Journal of Physiology (1991), **443**, *pp.* 727–738
With 7 figures
Printed in Great Britain

THE PEPTIDE FMRFamide ACTIVATES A DIVALENT CATION-CONDUCTING CHANNEL IN HEART MUSCLE CELLS OF THE SNAIL *LYMNAEA STAGNALIS*

BY B. L. BREZDEN, P. R. BENJAMIN* AND D. R. GARDNER†

From the Ottawa-Carleton Institute of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

(*Received 30 November 1990*)

SUMMARY

1. Isolated *Lymnaea stagnalis* heart ventricle cells contain cation-conducting channels with properties characteristic of Ca^{2+} channels. These channels, which carry inward Na^+ currents in the absence of Ca^{2+}, are activated by the molluscan cardioactive peptides FMRFamide and FLRFamide, and are blocked by Co^{2+} ions.

2. FMRFamide also activated inward Ba^{2+} currents at the cell's resting potential. These currents, which were also blocked by Co^{2+} ions, reversed at a membrane potential of +70 mV.

3. Both sodium and barium currents were initiated when the peptides were applied to the cell outside of the patch pipette indicating that a secondary messenger is likely to be involved in the FMRFamide response.

INTRODUCTION

The myogenic heart of *Lymnaea* is regulated by a variety of peptidergic and non-peptidergic motoneurones. Thus its neuronal control is comparable in complexity to the mammalian heart (Geraerts, de With, Vreugdenhil, van Hartingsveldt & Hogenes, 1984; Buckett, Dockray, Osborne & Benjamin, 1990*a*; Buckett, Peters & Benjamin, 1990*b*; Buckett, Peters, Dockray, Van Minnen & Benjamin, 1990*c*). The tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide; Price & Greenberg, 1977) is a potent cardioexcitatory agent in *Lymnaea* and it and/or related peptides have recently been localized in a pair of cardioactive motoneurones (Benjamin, Buckett & Peters, 1988; Buckett *et al.* 1990*c*) whose main cardioexciting effect appears to be mediated by FMRFamide.

Recent studies on the FMRFamide gene in *Lymnaea* (Linacre, Kellett, Saunders, Bright, Benjamin & Burke, 1990) have revealed a nucleotide sequence encoding a second type of tetrapeptide, Phe-Leu-Arg-Phe-NH_2 (FLRFamide). The cloned DNA (cDNA) encoding both FMRFamide and FLRFamide is expressed in central neurones (*in situ* hybridization studies, Linacre *et al.* 1990).

In so far as FMRFamide and FMRFamide-related peptides in *Lymnaea* are

* Present address: Neuroscience Interdisciplinary Research Centre at Sussex, School of Biological Sciences, University of Sussex, Brighton, Sussex BN1 9QG.

† To whom all correspondence should be addressed.

MS 9182

involved in the regulation of heart beat (Buckett *et al.* 1990*c*), it is reasonable to suppose that these agents might have an effect on some class of ion channel in the sarcolemma. Alterations in the active properties of such membrane-bound ion channels would be expected to lead to changes in the activity of the heart.

Several channel types which can be detected at the resting membrane potential in cell-attached membrane patches on dissociated *Lymnaea* heart cells have been identified. These include a stretch-sensitive K^+ channel (Brezden, Gardner & Morris, 1986), two distinct non-voltage-gated channels which carry Na^+ in the absence of Ca^{2+}, and a non-voltage-gated divalent cation channel which passes Ca^{2+} or Ba^{2+} (Gardner & Brezden, 1990). On the basis of their relative conductances, the two Na^+-conducting channels were labelled the 'SG' ('small conductance') channel and the 'LG' ('large conductance') channel (Brezden & Gardner, 1990).

Although the relationship between the Na^+-conducting channel and the divalent cation channel has not yet been completely established it is possible that either the SG or LG channel and the divalent cation channel are one and the same (Brezden & Gardner, 1990). This is supported by the evidence that the LG and SG Na^+ currents were reduced by micromolar levels of external Ca^{2+} (Gardner & Brezden, 1990). Such high selectivity for Ca^{2+} is also a characteristic of vertebrate voltage-gated calcium channels (Hess & Tsien, 1984; Tsien, Hess, McClesky & Rosenberg, 1987). However, as the SG and LG channels did not appear to be voltage-gated (being active over a wide range of membrane potentials), it was suggested that the SG and LG channels were either receptor-operated channels or secondary-messenger-operated channels (Gardner & Brezden, 1990).

In cell-attached membrane patches, the SG and LG channels usually became inactive shortly after the formation of a pipette–membrane seal. The classical molluscan neurotransmitters, acetylcholine and serotonin (5-HT), were ineffective at restoring channel activity, as were ATP, dibutyryl cyclic AMP and dibutyryl cyclic GMP (Gardner & Brezden, 1990). Now, however, we show that FMRFamide and its analogue, FLRFamide, are effective stimulants of SG and LG channels, and that FMRFamide activates a unitary Ba^{2+} conductance.

