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MULTIPLE ACTIONS OF A MOLLUSCAN CARDIOEXCITATORY NEUROPEPTIDE AND RELATED PEPTIDES ON IDENTIFIED *HELIX* NEURONES

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SUMMARY

1. The effects of the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRF amide) and related peptides (Price & Greenberg, 1977) were tested on *Helix aspersa* neurones.

2. Ionophoretic application of FMRFamide depolarized and excited some neurones, but hyperpolarized and inhibited others. In some neurones the sign of the response was dependent on the membrane potential.

3. Two responses resulted from an increase in membrane conductance, a depolarizing response mediated mainly by an increase in Na^+ ion permeability, and a hyperpolarizing response mediated by an increase in K^+ ion permeability.

4. In the C1 neurone a voltage-dependent response was observed, which only occurred when the neurone was depolarized from its resting level. This response was recorded as an inward current during voltage clamp and resulted from a decrease in K current(s), possibly Ca-activated K current.

5. More than one response may occur in a single neurone. In the C1 neurone, the K-mediated hyperpolarization occurred as well as the voltage-dependent response, while the depolarization seen in the F2 neurone was a combination of an increase in Na conductance and an increase in K conductance.

INTRODUCTION

Recent advances describing the occurrence and cellular localization of several neuropeptides have renewed interest in their possible roles and cellular actions in nervous tissue. The neurosecretory function of many of these compounds is well known, and some may be transmitter substances (see e.g. Dodd & Kelly, 1978; Jan, Jan & Kuffler, 1979; Hökfelt, Johansson, Ljungdahl, Lundberg & Schultzberg, 1980) whereas others may influence neuronal activity in other ways (Barker, 1977).

Ganglia of the clam *Macrocallista* contain the peptide Phe-Met-Arg-Phe-NH_2 or FMRFamide (Price & Greenberg, 1977). This compound has a marked stimulating effect on isolated heart preparations of a number of molluscan species and produces also, in very low concentrations, contractures of certain non-cardiac muscles (Greenberg & Price, 1979). A structurally similar peptide, having one or more additional

amino acids combined with the terminal free amino nitrogen, is present in the ganglia of the snail *Helix* (Price, 1982).

The present report describes the effects of FMRFamide, and of two homologous peptides, Phe-Nle-Arg-Phe-NH_2 (F*n*LRFamide) and Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH_2 (YGGFMRFamide), on a number of different specified neurones in the ganglia of *Helix aspersa*. Some of this work has been published in abstract (Cottrell, 1982*a*).

METHODS

Experiments were made on identified neurones in the cerebral, visceral and right parietal ganglia of *Helix aspersa*. The ganglia were pinned to the nylon base of a 0·8 ml recording chamber and the neurones were completely desheathed before penetration.

Intracellular recordings were made using micropipettes filled with either 2M-K acetate or 3M-KCl. In most experiments recordings were made using a Dagan 8100 single-electrode clamp system. This made both current- and voltage-clamp possible with a single micro-electrode (Wilson & Goldner, 1975). Some experiments were also made, however, with a two-electrode system which gave identical results.

FMRFamide, YGGFMRFamide (Peninsula Laboratories, San Carlos, CA) and F*n*LRFamide (kindly supplied by Dr J. Morley) were ionophoresed from pipettes containing 4–10 mM-peptide in distilled water. Physiological solution, flowing over the preparation at approximately 3 ml/min, had the following composition (mM): NaCl, 80; KCl, 5; $CaCl_2$, 7; $MgCl_2$, 5; buffered with Tris/HCl 5 mM, pH 7·7, or HEPES/NaOH 20 mM, pH 7·5. Both solutions gave identical results. The experiments were made at room temperature, 17–24 °C.

For the Ca injection experiments a second micropipette containing 0·5 M-$CaCl_2$ was inserted into the cell. Ca^{2+} ions were then ionophoresed into the cell during voltage clamp.

Single-channel recordings were made from the soma membrane of the C1 neurone using the high resolution patch-clamp techniques described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Desheathed C1 neurones in isolated cerebral ganglia preparations were exposed to physiological solution containing approximately 0·1 % trypsin (Sigma type IX) for 1 h, to render the membrane clean enough for 'giga seal' formation. Analogues of FMRFamide were applied by diffusion from the tip of a blunt micropipette containing a high concentration (10^{-4} M in physiological solution) of the peptide, positioned near to the cell soma.

RESULTS

Preliminary experiments showed that some neurones in the ganglia were excited whereas others were inhibited by FMRFamide. More detailed examination revealed the existence of at least three basic responses to the peptide: (1) a hyperpolarization resulting from an increase in conductance, (2) a depolarization resulting from an increase in conductance, (3) a more complicated response involving a voltage-dependent decrease in conductance. A combination of two or more of these basic responses occurred in many neurones, thereby complicating the analysis of ionic mechanisms.

Hyperpolarization resulting from an increase in conductance

Several neurones in the suboesophageal ganglia and the C1 neurone in each cerebral ganglion gave similar, hyperpolarizing responses to locally applied FMRFamide. Repeated application of the peptide did not lead to a desensitization of this response. The amplitude of electronic potentials resulting from hyperpolarizing current pulses was decreased during the response, suggesting that the peptide causes an increase in membrane conductance, presumably to Cl^- or K^+ ions. Under voltage clamp, an

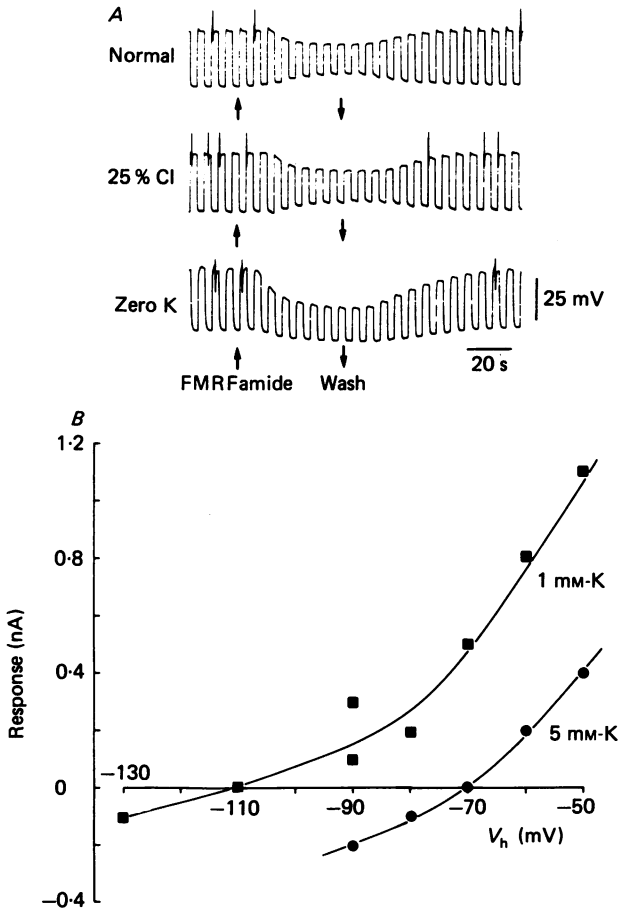

Fig. 1*A*, the C1 neurone was exposed to medium containing 8×10^{-6} M-FMRFamide during the period indicated on each record by the arrows. Throughout each recording, constant-current hyperpolarizing pulses were applied across the membrane to monitor changes in input resistance and reversal potential of the response. Evidence for a reduction in input resistance during FMRFamide application is seen in each record. In the absence of K, the reversal potential of the response was shifted in a negative direction. *B*, influence of holding potential (V_h) and extracellular K concentration on the magnitude and direction of the current response observed when the FMRFamide was ionophoretically applied onto a voltage-clamped C1 neurone. The reversal potential in normal medium was −70 mV. When the external K concentration was reduced to 1 mM, the reversal potential was shifted to −110 mV.

outward current was detected which could be reversed to an inward current at holding potentials −70 to −75 mV.

