FMRFamide Effects on Membrane Properties of Heart Cells Isolated From the Leech, Hirudo medicinalis

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SUMMARY AND CONCLUSIONS
1. The effects of the cardioactive peptide FMRFamide were tested on enzymatically dissociated muscle cells isolated from hearts of the leech. These cells were normally quiescent, with resting potentials near −60 mV.
2. Superfusion of FMRFamide induced a strong depolarization in isolated heart cells (e.g., >40 mV with 10−8 M FMRFamide). The depolarization was maintained in the continued presence of peptide and persisted long after its removal. Less frequently, FMRFamide superfusion elicited an episodic polarization rhythm.
3. The response of isolated heart cells to bath-applied FMRFamide showed a 1- to 2-min latency. The latency decreased with repeated applications of FMRFamide.
4. The FMRFamide response was diminished by Na+ replacement but persisted with Ca2+ channel blockade.
5. In voltage-clamped heart cells (−60 mV), superfusion of FMRFamide elicited a slow inward current with a transient and a sustained component.
6. Current-voltage (I-V) curves during FMRFamide superfusion in normal leech saline showed that FMRFamide also enhanced voltage-dependent outward currents activated at depolarized levels.
7. Under conditions in which K+ currents were substantially blocked, the FMRFamide-dependent I-V curve was net inward from −90 to +50 mV. A voltage-dependent component was blocked by Co2+ and a linear component by Na+ replacement.
8. We conclude that FMRFamide elicits a persistent inward current with a Na+ component and in addition modulates both voltage-dependent Ca2+ and K+ currents that may contribute to the normal myogenic activity of leech heart muscle cells.

INTRODUCTION
In the heartbeat system of the leech, Hirudo medicinalis, the peptide FMRFamide modulates both the central neural elements involved in controlling the heartbeat and the myogenic properties of the heart muscle. Biochemical and immunocytocchemical studies have localized FMRFamide or FMRFamide-like peptides to the motor neurons and modulatory neurons that innervate the leech heart and also to terminals on the heart (Evans and Calabrese 1989; Kuhlman et al. 1985a; Li and Calabrese 1987; Maranto and Calabrese 1984a). True FMRFamide and four related peptides have been isolated from the leech nerve cord and sequenced (Evans et al. 1991). In the heart muscle, FMRFamide application increases the strength of contractions, accelerates the rate of myogenic contractions, and can induce myogenic contractions in quiescent hearts (Calabrese and Maranto 1984; Kuhlman et al. 1985b; Li and Calabrese 1987). Furthermore, bath application of FMRFamide mimics many of the effects of electrical stimulation of the heart excitatory and modulatory neurons (Calabrese and Maranto 1984; Kuhlman et al. 1985b; Li and Calabrese 1987).

In this study we focused on the electrical basis of the excitatory effects of FMRFamide on leech heart muscle. We studied enzymatically dissociated muscle cells and, by way of control, compared our results with those obtained from intact lengths of the leech's heart tubes. Dissociation was necessary for voltage-clamp studies because in the leech the heart cells are electrically coupled (Maranto and Calabrese 1984a). We employed conventional intracellular recording and voltage-clamp techniques. Studies of intact lengths of heart required 10−4 M curare to block spontaneous cholinergic epsps (Calabrese and Maranto 1986). Part of this work has appeared previously in an abstract (Thompson and Calabrese 1988).

Since its initial identification as a cardiac excitor in a clam (Price and Greenberg 1977), the effects of FMRFamide have been examined in a variety of systems, notably in Aplysia. In Aplysia, FMRFamide inhibits contractions of the gut (Austin and Lukowiak 1983) and gills (Weiss et al. 1984) and causes contractions of the buccal musculature (Richmond et al. 1986). FMRFamide has been found to have diverse effects on central neurons in Aplysia. Recent studies utilizing voltage-clamp techniques have characterized several modulatory effects on Aplysia neurons, including activation of a slow, voltage-dependent Na current and modulation of the transient K+ current (Ia) (Ruben et al. 1984); activation of a Cl−-dependent inward rectifier and reduction of Ia (Thompson and Ruben 1988); activation of the K+ -mediated S-current (Brezina et al. 1987a); suppression of Ca2+ current (Brezina et al. 1987b); and activation of a slow Na+ current (Ichinose and McAdoo 1988). The effects found in the present study include at least three separate actions on muscle cells involving activation of a slow Na+—dependent conductance and modulation of voltage-dependent outward and Ca2+ currents.