METHODS

Detailed procedures for isolating and patch clamping *Lymnaea* heart ventricle cells have been described in Brezden *et al.* (1986) and Gardner & Brezden (1990). Briefly, cells were dissociated by digestion for 30 min in 0·25 % trypsin and, subsequently, for 2 h in 0·1 % collagenase in 0·5 mM-Ca^{2+} Leibovitz medium. Prior to patch clamping, the Leibovitz medium (with the ionic concentration modified for use with snails) was replaced with normal *Lymnaea* saline (Table 1).

Dissociated cells were plated onto cover-glasses in 35 mm Petri dishes. The tips of micropipettes, pulled from thick-walled Corning 7052 (Kovar) glass, were coated with Sylgard 184 elastomer (Dow Corning) and were heavily fire-polished. The tip openings were 1–1·5 μm in diameter. Channel currents were amplified with a List EPC-7 patch clamp amplifier (Medical Systems Corp.). The data were recorded on videotape with an Instrutech VR-10 digital recorder to be subsequently processed and analysed on a microcomputer with pCLAMP (Axon Instruments, Inc.). The analysis and fitting procedures for channel open times were as described in Brezden & Gardner (1990) and Sigurdson, Morris, Brezden & Gardner (1987). Data were filtered at 2 or 4 kHz with a 4-pole Bessel filter for dwell-time measurements. For amplitude measurements, the channel detection threshold was set half-way between the baseline and the open channel level. Amplitude data, measured by computer using the program pCLAMP, were assumed to conform to a Gaussian distribution. Closed dwell times were not analysed because there was always more than one channel present in the patches.

When it was desired to have only 59 mM-Na^+ in the patch pipette, a very low intra-pipette Ca^{2+} concentration was ensured by the addition of 1 mM-EGTA to the nominally 0 Ca^{2+} saline. This resulted in a calculated residual saline Ca^{2+} concentration of 3·2 nM (Brezden & Gardner, 1990). The pipette and bath saline compositions are given in Table 1.

It was very difficult to achieve acceptable glass–membrane seals on these molluscan heart cells with an elevated concentration of Ca^{2+} in the patch pipette. On the other hand, good seals were obtained when using 50 mM-Ba^{2+} as the permeant ion through divalent cation channels. Therefore, Ba^{2+}, which is often more permeant than Ca^{2+} in divalent cation channels (Tsien *et al.* 1987), was used to detect divalent cation channel openings.

TABLE 1. The dissociation media and pipette solutions

Concentration (mM)	Dissociation medium	Normal saline	0 Ca^{2+} saline	50 mM-Ba^{2+} saline
NaCl	59·0	50·0	59·0	0
KCl	1·6	1·6	0	0
$CaCl_2$	0·5	3·5	0	0
$MgCl_2$	2·0	2·0	0	0
$BaCl_2$	0	0	0	50·0
HEPES*	5·0	5·0	5·0	5·0
EGTA	0	0	1·0	0
Leibovitz's medium	33% (v/v)	0	0	0
Fetal calf serum	1·33% (v/v)	0	0	0
Gentamycin (10 mg/ml)	1·33 μg/ml	0	0	0

The Ca^{2+} concentration in the 0 Ca^{2+} saline was calculated using the dissociation constant of Owen (1976). To prepare cobalt saline, 5 mM-Co^{2+} was added to the 0 Ca^{2+}, 59 mM-Na^+ saline or the 50 mM-Ba^{2+} saline.

* Adjusted to pH 7·6 with 10 M-NaOH.

FMRFamide was obtained from Peninsula Laboratories Europe Ltd and Sigma Chemicals. FLRFamide was obtained from Cambridge Research Biochemicals Ltd.

FMRFamide or FLRFamide were administered to an area of the cell away from the patch pipette via a superfusion pipette ('extra-patch' superfusion). The superfusion pipettes, also made from Corning 7052 glass, had tip openings of about 1·5 μm in diameter. The pipettes were attached to a 1 ml gas-tight syringe (Hamilton) with polyethylene tubing. The pipette tip was positioned at distances ranging from about 20 to 100 μm from the cell. The contents were ejected manually in early experiments. In later experiments, superfusion was performed automatically with a pneumatic syringe pump which was controlled by a timing circuit. Using a marker dye, it was estimated that the superfusate travelled a distance of 100 μm in about 0·5 s.

Estimates of the potential difference across the membrane patch (V_m) were based on the data of Brezden *et al.* (1986), where the resting potential of isolated *Lymnaea stagnalis* heart ventricle cells was found to be −60 mV. Thus, V_m is approximated by $(-60 - V_p)$ mV, where V_p is the pipette potential. All data are from cell-attached patches and voltages are given as V_m.