Reduction in extracellular Cl concentration (80 mM-Na isethionate substituted for NaCl) did not affect the peptide-induced hyperpolarization, but a reduction in extracellular K concentration produced a marked shift, in the negative direction, of the reversal potential of the response (Fig. 1). The relationship between current response and membrane potential is shown in Figs. 1*B* and 2*B*. In the experiment

shown in Fig. 1*B*. there was a shift in E_{rev} of −40 mV when the external K concentration was reduced from 5 to 1 mM. This shift is very close to the theoretical value of −40·6 mV for a response mediated by K^+ ions alone, as calculated from the Nernst equation. The relationship between response and membrane potential is non-linear, and is similar to that found for the carbachol-induced K current in an *Aplysia* neurone observed by Ginsborg & Kado (1975).

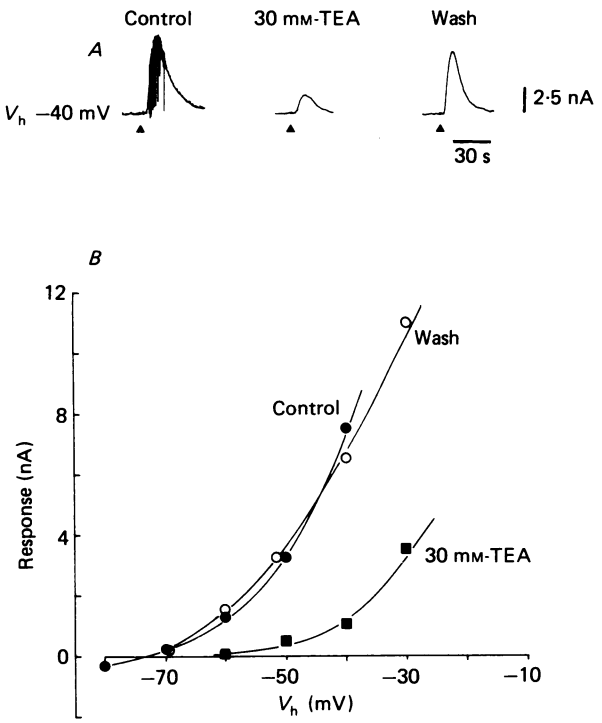

Fig. 2. The effect of TEA on the K response of a cell in the right parietal ganglion. *A*, current responses to ionophoresed FMRFamide (▲) with the cell clamped at −40 mV. Exposure to TEA (30 mM) reversibly reduced the response. *B*, influence of holding potential (V_h) on the response in the presence and absence of TEA.

Exposure of the preparation to 30 mM-tetraethylammonium (TEA) for 4–6 min reduced the response but did not completely block it (Fig. 2). TEA was substituted for NaCl, and hence controls for this and subsequent experiments reported where TEA was used were made in solution in which a corresponding amount of NaCl was substituted with sucrose. TEA is an agent known to depress K responses (Hille, 1970; Hermann & Gorman, 1981). Cooling the preparation to 5 °C, which is also known to suppress a number of K-mediated potentials in molluscan neurones (Kehoe, 1972; Swann & Carpenter, 1975), reduced the response.

Intracellular Cs^+ ions have been used to suppress K currents (Kehoe, 1972; Akaike, Lee & Brown, 1978; Tillotson & Horn, 1978). The effect of Cs on the FMRF amide-induced hyperpolarization in the C1 neurone was investigated using 2M-

CsCl-filled recording electrodes. Fig. 3 shows that the response to the peptide declined rapidly with time after impalement of the cell with a CsCl recording electrode. Under these conditions the outward K currents normally seen at depolarized potentials were considerably reduced. This property of Cs^+ ions was used to facilitate analysis of the fast depolarizing response observed in some neurones (see below).

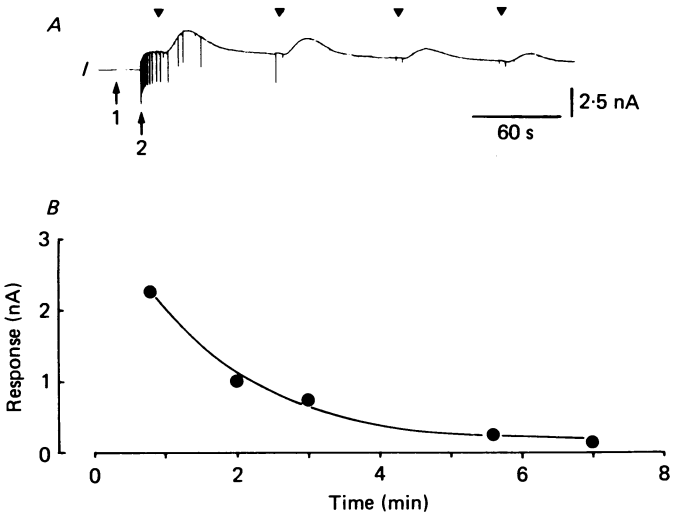

Fig. 3. *A*, current trace of a voltage-clamped C1 neurone impaled with a recording electrode containing 2M-CsCl. At point 1 the cell was impaled and at point 2 the neurone membrane was clamped to −40 mV (voltage trace not shown). The outward current response to ionophoretically applied FMRFamide (▼) rapidly diminishes with time. *B*, the results of a similar experiment in which the amplitude of the response to FMRFamide is plotted against time after impalement.

Depolarization resulting from an increase in conductance

Fewer neurones were observed to be depolarized than to be hyperpolarized by FMRFamide. One readily identifiable neurone which was depolarized by FMRF amide was located in the same position of the visceral ganglion as neurone E13 (see Kerkut, Lambert, Gayton, Loker & Walker, 1975). Repeated local applications of FMRFamide led to a desensitization of the response in this neurone (Fig. 4*A*).

When the neurone was voltage clamped, a fast inward current was observed following ionophoretic application of the peptide. During the response an increase in the amplitude of current pulses resulting from hyperpolarizing command pulses was observed, suggesting an increase in membrane conductance (Fig. 4*B*). Even when clamped to −10 mV, this inward current still persisted, suggesting a major contribution from a current carried by Na^+ or Ca^{2+} ions or both.

Co has been shown to block Ca current in *Helix* neurones (Akaike *et al.* 1978). When 1 mM-Co was present in the extracellular fluid the FMRFamide-induced response was unaffected. On the other hand, exposure of the preparation to Na-free medium (NaCl replaced with sucrose) for 12 min completely abolished the inward current over a

range of holding potentials from −70 to −30 mV. The response returned after a period of washing with normal medium (Fig. 5*A*). Physiological solution containing 30 mM-TEA had no effect on the response (Fig. 5*B*).

