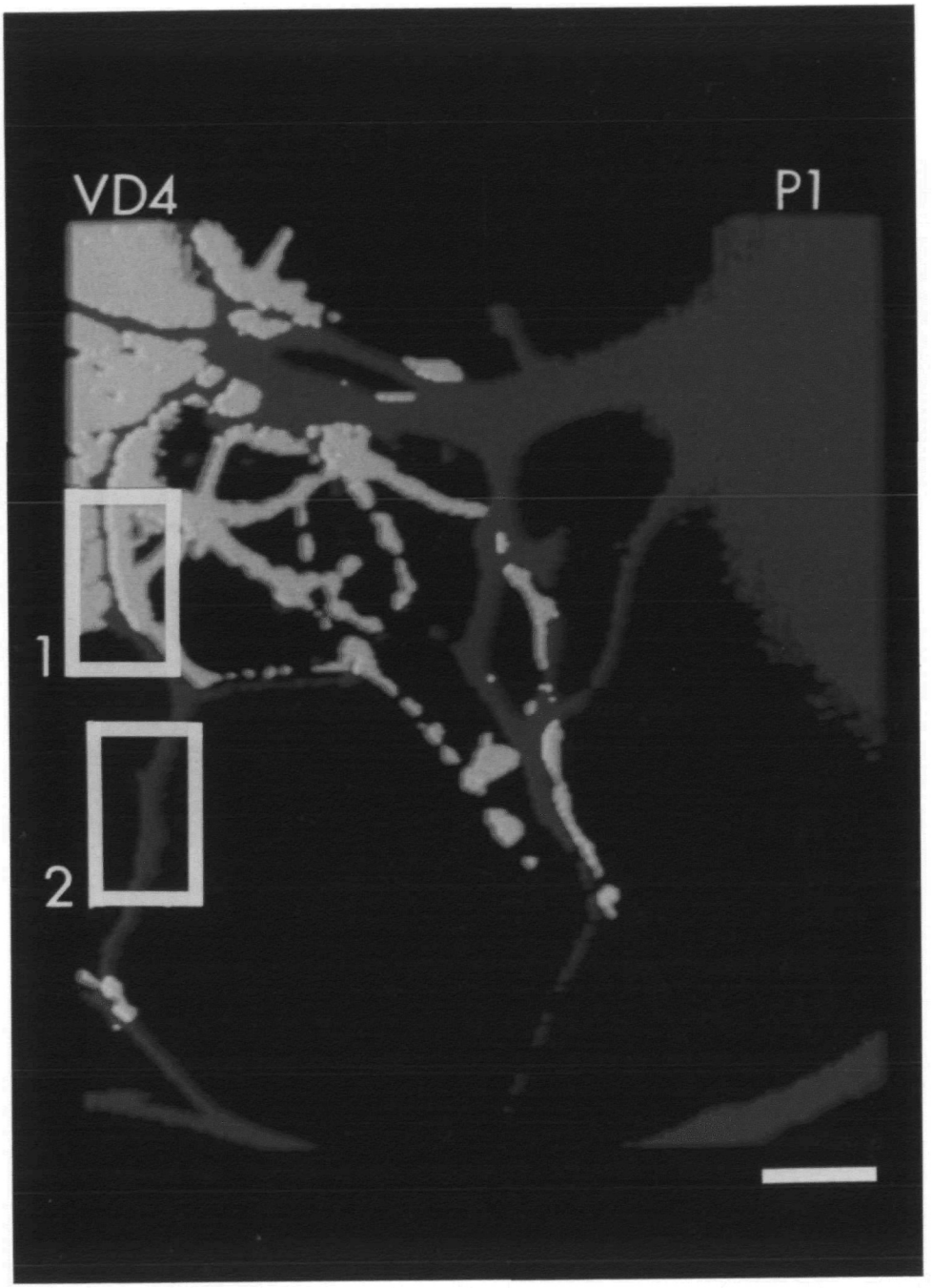

Fig. 6. Pseudocolour image of dye-filled neurones VD4 (pink=Lucifer Yellow) and P1 (blue=Fura-2). Region 1 shows an area of contact between the neurites of P1 and VD4. Region 2 delimits an area of P1 neurite without contact from VD4. Scale bar, 10 μm.