RESULTS

Effect of FMRFamide on Na^+ conductance in the absence of Ca^{2+}

With 59 mM-Na^+ and nominally 0 Ca^{2+} in the patch pipette, superfusion of the ventricle cell with 10^{-6} M-FMRFamide at a point about half a cell length (20–50 μm) away from the patch pipette (which did not contain FMRFamide), induced a large increase in channel activity in the patch 1–5 s after application. Simultaneous activation of several channels was reflected in the increased frequency of multiple openings (Fig. 1*A*), which was often, but not always, concurrent with a FMRFamide-induced contraction of the cell. At the resting membrane potential, measuring all inward currents and estimating the number of channels (based on the number of

multiple openings), the probability of the channels being open (P_o) was increased from a mean of 0·00008 ± 0·00002 (± S.D.) during a 30 s interval before the peptide-induced onset of channel openings to a mean of 0·126 ± 0·050 (± S.D.), measured over a 30 s interval following the onset of channel activity ($n = 6$ cells). The increased

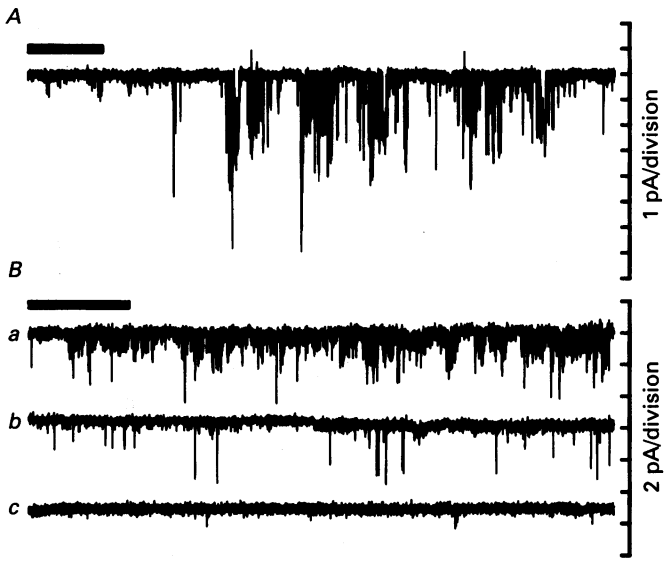

Fig. 1. *A*, increased ion channel activity in response to extra-patch superfusion with 10^{-6} M-FMRFamide. The delay between start of superfusion and onset of activity was 3 s. The events in this record are represented by a small conductance (SG) and a large conductance (LG) (see text). The SG events are those with an amplitude of about −1 pA. The LG events have an amplitude of about −2 to −3 pA. Deflections of about −6 to −8 pA are due to multiple openings. The occasional outward currents (upward deflections) are stretch-activated potassium channels which open spontaneously to conduct outward K^+ currents in the absence of external K^+ (Brezden *et al.* 1986). *B*, reduction in response of *Lymnaea* ventricle cells to repeated applications of 10^{-6} M-FMRFamide (applied to the cell by superfusion). The baseline activity before application of the peptide in *a* and *b* was as in *c*, with only occasional channel openings. The −2 pA events are LG channel currents or multiple openings. The −1 pA events are SG channel currents. *a*, response to the first application of FMRFamide, applied about 1 s before the beginning of the trace. P_o for the displayed trace = 0·160. *b*, 10 min after *a* (no intervening wash), reduced response to the second application of FMRFamide, applied about 7 s before the beginning of the trace. P_o for the displayed trace = 0·0008. *c*, 10 min after *b* (no intervening wash), no response to the third application of FMRFamide, applied at the beginning of the trace (P_o for the displayed trace = 0·0001). $V_m = -60$ mV. Scale bar = 5 s. Representative of data from eleven cells. Data for *A* and *B* are from different cells. Downward deflections represent inward currents. Filter frequency for *A* and *B* = 1 kHz.

activity persisted for 1–4 min, gradually declining until the patches became quiescent with only occasional single openings or brief bursts of openings evident for the remainder of the experiment (up to 1 h). The response to subsequent applications of FMRFamide was reduced, and was entirely lost by the third or fourth application (Fig. 1*B*).

With 59 mM-Na^+ and 5 mM-Co^{2+} in the patch pipette, FMRFamide-induced channel openings could not be detected ($n = 14$ cells).

Fig. 2. Increased ion channel activity in response to extra-patch superfusion with 10^{-6} M-FLRFamide. Delay between start of superfusion and onset of activity was usually about 1–5 s. *A*, first application at beginning of trace. No pressure was applied to the superfusion syringe. The FLRFamide was delivered to the cell membrane by diffusion from the superfusion pipette tip, which was about 2 μm from the cell membrane and about 30 μm from the patch pipette. P_o for the displayed trace = 0·080. *B*, second application about 2·5 min after the first (no intervening wash). P_o for the displayed trace = 0·0004. *C*, third application following a 1 min wash with normal saline about 5 min after the second application. P_o for the displayed trace = 0·0021. The −2 to −3 pA events are LG currents, while the −0·8 to −1·2 pA events are the SG currents. V_m = −60 mV. Scale bar = 5 s. Representative of data from seven cells. Downward deflections represent inward currents. Filter frequency for *A*, *B* and *C* = 1 kHz.