Impaling the neurone with a CsCl recording electrode resulted in a marked reduction of the outward K currents normally seen at depolarized holding potentials.

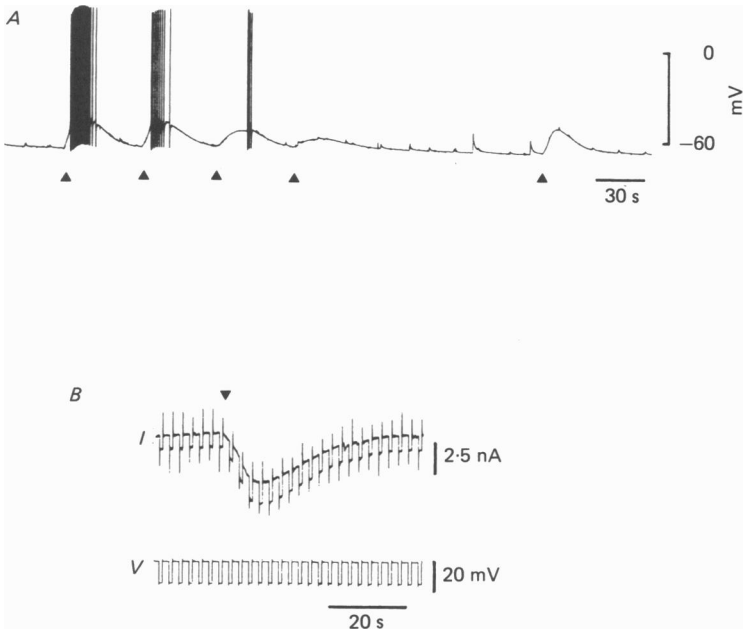

Fig. 4. The effect of ionophoresed FMRFamide (▲) on a neurone, E13, in the visceral ganglion. *A*, repeated application led to a desensitization of the depolarizing response. The response recovered after about 2 min in the absence of the peptide. *B*, the response recorded as an inward current when the membrane of the same cell was clamped at −50 mV. Constant amplitude hyperpolarizing command pulses were applied during the recording. The increase in the amplitude of the current deflexions during the response suggests that the peptide caused an increase in membrane conductance.

This enabled the cell to be clamped at more positive potentials. The presence of Cs^+ ions in the cell did not however affect the FMRFamide response. Fig. 6 shows the linearity of the relationship between current response and membrane potential. The peptide-induced current was still inward at +20 mV. Extrapolation of a line fitted by linear regression predicts a reversal potential of +40 mV. This value is lower than the calculated value of +85 mV for the Na equilibrium potential (E_{Na}) in these cells (Thomas, 1972).

These data taken together suggest that the fast depolarization caused by FMRF amide is mediated mainly, but not exclusively, by Na^+ ions.

Voltage-dependent depolarization resulting from a decrease in conductance

When the peptide-induced response was recorded at depolarized levels in the C1 neurone another response, inward in sign, became apparent (Cottrell, 1982*a*).

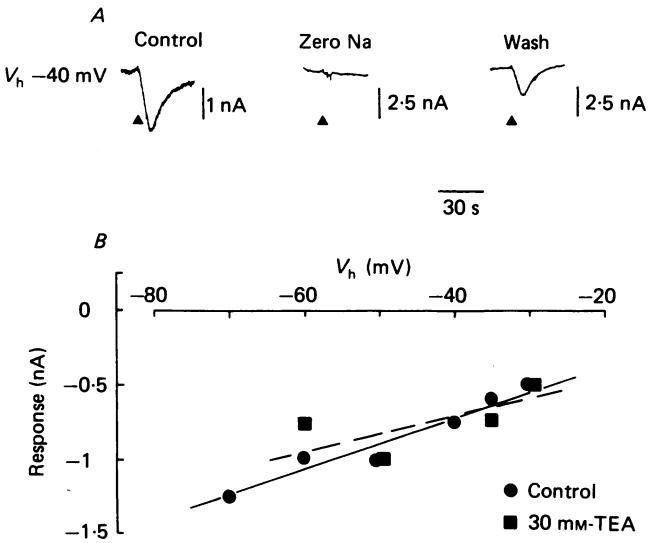

Fig. 5. FMRFamide responses of a neurone, E13, in the visceral ganglion. *A*, current recordings at a holding potential of −40 mV. Note the difference in scale. Exposure to zero Na medium abolished the inward current response, which recovered after washing. *B*, comparison of the response in control solution and solution containing 30 mM-TEA. TEA at this concentration had little or no effect on the response.

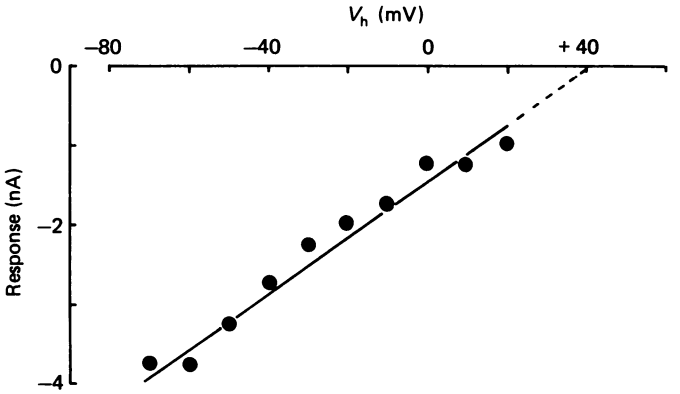

Fig. 6. The influence of holding potential on the fast depolarizing response to FMRFamide in the presence of intracellular Cs^+ ions (see text). The dashed line shows the extrapolation of the line fitted by linear regression to give an E_{rev} of +40 mV.

Responses at holding potentials from about −40 to −20 mV frequently comprised two components: (1) the outward K current described above and (2) an inward current response. At more depolarized levels, the inward current response predominated. The peptide appeared to cause a reduction in current passage across the membrane during the second response, suggesting that it could result from a reduction in conductance to an ion(s) or alternatively to a voltage-dependent increase in conductance to Na^+ or Ca^{2+} ions or both. This response could also be detected as a depolarization under current clamp if the membrane was sufficiently depolarized.

In some experiments, it was difficult to observe the inward current response with FMRFamide. Two other related peptides were tested, YGGFMRFamide and F*n*LRF amide. Both of those produced the inward and outward current response but the heptapeptide amide was more active in producing the inward current response. In some preparations, with the ionophoretic pipette fortuitously positioned, an inward current alone was elicited by YGGFMRFamide. This observation led us to use YGGFMRFamide to study the inward current response.