higher dose to test for the effects on calcium transients. Fig. 5 is a recording demonstrating the effects of bath-applied FMRFamide and GDPFLRFamide on the action-potential-evoked calcium transient in a region of the neurites of a P1 cell. In contrast to the minor effect of GDPFLRFamide on the macroscopic calcium current of P1 (7.7±6.4%, N=9; 10^{-5} mol l^{-1}, see Fig. 4C), the heptapeptide (N=10 from five cells; 10^{-5} mol l^{-1}) reversibly reduced the calcium transient by 60.4±8.1%. In addition to modulating calcium transients, application of all three FaRPs caused a simultaneous hyperpolarization of neurone P1.

VD4 stimulation modulates postsynaptic calcium influx

To determine whether VD4 stimulation also reduced the calcium transient in neurites of P1, pairs of VD4 and P1 neurones were plated into adhesive culture conditions and allowed to extend neurites to form cell–cell contacts. Neurone P1 was pressure-injected with Fura-2 and the calcium transients in its neurites were imaged using quantitative ratiometric imaging techniques. Fig. 6 shows a pair of synaptically connected neurones injected with fluorescent dyes. Two regions of interest in which ratiometric images were obtained are highlighted. The calcium levels of region 1 (an area of contact) are shown in Figs 7A and 8. Image pairs (340 nm, 380 nm excitation) were recorded at 4-s intervals and, after the third image pair had been acquired, neurone P1 was stimulated at a frequency of 4 Hz for 10 s to evoke 40 action potentials. Action potentials reliably increased the calcium level of P1 cell neurites. After recording three calcium transients (at 3-min intervals), peptidergic neurone VD4 was depolarized for 10 s to evoke a train of

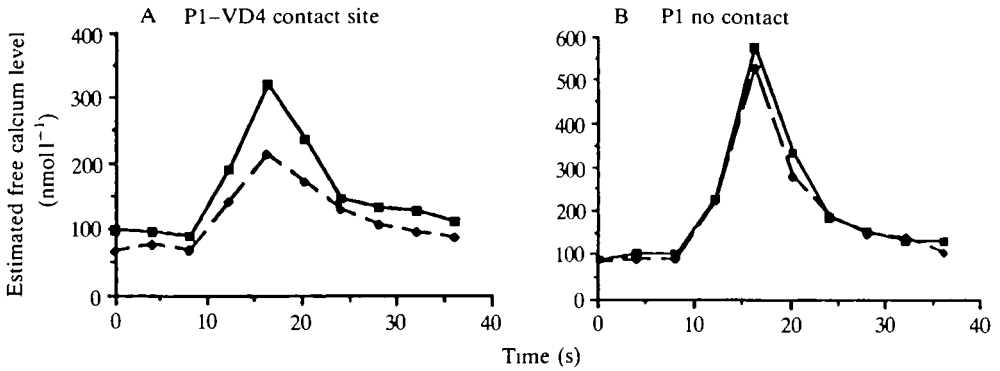

Fig. 7. VD4 stimulation modulates the calcium influx in neurites of P1. The calcium levels of neurone P1 in regions 1 (A) and 2 (B) of Fig. 6 are depicted. Neurone P1 was stimulated after the third calcium measurement in each data set, resulting in a transient rise in calcium level. Solid lines, P1 stimulation alone. Dashed lines, P1 stimulation following VD4 stimulation. (A) The stimulation of neurone VD4 caused a 30% reduction in the calcium influx, compared with that in response to P1 stimulation alone, in the neurites of P1 at a site of contact between neurones VD4 and P1. (B) Stimulation of neurone VD4 had a minimal effect (7% reduction) on the calcium influx in response to P1 stimulation at a site on P1 cell neurites where there was no VD4 contact.