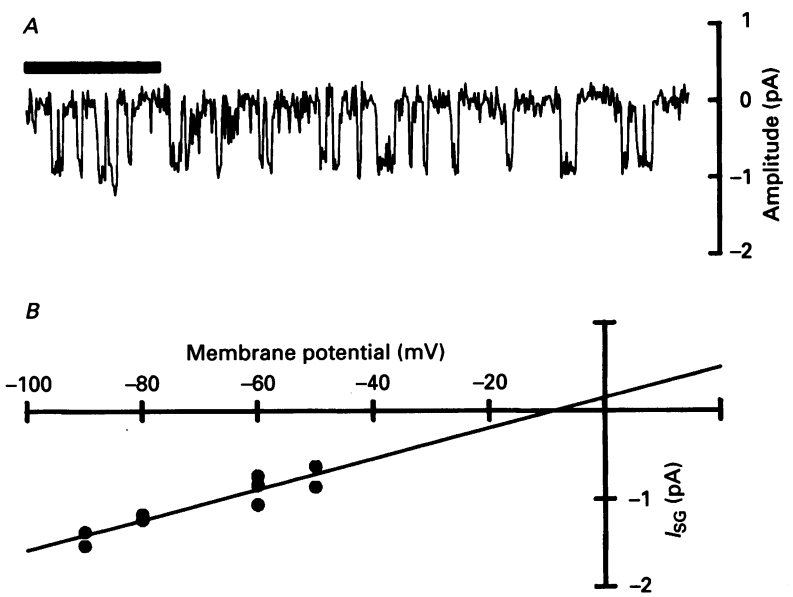

Fig. 3. *A*, FMRF-activated SG events. V_m = −60 mV. Scale bar = 5 ms. *B*, current–voltage relationship for the smaller amplitude (SG) unitary events evoked by superfusion with 10^{-6} M-FMRFamide. The data are fitted by a first-order linear regression. Slope conductance = 16 pS. Extrapolated reversal potential = −9 mV. Data from three patches on three cells were combined. Filter frequency = 1 kHz.

Effect of FLRFamide on Na^+ conductance in the absence of Ca^{2+}

The response to 10^{-6} M-FLRFamide (Fig. 2) was similar to the response to FMRFamide. After a delay of 1–5 s, the inward Na^+ current (59 mM-Na^+, nominally 0 Ca^{2+} in the patch pipette) was increased to well above pre-treatment levels. The P_o

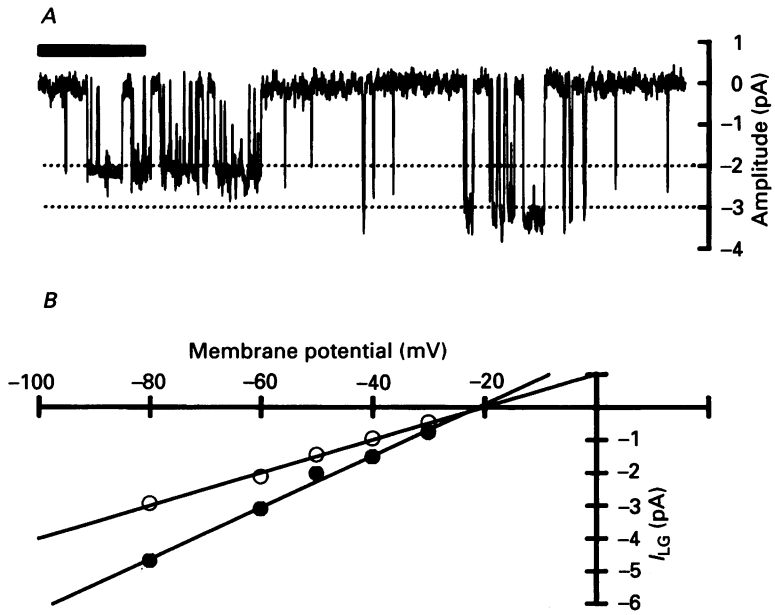

Fig. 4. *A*, FMRF-activated LG events. Note two amplitude levels. $V_m = -60$ mV. Scale bar = 50 ms. Representative of data from eleven cells. Downward deflections represent inward currents. *B*, current–voltage relationship for two putative subconductance states of the larger amplitude (LG) FMRFamide-evoked unitary currents. Data are from a single patch and are fitted by a first-order linear regression. Slope conductance for the smaller amplitude events = 49 pS. Slope conductance for the larger amplitude events = 78 pS. The extrapolated reversal potential for both conductance states is about −20 mV. Filter frequency = 2 kHz.

(calculated as for FMRFamide, see above) increased from $0{\cdot}00007 \pm 0{\cdot}000002$ (±S.D.) to $0{\cdot}100 \pm 0{\cdot}023$ (±S.D.) measured over 30 s intervals before and after the application of peptide ($n = 5$ cells). As in the response to FMRFamide, the response to subsequent applications of 10^{-6} M-FLRFamide was also diminished (Fig. 2*B*), and generally disappeared by the third or fourth application. The response to further application was partially restored by washing the cells for 20 min with normal saline (Fig. 2*C*).

With a peptide concentration of 10^{-6} M, it was not always necessary to apply pressure to the superfusion pipette in order to evoke a response; channel activity could be stimulated by simply approaching the cell membrane within 2–5 μm with the superfusion pipette (Fig. 2*A*).

In most patches with nominally 0 Ca^{2+} and 59 mM-Na^+ in the pipette, two types of peptide-activated channel currents could be distinguished. At the resting membrane potential (approximately −60 mV), the smaller unitary currents (Fig. 3*A*) were characterized by an amplitude of $-1{\cdot}0 \pm 0{\cdot}04$ pA (±S.D., data from three

cells), a slope conductance of 16 pS and a reversal potential of −9 mV (Fig. 3*B*). The open time distribution was best fitted by a single exponential with an open dwell-time constant of 1·1±0·03 ms (±S.E.M., 926 events from two cells) (Fig. 5*A*).