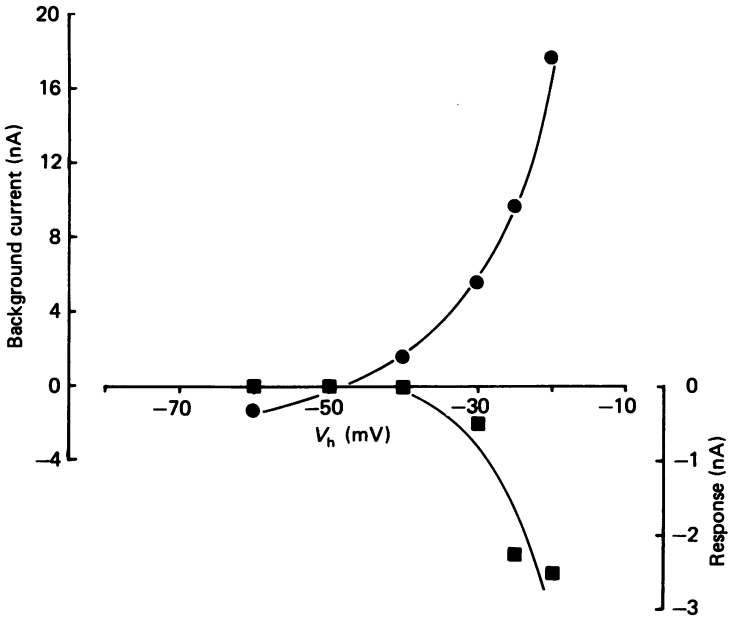

Fig. 7. Relationship between the amplitude of the voltage-dependent response and membrane potential in a C1 neurone compared to the steady-state *I–V* curve of the same cell. ■, response current; ●, background current. The background current was measured just before application of the peptide.

In Fig. 7 the voltage dependence of the response is compared with the *I–V* curve of the C1 neurone. The response began to occur at about the same potential at which the delayed rectification was activated. The ionic mechanism of this response was studied by Cottrell (1982*a*), and therefore only a summary of those results previously described will be given here, along with results from some additional experiments.

When the extracellular Na and Cl concentrations were altered, there was little or no change in the response, suggesting that neither Na^+ ions nor Cl^- ions are involved. An involvement of Ca did seem likely, as addition of 1 mM-$CoCl_2$ markedly reduced the response. It is known that Ba can carry the Ca current in snail neurones (Akaike *et al.* 1978). When $BaCl_2$ was substituted for most of the $CaCl_2$ (6 mM-$BaCl_2$, 1 mM-$CaCl_2$), the response was also abolished. This result suggested that the response was not simply due to an influx of Ca^{2+} ions but that it also involved a suppression of a K current because Ba^{2+} ions are known to block K currents (Werman & Grundfest, 1961).

The involvement of K was also suggested in preparations in which the inward current alone was detected. In these preparations, the inward current response recorded at −30 mV was increased on reducing the external K concentration from 5 to 1 mM. These observations suggested that the inward current response could therefore result from a suppression of a Ca-activated K current.

TEA at 30 mM could be used to unmask the inward current when accompanied with the outward current response. There is some variability in the sensitivity of the delayed K and the Ca-activated K currents to different concentrations of TEA in molluscan neurones. In some neurones, both currents are equally affected whereas in others the Ca-activated K current seems to be less susceptible (Meech & Standen, 1975; Thompson, 1977; Hermann & Gorman, 1981).

An action of the peptides on the Ca-activated K current was investigated further by testing their effect on the increased K current evoked by intracellular injection of Ca^{2+} ions. In this extensive series of experiments, Ca^{2+} ions were injected ionophoretically. An electrode containing 0·5 M-$CaCl_2$ was inserted in the neurone and a positive current of about 40 nA passed for 5–15 s with respect to another micropipette placed in the medium. The neurone was voltage clamped during the injection and the membrane potential therefore remained unchanged or was only shifted by about 5 mV during the injection period. The increased current evoked by Ca injection was reversed in sign between −70 and −80 mV in normal medium and at more negative potentials in medium with reduced KCl, as previously observed in other snail neurones (Meech, 1974). This established that the current evoked by Ca injection was carried by K^+ ions.

In eight C1 neurones, it was observed that the inward current elicited by either FMRFamide, F*n*LRFamide or YGGFMRFamide was increased in amplitude when the background outward current was increased by injection of Ca. Demonstration of this effect depended on whether the increased outward current persisted for sufficiently long to test the effect of applied peptide, which in turn presumably related to a number of factors that influence the level of free Ca^{2+} ions close to the inner surface of the membrane (Barish & Thompson, 1983). Fig. 8 shows the effect of Ca injection in one cell clamped at −40 mV. The increase in the amplitude of the response to the peptide observed after increasing the size of the outward directed Ca-activated K current suggests that an action of YGGFMRFamide in producing this response is to suppress a Ca-activated K current.

Single-channel recordings

Recordings made from cell-attached membrane patches showed single or multiple unitary outward current events when held at depolarized potentials. Two different sizes of current were commonly seen with slope conductances of about 19 and 52 pS when the patch of membrane was clamped to potentials of +10 mV. Since the larger currents predominated, we restricted our study to possible effects on these currents. The larger single-channel currents were measured with cell-attached patches on several C1 neurones. Currents were only observed when the electrode was clamped at negative potentials, i.e. with the membrane held at depolarized levels. A graph showing the relationship between single-channel current and holding potential on one patch is shown in Fig. 9. The resting membrane potential has been assumed to be

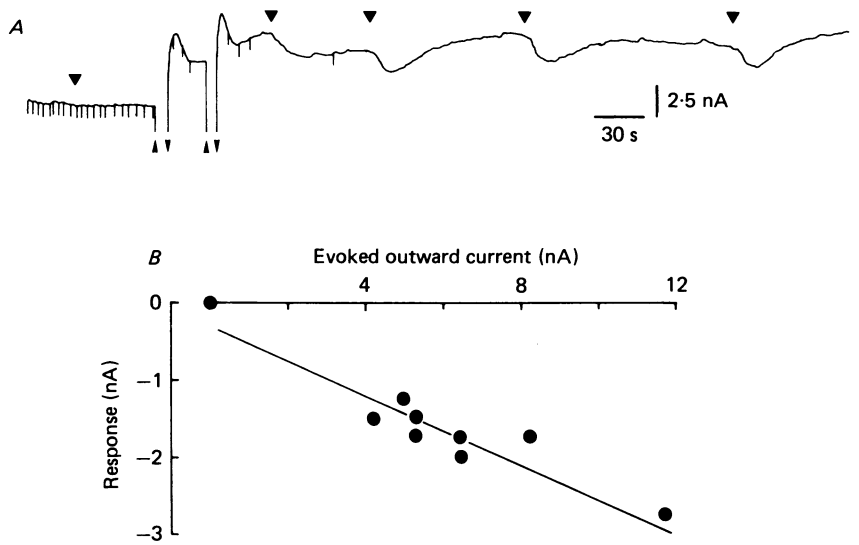

Fig. 8. Effect of YGGFMRFamide on the increased K current evoked by intracellular application of Ca^{2+} ions. *A*, current record of a C1 neurone clamped to −40 mV. The peptide was applied locally to the neurone. Before Ca injection, only a small inward current was seen in response to the peptide. After two Ca injections of 60 nA (seen as downward deflexions in the current record), there was an increase in the background K current and repeated application of the peptide produced much larger responses than before. Ionophoretic artifacts have been removed from this record. *B*, a graphical presentation of the data. The abscissa is the increased outward current caused by the Ca injection, the ordinate is the amplitude of the inward current response to YGGFMRFamide. The size of the response appears to be directly proportional to the amplitude of the evoked Ca-activated K current. These experiments were performed in normal physiological solution.