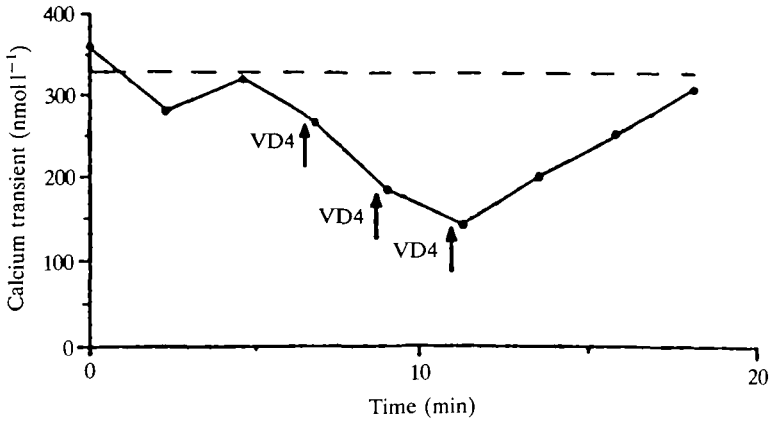

Fig. 8. Repeated stimulation of neurone VD4 promotes a cumulative decrease in the calcium transient in neurites of P1 in a region of contact between VD4 and P1 cells. The dashed line indicates the average calcium transient prior to the first stimulation of VD4 (321 nmol l^{-1}). Neurone VD4 was depolarized for 10-s periods (arrows) immediately before the activity-dependent calcium transient in P1 was recorded.

action potentials. In all preparations (N=4 cell pairs) the subsequent calcium transient in P1 was reduced in magnitude (Figs 7A, 8; N=8 sites of contact). In the example shown in Fig. 7, stimulation of P1 elevated the calcium level of region 1 (control conditions) from 87 to 322 nmol l^{-1} (Fig. 7A). Following VD4 stimulation, action potentials in P1 only raised the calcium level from 70 to 214 nmol l^{-1}. Thus, the calcium transient was reduced from 235 to 144 nmol l^{-1} following stimulation of peptidergic neurone VD4. Fig. 8 shows the magnitude of the calcium transient over time and the modulatory action of VD4. Multiple stimuli applied to VD4 at 3-min intervals caused a cumulative reduction in the calcium transient of P1, which reversed after cessation of VD4 stimulation.

Data were recorded from regions of P1 cell neurites that had direct physical contact with VD4 processes and from regions in which there was no discernible contact. VD4 stimulation reduced the calcium transient in P1 cell neurites by 30.6±2.79 % (mean±S.E.M.) at regions of contact with VD4 (N=8 regions from four cells). In contrast, VD4 stimulation had no significant effect on calcium transients (3.1±5.1 % reduction) in regions of non-contact (N=7 regions from four cells). This observation is illustrated in region 2 of the preparation in Fig. 6, where simultaneous dye-fills of P1 and VD4 were achieved. Stimulation of VD4 did not appreciably modulate the calcium transient in the non-contact region of P1 (7 % reduction; Fig. 7B), whereas the transient was reduced by 37 % in the region of contact (Fig. 7A).

Discussion

The exogenous application of synthetic peptides has been used, in the past, to

study peptidergic modulation of identified neurones. Through this approach the importance of peptides in the modulation of several ionic currents (Walker *et al.* 1988; Taussig *et al.* 1989; Bley and Tsien, 1990) and in the regulation of the secretory machinery (Man-Son-Hing *et al.* 1989; Dale and Kandel, 1990) has been demonstrated. However, global application of neuropeptides may not simulate the true nature of synaptic peptide release. In the present study we have established that neurone VD4 contains multiple peptides and that this neurone is the presynaptic element of an inhibitory connection with neurone P1 in the central nervous system of *H. trivolvis*. By successfully isolating the synapse in culture, we have been able to use this system to investigate the nature of the modulatory actions of a peptidergic neurone on its target.

Although VD4 exhibited FMRFamide-like immunoreactivity, the precise FaRP complement was unknown. Previous work on the CNS of *H. trivolvis* has identified FMRFamide, FLRFamide and GDPFLRFamide as the main congeners of FMRFamide (Bulloch *et al.* 1988). In *Lymnaea stagnalis* the mRNA encoding the tetrapeptides (FLRFamide and FMRFamide) is found in neurones distinct from those expressing heptapeptide-encoding mRNA (J. F. Burke, personal communication). The high levels of FMRFamide and FLRFamide found in VD4 of *H. trivolvis* suggest that this neurone is primarily a 'tetrapeptide' neurone. However, the existence of a small peak at the GDPFLRFamide elution position indicates that, in *H. trivolvis*, individual neurones may express both tetrapeptides and extended FaRPs.