The larger unitary currents were often distinguished by two different amplitude levels of about −2 and −3 pA at the resting membrane potential (Fig. 4*A*). The

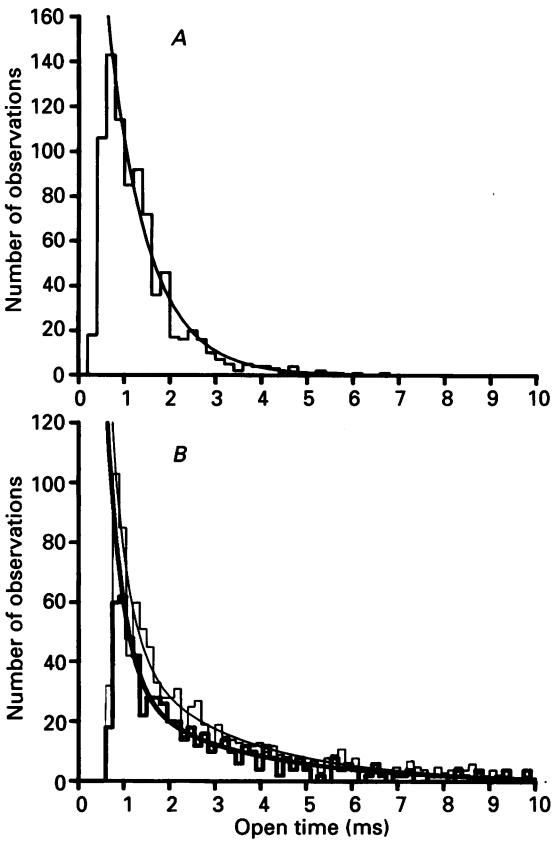

Fig. 5. *A*, open time distribution for the SG unitary events evoked by superfusion with FMRFamide. The fitted curve is a single exponential. $V_m = -60$ mV. $\tau_o = 1{\cdot}1 \pm 0{\cdot}03$ ms (±S.E.M.). Data from patches on two cells were combined; $n = 926$ events. *B*, comparison of the open dwell-times for events of different conductances. Light lines represent the smaller conductance (49 pS) events with $\tau_{o1} = 0{\cdot}37 \pm 0{\cdot}07$ ms (±S.E.M.) and $\tau_{o2} = 2{\cdot}74 \pm 0{\cdot}37$ ms (±S.E.M.); $n = 879$ events. Heavy lines represent the larger conductance (78 pS) events with $\tau_{o1} = 0{\cdot}38 \pm 0{\cdot}07$ ms (±S.E.M.) and $\tau_{o2} = 2{\cdot}58 \pm 0{\cdot}35$ ms (±S.E.M.); $n =$ 626 events. $V_m = -60$ mV. Data are from the same patch. Filter frequency = 2 kHz.

slope conductances for the −2 and −3 pA events were 49 and 78 pS, respectively, over a −30 to −80 mV range of membrane potentials (V_m). The extrapolated reversal potentials were about −20 mV for both types of event (Fig. 4*B*), whilst both their open dwell-time distributions at the resting potential were best fitted by two exponential terms with practically identical time constants (τ) of $\tau_{o1} = 0{\cdot}37 \pm 0{\cdot}07$ ms (±S.E.M.) and $\tau_{o2} = 2{\cdot}74 \pm 0{\cdot}37$ ms (±S.E.M.) for the 2 pA events ($n = 879$ events), and $\tau_{o1} = 0{\cdot}38 \pm 0{\cdot}07$ ms (±S.E.M.) and $\tau_{O2} = 2{\cdot}58 \pm 0{\cdot}35$ ms (±S.E.M.) for the 3 pA events ($n = 626$ events; filter frequency = 2 kHz; (Fig. 5*B*).

On the basis of the similarity in their open dwell-time constants and reversal potentials, it is possible that the 49 pS conductance and the 78 pS conductance represent subconductance states of the same channel. However, transitions from one state to the other were not seen in the presence of FMRFamide, although such