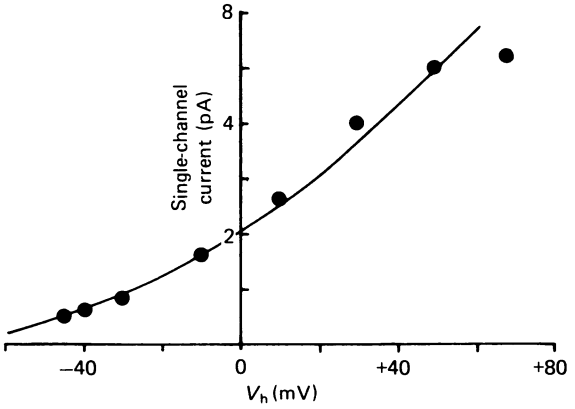

Fig. 9. Graph showing single-channel current amplitudes (●) plotted against patch holding potential for channels activated by depolarization in the C1 neurone. The K concentration in the patch pipette was 5 mM. The curve shows a theoretical fit to the data calculated from the Goldman–Hodgkin–Katz equation on the basis of a K channel permeability of $25{\cdot}1 \times 10^{-14}$ cm^3 s^{-1} and $E_m = -50$ mV (see text).

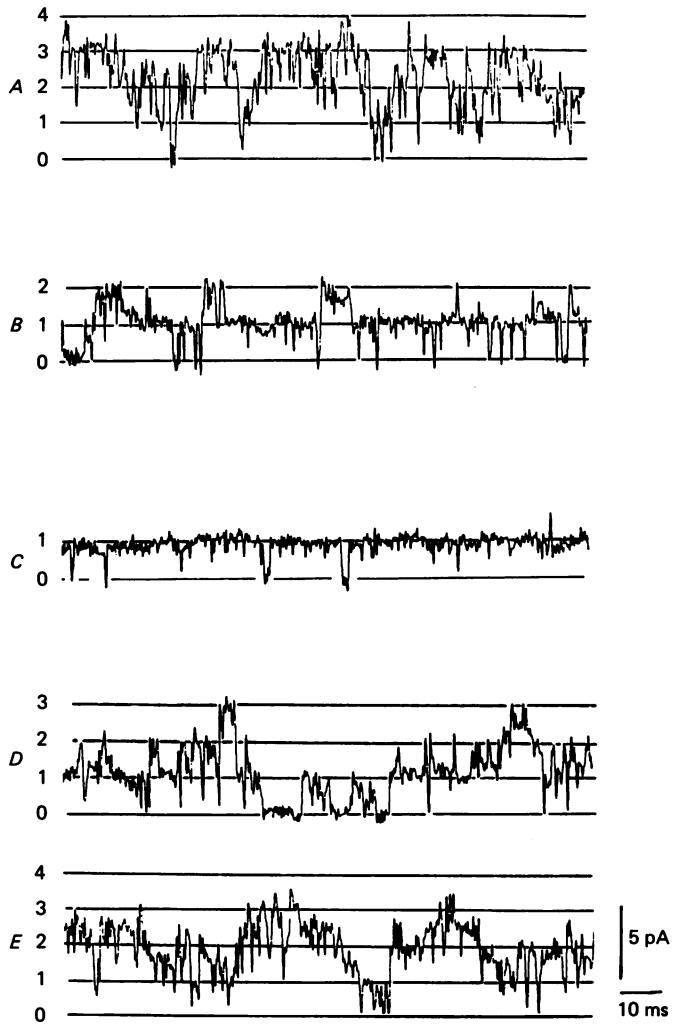

Fig. 10. Patch-clamp recordings from the C1 neurone. This cell-attached patch was clamped to a potential of approximately +20 mV. In the control record (*A*), multiple unitary current steps are seen (0 is the closed state and upward deflexions corresponds to outward currents). Recording *B* was obtained 30 s after positioning a blunt micropipette containing 10^{-4} M-F*n*LRFamide near to the neurone soma. In comparison to *A* the number of step openings has been reduced to single or occasional double events only. After 1 min (trace *C*) there was a further reduction in the number of events with some periods of total closure interrupted mainly by single openings. The effect was reversible, with traces *D* and *E* being obtained 1 min and 2 min respectively following removal of the peptide-containing micropipette. K^+ ion concentration in the patch pipette was 5 mM.

−50 mV. This is the average value obtained in more than 100 neurones using intracellular recording methods; a value close to −50 mV was also obtained in several of the patch-clamp experiments when the neuronal membrane was ruptured inadvertently and the patch electrode entered the cell. The continuous line in Fig. 9 is the relationship predicted by the Constant Field equation (Hodgkin & Katz,

1949), with a pK = $25{\cdot}1 \times 10^{-14}$ cm^3/s and $[K]_i$ = 98 mM (cf. Alvarez-Leefmans & Gamiño, 1982).

The amplitude of the single current was dependent on the concentration of K^+ ions in the patch pipette. For example, average values obtained at a pipette potential of −70 mV (equivalent to a membrane potential of approximately +20 mV: see above) were 3·44 pA (n = 2) in 1 mM-K, 2·63 pA (n = 3) in 5 mM-K and 1·54 (n = 2) in 20 mM-K. These data suggest that the larger currents are K currents.

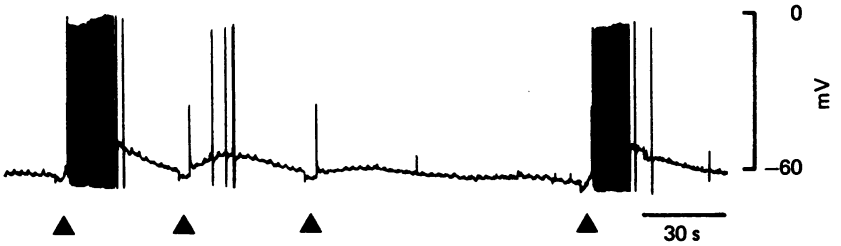

Fig. 11. Repeated application of FMRFamide (▲) onto the F2 neurone led to rapid desentization of the depolarizing response. The response recovered after about 90 s in the absence of the peptide.

The method of application of the FMRFamide analogue allowed access of the peptides to the whole of the C1 neuronal membrane other than the patch under the recording electrodes. In four recordings from the C1 neurone at depolarized holding potentials it was possible repeatedly and reversibly to reduce the number of channel openings observed when the neuropeptide containing electrode was positioned near to the cell soma. The onset of the effect was slow, the maximum effect being seen after a delay of 30 s–1 min. The effect is illustrated in Fig. 10. The top trace shows a control recording obtained before application of the peptide; several channels were present under this patch with up to four different current steps being observed. On positioning another electrode containing 10^{-4} M-F*n*LRFamide near to the cell soma there was a gradual reduction in the number of steps to the higher current levels, so that after a period of 1 min only single or occasionally double steps were observed. The effect was slowly reversed over a period of 2 min, as is shown in the lower two traces. Similar effects were seen with YGGFMRFamide. The method of application of the peptide suggests an indirect mechanism for the observed reduction in unitary current events.

(With normal physiological solution present in the electrode, no unitary currents were seen at the resting potential of the cell. When peptide was included in the patch electrode, again no channels were observed at the resting potential to account for the hyperpolarizing response of the neurone to FMRFamide described above. The absence of single-channel currents under these conditions suggests that receptors responsible for the outward current response were not present in the patches of membrane from which recordings were made, or that the current was too small to be detected.)