The reconstruction of the VD4/P1 synapse in culture provides an accessible system for investigating the consequences of peptide release from neurone VD4 onto its target. Stimulation of VD4 has multiple modulatory actions on neurone P1. First, a hyperpolarization occurs in P1 as a result of activity in VD4. The effect of this action of VD4 stimulation is to reduce the excitability of P1. The ionic basis of the hyperpolarization in P1 has not been fully characterized, but preliminary evidence suggests that it is mediated by an increase in a potassium conductance (J. E. Richmond, unpublished observations). Such increases in potassium conductance due to exogenous FMRFamide application have been reported in other molluscan neurones (Belardetti *et al.* 1987; Taussig *et al.* 1989). Second, the activity-dependent calcium transient in P1 is reduced following stimulation of VD4. This decrease in calcium entry may be attributable, in part, to a reduction in the voltage-activated calcium current of P1 by the release of FMRFamide and FLRFamide, as demonstrated by the exogenous application of FaRPs to voltage-clamped spherical P1 neurones. However, it is likely that the modulation of the calcium transient is also due to additional mechanisms, since the heptapeptide GDPFLRFamide, which had little effect on the calcium current, reliably reduced the calcium transient. We propose that the FaRPs endogenous to neurone VD4 have a compound action on calcium influx (1) through a reduction of the HVA calcium current and (2) through a hyperpolarization (possibly due to an increased potassium conductance), causing a further reduction in calcium influx during P1 cell action potentials.

We wish to thank Drs Mark Zoran, Fred Bahls and Helen Man-Son-Hing, and Trent Basarsky, for constructive criticism of the manuscript, and Drs A. Don Murphy, N. Syed and J. F. Burke for personal communications. This work was supported by NIH grants NS26650 and NS24233 (P.G.H.) and NIH grant HL28440 (D.A.P.) and the Iowa State University Biotechnology Council. P.G.H. is an Alfred P. Sloan Fellow.

References

BELARDETTI, F., KANDEL, E. R. AND SIEGELBAUM, S. A. (1987). Neuronal inhibition by the peptide FMRFamide involves opening of S K^+ channels. *Nature* **325**, 153–156.

BENJAMIN, P. R. (1984). Interneuronal network acting on snail neurosecretory neurones (yellow cells and yellow-green cells of *Lymnaea*). *J. exp. Biol.* **113**, 165–185.

BEWICK, G. S., PRICE, D. A. AND COTTRELL, G. A. (1990). The fast response mediated by the C3 motoneurone of *Helix* is not attributable to the contained FMRFamide. *J. exp. Biol.* **148**, 201–219.

BLEY, K. R. AND TSIEN, R. W. (1990). Inhibition of Ca^{2+} and K^+ channels in sympathetic neurones by neuropeptides and other ganglionic transmitters. *Neurone* **2**, 379–391.

BREZINA, V., ECKERT, R. AND ERXLEBEN, C. (1987). Suppression of calcium currents by an endogenous neuropeptide in neurones of *Aplysia californica*. *J. Physiol., Lond.* **388**, 565–595.

BULLOCH, A. G. M., PRICE, D. A., MURPHY, A. D., LEE, T. D. AND BOWES, H. N. (1988). FMRFamide peptides in *Helisoma*: Identification and physiological actions at a peripheral synapse. *J. Neurosci.* **8**, 3459–3469.

CHRONWALL, B. M., OLSCHOWKA, J. A. AND O'DONOHUE, T. L. (1984). Histochemical localization of FMRFamide-like immunoreactivity in the rat brain. *Peptides* **5**, 569–584.

COATES, C. J. AND BULLOCH, A. G. M. (1985). Synaptic plasticity in the molluscan peripheral nervous system: Physiology and role for peptides. *J. Neurosci.* **5**, 2677–2684.