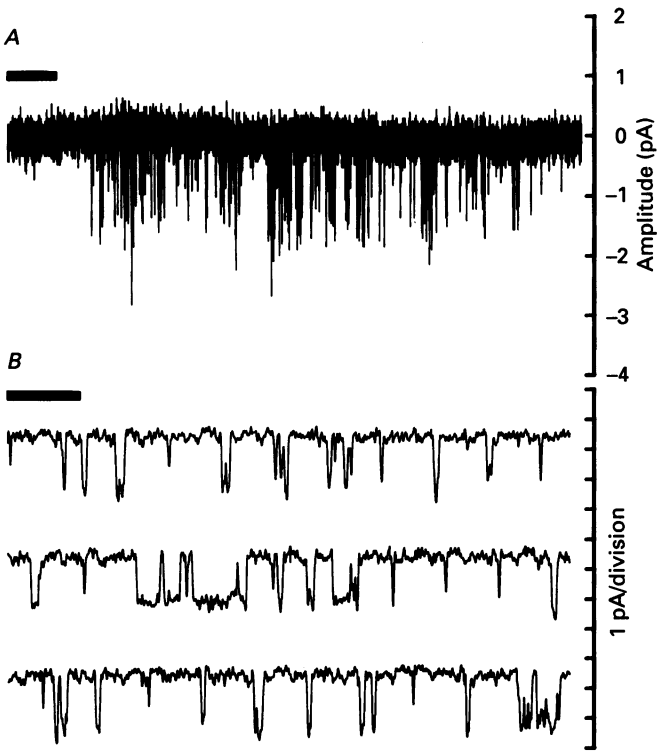

Fig. 6. *A*, activation of unitary currents by superfusion with 10^{-8} M-FMRFamide; 50 mM-Ba^{2+} in the patch pipette. The cell was superfused about 50 μm from the patch pipette. Channel activity started 6 s after the onset of superfusion with FMRFamide. $V_m = -60$ mV. Scale bar = 5 s. Filter frequency = 2 kHz. *B*, FMRFamide-activated Ba^{2+} currents shown on a shorter time scale. $V_m = -60$ mV. Scale bar = 5 ms. Downward deflections represent inward currents. Representative of data from eighteen cells. Filter frequency = 1 kHz.

transitions, though rare, have been previously observed in spontaneous openings of this channel (Brezden & Gardner, 1990).

The amplitudes, open time constants and reversal potentials of the 16 pS conductance peptide-induced events were similar to those of SG channel currents (amplitude = 1 pA at $V_m = -60$ mV, slope conductance = 15 pS, $\tau_o = 1{\cdot}0$ ms, extrapolated reversal potential = −10 mV). The 49 and 78 pS conductance peptide-induced events were similar to those measured for the LG channel currents (amplitude = −2 or −3 pA at $V_m = -60$ mV, slope conductance = 50 and 72 pS, $\tau_{o1} = 0{\cdot}5$ ms, $\tau_{o2} = 1{\cdot}9$ ms, extrapolated reversal potential = −20 mV). The SG and LG channels have been previously described in *Lymnaea* heart cells (Brezden & Gardner, 1990; Gardner & Brezden, 1990).

Effect of FMRFamide on Ba^{2+} conductance

With 50 mM-Ba^{2+} in the patch pipette, a 2 s extra-patch superfusion with 10^{-8} M-FMRFamide initiated a burst of unitary Ba^{2+} currents (Fig. 6*A*). In some cells the FMRFamide-activated channel activity lasted for only a few seconds, while in

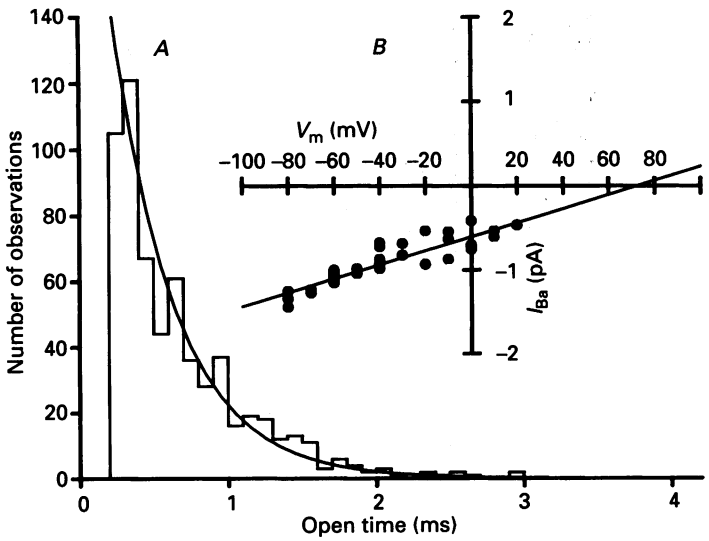

Fig. 7. *A*, open time distribution for the Ba^{2+} unitary events evoked by superfusion with FMRFamide. The fitted curve is a single exponential. $V_m = -60$ mV. $\tau_o = 0{\cdot}42 \pm 0{\cdot}03$ ms (±S.E.M.). Data are from patches on two cells, $n = 1679$ events. *B*, current–voltage relationship for the unitary Ba^{2+} events evoked by superfusion with FMRFamide. Slope conductance = 8·3 pS. Extrapolated reversal potential = +70 mV. Data from three patches on three cells were combined. Filter frequency = 4 kHz.

others, occasional channel openings persisted for several minutes. The P_o calculated as above, increased from $0{\cdot}00006 \pm 0{\cdot}00003$ (±S.D.) to $0{\cdot}0268 \pm 0{\cdot}0134$ (±S.D.) measured over 30 s intervals before and after the application of peptide ($n = 14$ cells).

The open dwell-time distribution for the Ba^{2+} currents was best fitted by a single exponential with a time constant of $0{\cdot}42 \pm 0{\cdot}03$ ms (±S.E.M.; filter frequency = 4 kHz; $n = 1679$ events; Fig. 7*A*). The amplitude of these Ba^{2+} currents averaged $-1{\cdot}29 \pm 0{\cdot}20$ pA (±S.D.) at a membrane potential of −60 mV, with a slope conductance of 8·3 pS, and an extrapolated reversal potential of +70 mV (Fig. 7*B*).