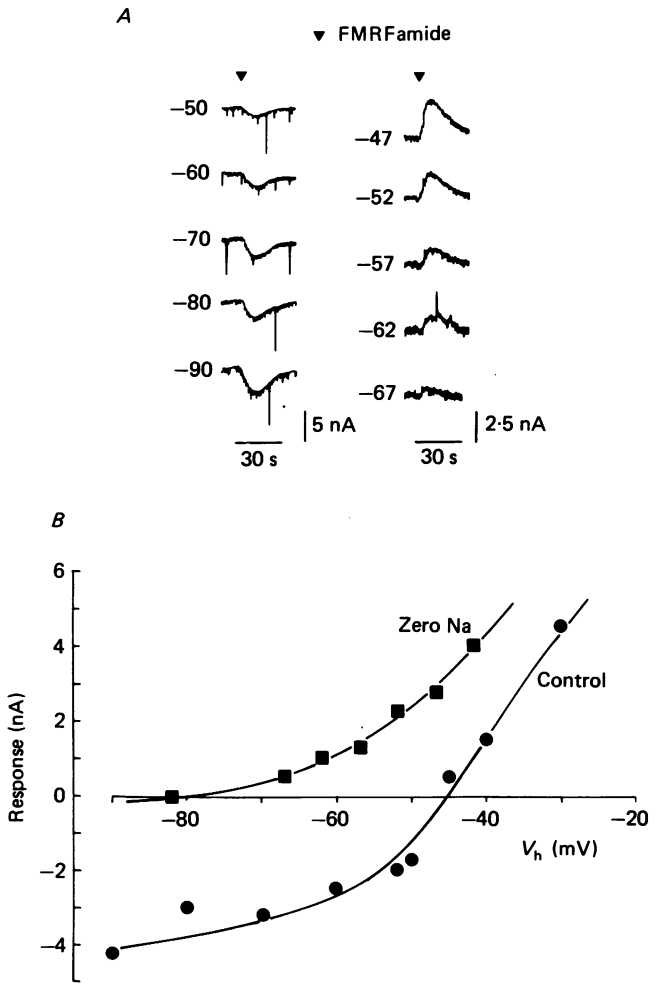

Fig. 12. Current responses observed in normal and zero-Na medium when FMRFamide was applied locally to the F2 neurone. *A*, exposure to zero-Na medium revealed an outward current component of the response. *B*, the influence of holding potential on the response in both normal and zero-Na medium.

Neurones showing multiple responses

Ionophoretic application of FMRFamide onto the membrane of many neurones led to a combination of the above response. In the previous section a combination of the K-mediated hyperpolarization and the voltage-dependent depolarization was described in the C1 neurone (Cottrell, 1982*a*).

The other major combination of responses was seen in an identified neurone in the right parietal ganglion, the F2 neurone. Application of FMRFamide at the resting potential depolarized and excited this neurone. Repeated application led to a desensitization of the response; recovery occurred after a short period in the absence of the peptide (Fig. 11). E_{rev} varied from −50 to −25 mV in F2 neurones in different

preparations; the relationship between holding potential and response was non-linear (Fig. 12*B*).

Exposure of the preparation to zero-Na solution abolished the inward current component and revealed an outward component which did not invert until about −80 mV (Fig. 12). As with the previously described hyperpolarization, this outward

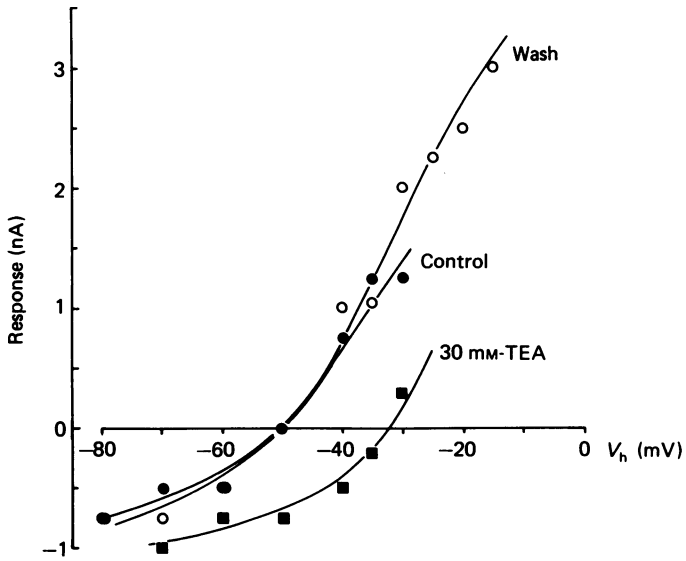

Fig. 13. The effect of TEA on the response of the F2 neurone to FMRFamide. TEA (30 mM) reduces the outward K component and therefore increases the net inward current component of the response at varying holding potentials. TEA was substituted for Na, therefore both control response and the responses after washing were obtained in low (50 mM)-Na medium.

component is likely to be due to an increase in permeability to K^+ ions because the E_{rev} of the response is similar to E_K. A major involvement of Cl is unlikely because the outward component was observed in solutions greatly depleted of Cl (NaCl substituted with sucrose) in which E_{Cl} was much more positive than E_K.

Physiological solution containing 30 mM-TEA increased the amount of inward current seen and made E_{rev} about 18 mV more positive (Fig. 13). This result, together with the different E_{rev} seen in different cells, is consistent with the situation in which two separate populations of channels, rather than one less selective population of channels, are opened by the peptide. Further evidence for there being two separate channels was provided by the action of intracellular Cs^+ ions. When Cs^+ ions were present in the F2 neurone the outward component was blocked but the inward current remained unaltered. Under these conditions the inward current in the F2 neurone appeared identical to that of the fast depolarization observed in neurone E13.

DISCUSSION

Ionic mechanisms of action

FMRFamide exerts at least three different actions on snail neurones. Two of these actions involve an increase in membrane conductance. In one case this leads to inhibition by increasing the permeability to K^+ ions and in the other to excitation by increasing the permeability predominantly but not exclusively to Na^+ ions. Similar actions have been observed in gastropod neurones in response to the transmitter substances acetycholine (Kehoe, 1972), 5-hydroxytryptamine (Gerschenfeld & Paupardin-Tritsch, 1974) and dopamine (Ascher, 1972; Berry & Cottrell, 1975).

The current mediating the hyperpolarizing response shows a voltage dependence similar to that of the response produced by carbachol on *Aplysia* neurones (Ginsborg & Kado, 1975). This response reverses between -70 and -80 mV, is markedly reduced by TEA and almost completely abolished by intracellular Cs^+ ions (Kehoe, 1972). The effect of low-K medium on this response, as recorded from the C1 neurone, was to shift E_{rev} by an amount which agrees closely with the theoretical change in E_K. This would be expected of a response mediated by K^+ ions alone.