COHAN, C. S., CONNOR, J. A. AND KATER, S. B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. *J. Neurosci.* **7**, 3588–3599.

COLOMBAIONI, L., PAUPARDIN-TRITSCH, D., VIDAL, P. P. AND GERSCHENFELD, H. M. (1985). The neuropeptide FMRF-amide decreases both the Ca^{2+} conductance and a cyclic 3′,5′-adenosine monophosphate-dependent K^+ conductance in identified molluscan neurones. *J. Neurosci.* **5**, 2533–2538.

COTTRELL, G. A., DAVIES, N. W. AND GREEN, K. A. (1984). Multiple actions of a molluscan cardioexcitatory neuropeptide and related peptides on identified *Helix* neurones. *J. Physiol., Lond.* **356**, 315–333.

DALE, N. AND KANDEL, E. R. (1990). Facilitatory and inhibitory transmitters modulate spontaneous transmitter release at cultured *Aplysia* sensorimotor synapses. *J. Physiol., Lond.* **421**, 203–222.

GRYNKIEWKZ, G., POENIE, M. AND TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260**, 3440–3450.

HAYDON, P. G. (1988). The formation of chemical synapses between cell-cultured neuronal somata. *J. Neurosci.* **8**, 1032–1038.

HAYDON, P. G., COHAN, C. S., MCCOBB, D. P., MILLER, H. R. AND KATER, S. B. (1985). Neurone-specific growth cone properties as seen in identified neurones of *Helisoma*. *J. Neurosci. Res.* **13**, 285–300.

HAYDON, P. G. AND MAN-SON-HING, H. (1988). Low- and high-voltage activated calcium currents: Their relationship to the site of neurotransmitter release in an identified neurone of *Helisoma*. *Neuron* **1**, 919–927.

KRAMER, R. H., LEVITAN, E. S., CARROW, G. M. AND LEVITAN, I. B. (1988). Modulation of a subthreshold calcium current by the neuropeptide FMRFamide in *Aplysia* neuron R15. *J. Neurophysiol.* **60**, 1728–1738.

MAN-SON-HING, H., ZORAN, M. J., LUKOWIAK, K. AND HAYDON, P. G. (1989). A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. *Nature* **341**, 237–239.

MURPHY, A. D., LUKOWIAK, K. AND STELL, W. K. (1985). Peptidergic modulation of patterned motor activity in identified neurones of *Helisoma*. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7140–7144.

O'DONOHUE, T. L., BISHOP, J. F., CHRONWALL, B. M., GROOME, J. AND WATSON, W. H. (1984). Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system. *Peptides* **5**, 563–568.

PRICE, D. A. (1986). Evolution of a molluscan cardioregulatory neuropeptide. *Am. Zool.* **26**, 1007–1015.

PRICE, D. A. AND GREENBERG, M. J. (1977). Structure of a molluscan cardioexcitatory peptide. *Science* **197**, 670–671.

PRICE, D. A., LESSER, W., LEE, T. D., DOBLE, K. E. AND GREENBERG, M. J. (1990). Seven FMRFamide-related and two SCP-related cardioactive peptides from *Helix*. *J. exp. Biol.* **154**, 421–437.

SYED, N. I., BULLOCH, A. G. M. AND LUKOWIAK, K. (1990). *In vitro* reconstruction of the respiratory central pattern generator of the mollusc *Lymnaea*. *Science* **250**, 282–285.

TAUSSIG, R., SWEET-CORDERO, A. AND SCHELLER, R. H. (1989). Modulation of ionic currents in *Aplysia* motor neurone B15 by serotonin, neuropeptides and second messengers. *J. Neurosci.* **9**, 3218–3229.

WALKER, M. W., EWALD, D. A., PERNEY, T. M. AND MILLER, R. J. (1988). Neuropeptide Y modulates neurotransmitter release and Ca^{2+} currents in rat sensory neurones. *J. Neurosci.* **8**, 2438–2446.

WONG, R. G., HADLEY, R. D., KATER, S. B. AND HAUSER, G. C. (1981). Neurite outgrowth in molluscan organ and cell cultures: The role of conditioning factor(s). *J. Neurosci.* **1**, 1008–1021.