With 50 mM-Ba^{2+} and 5 mM-Co^{2+} in the patch pipette, FMRFamide-induced channel openings could not be detected ($n = 17$ cells).

DISCUSSION

Activation of Na^+ flux through SG and LG channels

When the patch pipette contained only Na^+, Cl^- and HEPES buffer, the SG and LG channel currents were assumed to arise from the influx of sodium ions. These currents, which were inward at $V_m = -60$ mV, could not have been due to an efflux

of chloride ions because the reversal potentials were much more positive than the Cl^- equilibrium potential, which is near the resting potential in these cells (Brezden & Gardner, 1990).

In quiescent cells transient SG and LG channel activity was induced by applying FMRFamide to the cell outside the patch pipette. As there was no apparent effect on other channels (stretch-activated potassium, stretch-activated sodium or voltage-gated which are also present in these cells (Gardner & Brezden, 1990), FMRFamide and FLRFamide appear to specifically activate only SG and LG channels under conditions where Na^+ is the charge carrier.

The *Lymnaea* ventricle cells were very sensitive to FMRFamide and FLRFamide. With a concentration of 10^{-6} M in the superfusion pipette, it was often not necessary to apply pressure to the pipette to evoke a response. Bringing the superfusion pipette to within a few micrometers of the cell surface often sufficed to produce a large increase in channel activity. Thus the small amount of FMRFamide or FLRFamide which diffused from the pipette tip to the cell was an effective stimulant.

In the absence of the cardioactive peptides, spontaneous SG and LG channel activity is only occasionally seen in isolated *Lymnaea stagnalis* heart ventricle cells. This activity persists for only a short time following patch formation, typically disappearing within seconds to minutes after formation of a gigaseal. It may be that a distortion of the membrane by suction, applied to the pipette during patch formation, transiently stimulates these channels. That SG and LG channel activity subsides in the absence of further stimulus (Brezden & Gardner, 1990) suggests that these channels are not very active in the resting muscle cell.

The close correspondence between the FMRFamide-activated Na^+ currents and the previously described LG and SG channel currents (Brezden & Gardner, 1990) suggests that these spontaneously active channels and peptide-activated channels are the same. On the other hand, the properties of FMRFamide-induced Ba^{2+} channel currents reported here, with a slope conductance of 8·3 pS, an open time constant of 0·42 ms and a reversal potential of +70 mV, are different from the previously reported open time constant of 1·4 ms, a slope conductance of 12 pS and a reversal potential of +55 mV for the spontaneous Ba^{2+} (BaP) channel currents (Brezden & Gardner, 1990). Since the kinetics of the rarely observed spontaneous Ba^{2+} currents were similar to the kinetics of the spontaneous SG currents, it was suggested that both BaP and SG currents might be passing through the same channel (Brezden & Gardner, 1990). However, the FMRFamide-activated Ba^{2+} channel currents reported here could be much more reliably measured. Since their kinetic properties were different from those of the previously reported Ba^{2+} currents, it would appear that the FMRFamide-activated Ba^{2+} channels are not the same as the SG channels.

It has been previously reported that Co^{2+} blocked the spontaneous inward Na^+ currents through SG and LG channels. The peptide-activated Na^+ currents reported here were also blocked by Co^{2+}. This supports the evidence that the SG and LG channels are FMRFamide-sensitive divalent cation channels (Gardner & Brezden, 1990). The FMRFamide-activated Ba^{2+} currents were also blocked by Co^{2+} which suggests that these currents pass through a divalent cation channel as well. However, it is not possible to determine whether both Na^+ and Ba^{2+} ions pass through the same channel on the basis of the available evidence.

Is the FMRFamide response mediated by secondary messengers?

The cardioactive agents examined here activated channel activity *within* the membrane patch although they were applied to the cell membrane *outside* the patch pipette. It is not likely that these agents could have diffused through the electrically tight seal between the cell membrane and the pipette glass to activate the receptors directly. There is also a delay of several seconds before the onset of activity. Consequently, we suggest that FMRFamide and related agents exert their effect through one of the several possible secondary messenger systems available to these cells. The most likely candidates are Ca^{2+}-activated Ca^{2+} release from the intracellular calcium stores (Ford & Podolsky, 1970), the cyclic AMP/cyclic GMP system, or the phosphatidyl inositol system. For instance, it has been reported that FMRFamide exerts a modulatory effect on S-type K^+ channels in *Aplysia* neurones via a G protein-mediated activation of arachidonic acid release (Piomelli, Volterra, Dale, Siegelbaum, Kandel, Schwartz & Belardetti, 1987; Volterra & Siegelbaum, 1988).

The reduction in response to repeated applications of cardioactive peptides

The response of the heart cells to 10^{-6} M-FMRFamide or 10^{-6} M-FLRFamide was diminished with each consecutive application. However, the muscle cells remained viable as they were subsequently able to contract when their cell membranes were intentionally damaged with the micropipettes. Possibly, the reduction in response to FMRFamide was due to a depletion of an intermediate substrate in a secondary messenger pathway. For example, Berridge & Fain (1979) demonstrated that the loss of secretory response to 5-hydroxytryptamine in the blowfly salivary gland was due to a depletion of inositol.