The current–voltage relationship of the depolarizing response was linear over the range of potentials tested. Although intracellular Cs^+ ions did not seem to affect the response it proved useful in eliminating the voltage-dependent K currents and therefore made it possible to clamp the cell membrane at more positive potentials. By extrapolation of the relationship between current response and membrane potential, E_{rev} appeared to be around $+40$ mV. This, together with the observation that the response was abolished by exposure to Na-free medium, is consistent with a major part of the current being carried by Na^+ ions. Recently Dingledine (1983) has observed that *N*-methyl aspartate activates a Ca current in hippocampal neurones and that this is blocked by Co. The fast depolarization observed here was not, however, affected by 1 mM-$CoCl_2$.

The other, inward, current response to FMRFamide observed in the C1 neurone was only seen when the membrane was depolarized. Analysis of this response has proved more difficult, partly because it is complicated by the presence of the K-dependent, hyperpolarizing response. However, the related peptide YGGFMRFamide was found to be more potent at producing this inward current response and was therefore used in many cases to facilitate analysis of the ionic mechanism. The experiments in which the external Na concentration was reduced suggested that the response is unlikely to result from a voltage-dependent increase in permeability to Na^+ ions. Other possible mechanisms include a voltage-dependent increase in Ca^{2+} ion conductance, a reduction in conductance to K^+ ions, or a combination of the two processes.

The voltage-dependent nature of the response could be related to the outward rectification, the occurrence of which is paralleled by the appearance of the response. The delayed outward currents recorded on depolarization are primarily carried by K^+ ions. The blockade of the response by the Ca-channel blocker, Co, suggested the involvement of Ca^{2+} ions and therefore an increase in an inward Ca current or a reduction in a Ca-activated K current. The enhancement of the response following

injection of Ca^{2+} ions provided evidence for a reduction in a K current sensitive to internal Ca^{2+} ions. The single-channel experiments confirmed the view that the peptides were suppressing at least one K current. Further experiments will be required to test whether the single channels which were studied are indeed sensitive to Ca and to determine whether the larger currents alone are suppressed by the peptides.

Other voltage-dependent responses, inward in sign, have been observed with 5-hydroxytryptamine (Pellmar & Wilson, 1977; Pellmar & Carpenter, 1980; Paupardin-Tritsch, Deterre & Gerschenfeld, 1981; Siegelbaum, Camardo & Kandel, 1982) and following activation of an identified serotonergic input (Cottrell, 1981). In bull-frog sympathetic ganglion cells, luteinizing hormone releasing factor, and also muscarinic agonists, suppress a voltage-sensitive K current which is not sensitive to Ca^{2+} ions (Adams & Brown, 1980; Brown & Adams, 1980). In hippocampal neurones, histamine and noradrenaline decrease a Ca-activated K conductance (Haas & Konnerth, 1983). Also muscarinic agonists and 5-hydroxytryptamine suppress a Ca-activated K conductance in guinea-pig myenteric neurones (Grafe, Mayer & Wood, 1980; Morita, North & Tokimasa, 1982; North & Tokimasa 1983). Further experiments on the *Helix* C1 neurone are required to define more fully the properties of the K current or currents which are suppressed by the FMRFamide peptides, for comparison with those described in other cells.

Receptor–channel complexes

The response of the F2 neurone to FMRFamide comprises two components, a hyperpolarizing and a depolarizing part. The hyperpolarization is mediated by K^+ ions and the depolarization mainly by Na^+ ions. There are at least four different mechanisms possible to explain the total response: (1) a single receptor associated with a single channel permeable to both Na^+ and K^+ ions, (2) a single receptor coupled to two different channels which are activated simultaneously by the receptor, (3) two different channels associated with one receptor type, one channel being permeable mainly to Na^+ ions and the other to K^+ ions, (4) two different receptors, one type associated with a channel mainly permeable to Na^+ ions and the other with a K channel.

The E_{rev} of the response varied between -50 and -25 mV in F2 neurones from different animals. This is evidence against possibilities (1) and (2) as in both cases E_{rev} should be similar in all F2 neurones studied. The suppression of the K component by TEA and by Cs^+ ions also suggests that the responses cannot be explained by a single channel through which both ions pass. If this were the case, then TEA and Cs^+ ions, being channel blockers, would be expected to affect both components to the same extent. Instead a shift in E_{rev} to less negative values occurs.

The most plausible explanation is that the F2 neurone has one receptor complex which mediates the hyperpolarizing response and another complex which mediates the depolarizing response. Further experiments are required to test whether the receptors associated with each complex are different.

Physiological role of the responses

Two of the actions of FMRFamide on the neurones may be considered to be 'transmitter-like' in so far as they are mediated by an increase in membrane conductance. These responses suggest that FMRFamide and related peptides could have both excitatory and inhibitory transmitter roles in molluscan nervous systems. The reason for a combination of both excitatory and inhibitory responses occurring on the same neurone (e.g. the F2 neurone) is not known, but it is commonly encountered with several transmitter substances (see Gerschenfeld, 1973).

The voltage-dependent action seen in the C1 neurone could have a regulating or modulating role. An action of 5-hydroxytryptamine resulting in lengthening of the spike in sensory neurones, which is thought to be important in behavioural sensitization, probably also involves a similar mechanism of action (Klein & Kandel, 1978; Siegelbaum *et al.* 1982). Serotonergic inputs may also influence accommodation in follower neurones (Cottrell, 1982*b*). Other reported examples of voltage-dependent actions of endogenous neuroactive compounds include that of enkephalin in reducing spike duration by an apparent reduction of Ca conductance (Mudge, Leeman & Fischbach, 1979), and the effect of luteinizing hormone-releasing factor in bull-frog ganglion cells mentioned above. The physiological importance of such actions is not fully understood but some of them could, if occurring at terminal release sites, alter transmitter output because of the known relationship between spike duration and transmitter release (Katz & Miledi, 1967).

A FMRFamide-like peptide has been detected in extracts of *Helix* ganglia using biological and immunological assays. However the *Helix* peptide is not identical to FMRFamide; fractionation on CS-Sephadex columns shows that it is less polar than FMRFamide itself. Because the material has very similar pharmacological properties to FMRFamide and cross-reacts with antibodies directed against the sequence -Met-Arg-Phe-NH_2, it probably contains the sequence -Met-Arg-Phe-NH_2 or -Leu-Arg-Phe-NH_2, most probably with three or four additional amino acids at the *N*-terminal (Price, 1982). Recently the *Helix* peptide has been detected in an identified neurone, the C3 neurone, in each cerebral ganglion of *Helix aspersa* (Cottrell, Schot & Dockray, 1983). Studies on the role of this neurone may help to elucidate the functions of FMRFamide peptides and their physiological action.

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REFERENCES

ADAMS, P. R. & BROWN, D. A. (1980). Leuteinizing hormone-releasing factor and muscarinic agonists act on the same voltage-sensitive K^+-current in bullfrog sympathetic neurones. *British Journal of Pharmacology* **68**, 353–355.

AKAIKE, N., LEE, K. S. & BROWN, A. M. (1978). The calcium current of *Helix* neurone. *Journal of General Physiology* **71**, 509–531.

ALVAREZ-LEEFMANS, F. J. & GAMIÑO, S. M. (1982). Intracellular free potassium concentration in snail neurones measured with ion-selective microelectrodes. Neuroscience Abstracts **8**, 684.

ASCHER, P. (1972). Inhibitory and excitatory effects of dopamine on *Aplysia* neurones. *Journal of Physiology* **225**, 173–209.

BARISH, M. E. & THOMPSON, S. H. (1983). Calcium buffering and slow recovery kinetics of calcium-dependent outward current in molluscan neurones. *Journal of Physiology* **237**, 201–219.

BARKER, J. L. (1977). Physiological roles of peptides in the nervous system. In *Peptides in Neurobiology*, ed. GAINER, H. New York: Plenum Press.

BERRY, M. S. & COTTRELL, G. A. (1975). Excitatory, inhibitory and biphasic synaptic potential mediated by an identified dopamine-containing neurone. *Journal of Physiology* **24**, 589–612.

BROWN, D. A. & ADAMS, P. R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+-current in a vertebrate neurone. *Nature* **283**, 673–676.

COTTRELL, G. A. (1981). An unusual synaptic response mediated by a serotonin neurone. *Quarterly Journal of Experimental Physiology* **66**, 475–485.

COTTRELL, G. A. (1982*a*). FMRFamide neuropeptides simultaneously increase and decrease K^+ currents in an identified serotonin-containing neurone. *Nature* **296**, 87–89.

COTTRELL, G. A. (1982*b*). Physiological role of a slow, voltage-sensitive, synaptic response mediated by an identified serotonin-containing neurone. *Quarterly Journal of Experimental Physiology* **67**, 179–183.

COTTRELL, G. A., SCHOT, L. P. C. & DOCKRAY, G. J. (1983). Identification and probable role of a single neurone containing the neuropeptide *Helix* FMRFamide. *Nature* **304**, 638–640.

DINGLEDINE, R. (1983). *N*-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells. *Journal of Physiology* **343**, 384–405.

DODD, J. & KELLY, J. S. (1978). Is somatostatin an excitatory transmitter in the hippocampus? *Nature* **273**, 674–675.

GERSCHENFELD, H. M. (1973). Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiological Reviews* **53**, 1–119.

GERSCHENFELD, H. M. & PAUPARDIN-TRITSCH, D. (1974). Ionic mechanisms and receptor properties underlying the response of molluscan neurones to 5-hydroxytryptamine. *Journal of Physiology* **243**, 427–456.

GINSBORG, B. L. & KADO, R. T. (1975). Voltage–current relationship of a carbachol-induced potassium-ion pathway in *Aplysia* neurones. *Journal of Physiology* **245**, 713–725.

GRAFE, P., MAYER, C. J. & WOOD, J. D. (1980). Synaptic modulation of calcium-dependent potassium conductance in myenteric neurones in the guinea-pig. *Journal of Physiology* **305**, 235–248.

GREENBERG, M. J. & PRICE, D. A. (1979). FMRFamide, a cardio-excitatory neuropeptide of molluscs: an agent in search of a mission. *American Zoologist*, **19**, 163–174.

HAAS, H. L. & KONNERTH, A. (1983). Histamine and noradrenaline decrease calcium-activated potassium conductance in hippocampal pyramidal cells. *Nature* **302**, 432–434.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HERMANN, A. & GORMAN, A. L. F. (1981). Effects of TEA on potassium currents in a molluscan neurone. *Journal of General Physiology* **78**, 87–110.

HILLE, B. (1970). Ionic channels in nerve membranes. *Progress in Biophysics and Molecular Biology* **21**, 1–23.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *Journal of Physiology* **108**, 37–77.

HÖKFELT, T., JOHANSSON, O., LJUNGDAHL, A., LUNDBERG, J. M. & SCHULTZBERG, M. (1980). Peptidergic neurones. *Nature* **284**, 515–521.

JAN, Y. N., JAN, L. Y. & KUFFLER, S. W. (1979). A peptide as a possible transmitter in sympathetic ganglia of the frog. *Proceedings of the National Academy of Sciences of the U.S.A.* **76**, 1501–1505.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *Journal of Physiology* **192**, 407–436.

KEHOE, J. (1972). Ionic mechanisms of a two-component cholinergic inhibition in *Aplysia* neurones. *Journal of Physiology* **225**, 85–114.

KERKUT, G. A., LAMBERT, J. D. C., GAYTON, R. J., LOKER, J. E. & WALKER, R. J. (1975). Mapping of nerve cells in suboesophageal ganglia of *Helix aspersa*. *Comparative Biochemistry and Physiology* **50A**, 1–25.

KLEIN, M. & KANDEL, E. R. (1978). Presynaptic modulation of a voltage dependent Ca current: mechanism for behavioural sensitization in *Aplysia californica*. *Proceedings of the National Academy of Sciences of the U.S.A.* **75**, 3512–3516.

MEECH, R. W. (1974). The sensitivity of *Helix aspersa* neurones to injected calcium ions. *Journal of Physiology* **249**, 259–277.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation in *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx. *Journal of Physiology* **249**, 211–239.

MORITA, K., NORTH, R. A. & TOKIMASA, T. (1982). Muscarinic agonists inactivate potassium conductance of guinea-pig myenteric neurones. *Journal of Physiology* **333**, 125–139.

MUDGE, A. W., LEEMAN, S. E. & FISCHBACH, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurones in culture and decreses action potential duration. *Proceedings of the National Academy of Sciences of the U.S.A.* **76**, 526–530.

NORTH, R. A. & TOKIMASA, T. (1983). Depression of calcium-dependent potassium conductance of guinea-pig myenteric neurones. *Journal of Physiology*, **342**, 253–266.

PAUPARDIN-TRITSCH, D., DETERRE, P. & GERSCHENFELD, H. M. (1981). Relationship between two voltage dependent serotonin responses of molluscan neurones. *Brain Research* **217**, 201–206.

PELLMAR, T. C. & CARPENTER, D. O. (1980). Serotonin induces a voltage-sensitive calcium current in neurones of *Aplysia californica*. *Journal of Neurophysiology* **44**, 423–439.

PELLMAR, T. C. & WILSON, W. A. (1977). Unconventional serotonergic excitation in *Aplysia*. *Nature* **269**, 76–78.

PRICE, D. A. (1982). The FMRFamide-like peptide of *Helix*. *Comparative Biochemistry and Physiology* **72C**, 325–328.

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

SIEGELBAUM, S. A., CAMARDO, J. S. & KANDEL, E. R. (1982). Serotonin and cyclic AMP close single K channels in *Aplysia* sensory neurones. *Nature* **299**, 413–417.

SWANN, J. W. & CARPENTER, D. O. (1975). Organization of receptors for neurotransmitters on *Aplysia* neurones. *Nature* **258**, 751–754.

THOMAS, R. C. (1972). Intracellular sodium activity and the sodium pump in snail neurones. *Journal of Physiology*, **220**, 55–71.

THOMPSON, S. H. (1977). Three pharmacologically distinct K channels in molluscan neurones. *Journal of Physiology* **265**, 465–488.

TILLOTSON, D. & HORN, R. (1978). Inactivation without facilitation of calcium conductance in caesium-loaded neurones of *Aplysia*. *Nature* **273**, 312–314.

WERMAN, R. & GRUNDFEST, J. (1961). Graded and all-or-none electrogenesis in arthropod muscle II. The effects of alkali-earth and onium ions on lobster muscle fibres. *Journal of General Physiology* **44**, 997–1027.

WILSON, W. A. & GOLDNER, M. M. (1975). Voltage clamping with a single microelectrode. *Journal of Neurobiology* **6**, 411–422.