A reduction in the response was not as pronounced when the FMRFamide concentration was reduced to 10^{-8} M. This would be expected if this lower concentration of FMRFamide activated fewer receptors and thus put less demand on the substrates required by the secondary messenger pathway.

D.R.G. thanks the Natural Sciences and Engineering Research Council of Canada for an operating grant (A-4290) in support of this project. P.R.B. thanks the British Medical Research Council for financial support for this project and the Royal Society for a study grant. The authors thank Dr Corné J. Kros for his comments and suggestions regarding this manuscript.

REFERENCES

BENJAMIN P. R., BUCKETT, K. J. & PETERS, M. (1988). Neurones containing FMFRamide-like peptides in the model invertebrate system, *Lymnaea*. In *Neurobiology of Invertebrates, Transmitters, Modulators and Receptors*, ed. SALANKI, J. & S.-ROSZA, K., pp. 247–259. Akademia; Kiadó, Budapest.

BERRIDGE, M. J. & FAIN, J. N. (1979). Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. *Biochemical Journal* **178**, 59–69.

BREZDEN, B. L. & GARDNER, D. R. (1984). The ionic basis of the resting potential in a cross-striated muscle of the aquatic snail *Lymnaea stagnalis*. *Journal of Experimental Biology* **108**, 305–314.

BREZDEN, B. L. & GARDNER, D. R. (1990). Non-voltage-gated calcium channels in snail heart ventricle cells. *Journal of Experimental Biology* **150**, 187–203.

BREZDEN, B. L., GARDNER, D. R. & MORRIS, C. E. (1986). A potassium-selective channel in isolated *Lymnaea stagnalis* heart muscle cells. *Journal of Experimental Biology* **123**, 175–189.

BUCKETT, K. J., DOCKRAY, G. J., OSBORNE, N. N. & BENJAMIN, P. R. (1990*a*). Pharmacology of the myogenic heart of the pond snail, *Lymnaea stagnalis*. *Journal of Neurophysiology* **63**, 1413–1425.

BUCKETT, K. J., PETERS, M. & BENJAMIN, P. R. (1990*b*). Excitation and inhibition of the heart of the snail, *Lymnaea*, by non-FMRFamidergic motor neurons. *Journal of Neurophysiology* **63**, 1436–1447.

BUCKETT, K. J., PETERS, M., DOCKRAY, G. J., VAN MINNEN, J. & BENJAMIN, P. R. (1990*c*). Regulation of heart beat in *Lymnaea* by motoneurons containing FMRFamide-like peptides. *Journal of Neurophysiology* **63**, 1426–1435.

FORD, L. E. & PODOLSKY, R. J. (1970). Regenerative calcium release within muscle cells. *Science* **167**, 58–59.

GARDNER, D. R. & BREZDEN, B. L. (1990). Single ion channels in snail ventricle heart cells. *Comparative Biochemistry and Physiology* **96A**, 79–85.

GERAERTS, W. P. M., DE WITH, N. D., VREUGDENHIL, E., VAN HARTINGSVELDT, W. & HOGENES, T. M. (1984). Studies on the physiological role of a partially purified small cardioactive neuropeptide of *Lymnaea stagnalis*. *Journal of Comparative Physiology* B **154**, 29–34.

HESS, P. & TSIEN, R. W. (1984). Mechanism of ion permeation through calcium channels. *Nature* **309**, 453–456.

LINACRE, A., KELLETT, E., SAUNDERS, S., BRIGHT, K., BENJAMIN, P. R. & BURKE, J. F. (1990). Cardioactive neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) and novel related peptides are encoded in multiple copies by a single gene in the snail *Lymnaea stagnalis*. *Journal of Neuroscience* **10**, 412–419.

OWEN, J. D. (1976). The determination of the stability constant for calcium-EGTA. *Biochimica et Biophysica Acta* **451**, 321–325.

PIOMELLI, D., VOLTERRA, A., DALE, N., SIEGELBAUM, S. A., KANDEL, E. R., SCHWARTZ, J. H. & BELARDETTI, F. (1987). Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of *Aplysia* sensory cells. *Nature* **328**, 38–43.

PRICE, D. A. & GREENBERG, M. J. (1977). Pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. *General Pharmacology* **11**, 237–241.

SIGURDSON, W. J., MORRIS, C. E., BREZDEN, B. L. & GARDNER, D. R. (1987). Stretch activation of a K^+ channel in molluscan heart cells. *Journal of Experimental Biology* **127**, 191–209.

TSIEN, R. W., HESS, P., MCCLESKEY, E. W. & ROSENBERG, R. L. (1987). Calcium channels: mechanism of selectivity, permeation, and block. *Annual Reviews of Biophysics and Biophysical Chemistry* **16**, 265–290.

VOLTERRA, A. & SIEGELBAUM, S. A. (1988). Role of two different guanine nucleotide-binding proteins in the antagonistic modulation of the S-type K^+ channel by cAMP and arachidonic acid metabolites in *Aplysia* sensory neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 7810–7814.