METHODS
Animals
Leeches were obtained from suppliers in Germany and France (Blutegelimport and Versand, Ricarimpex, and Biopharm). They were kept in artificial pond water containers in a dark incubator at 15°C for up to 4 mo. Occasionally leeches were fed defibrinated bovine blood.
Muscle cell isolation

Both hearts were dissected from the leech in normal saline under cold anesthesia. They were then subjected to gentle trituration in 1 mg/ml collagenase (type II, Sigma) for 30 min, followed by 2 mg/ml protease (type XIV, Sigma) for 30–45 min. This process dispersed the hearts into individual cells. The heart cell suspension was spun in a centrifuge to form a pellet of heart cells, and the supernatant was removed. Fresh saline was added and the resuspended cells were spun again at least four times. After the cells were repeatedly rinsed in this manner, they were stored in test tubes in solutions of L15 (GIBCO) culture medium (supplemented with 10 mM glucose and 2% vol/vol fetal bovine serum). This method is a modification of the procedure developed by Maranto and Calabrese (1984b).

The results of this study were obtained from dissected hearts of >70 leeches. For recordings, a suspension of isolated cells in culture medium was pipetted into a dish of saline. The dispersed cells sank to the bottom of the dish, where they could be easily penetrated with one or two microelectrodes. Most experiments were conducted the same day or early the next day after cell isolation. Cell cultures were stored at 4°C. There was no observable difference in cell properties whether the cells were stored at 4 or 15°C (the temperature at which the leeches were kept), except that the cultures became contaminated more quickly when kept at 15°C. Although we ordinarily used heart cells within the 1st day after isolation, isolated leech heart cells survived in short-term cell cultures for up to 5 days. They retained their normal morphology (examined at the light level) and also the ability to contract on depolarization. The cells were spindle shaped, ~200 μm long and 15 μm wide. The length constant, calculated from cable equation modeling, with the slope resistance at a depolarized condition, gives a value greater than the entire length of an individual fiber (335 μm).

Voltage-clamp measurements

Conventional two-electrode voltage-clamp techniques were employed. An Axoclamp-2 (Axon Instruments) voltage clamp and Pclamp software were used in this study. Glass microelectrodes had resistances of 30–70 MΩ when filled with K+ acetate (4 M). In some experiments the interior of the cell was loaded with Cs+. This was accomplished by using Cs acetate solution (2 M) or CsSO₄ (3.5 M) to fill the current and voltage electrodes. Microelectrode setting time for steps was on the order of 2 ms, which did not interfere with the steady-state and ramp measurements that were used in this study.

Solutions

Normal leech saline contains the following (in mM): 115 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 10 mM glucose, and 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer, adjusted to pH 7.4. Bath application of FMRFamide (Bachem) was with superfusion in a steady flow system (2.0 ml/min). The peptide was dissolved in normal or experimental salines. The constituents of experimental salines are discussed in the relevant sections of results. In general, saline constituents were as in normal saline except where indicated. NaCl was fully replaced by either N-methyl-D-glucamine (pH adjusted with HCl) or a combination of BaCl₂ and tetrathylammonium (TEA) or partially replaced by BaCl₂, CoCl₂, or MnCl₂. In addition, in experiments with CuCl₂ or MnCl₂, CaCl₂ was absent.

RESULTS

Characteristics of isolated heart cells

After enzymatic dissociation, isolated heart cells were usually quiescent, with resting potentials near -60 mV. In other systems where isolated mature heart cells of the rabbit were studied, the same phenomenon (of quiescence) was also observed (DiFrancesco et al. 1986). Extremely rarely in this study (5 of ~600), isolated leech heart cells spontaneously produced a polarization rhythm in normal saline (Fig. 1A). In the more typical quiescent heart cells, depolarizing pulses of current elicited Na+-dependent action potentials; Ca²⁺-dependent plateaus; and, after trains of stimuli, pronounced afterhyperpolarizations (Fig. 1B–D). Each of these active responses may be involved in the polarization rhythm of muscle cells in the intact heart. Preliminary ion-substitution experiments indicate that the action potentials are primarily carried by Na⁺ (full size and graded action potentials were abolished in Na⁺-free saline, not shown). The afterhyperpolarization is characteristic of cells with a Ca²⁺-activated K⁺ current and was blocked by 10 mM CoCl₂ (not shown). The plateaus appear to be Ca²⁺-dependent. They were blocked by 50 μM Cd²⁺, 5 mM Mn²⁺, and 10 mM CoCl₂; persisted in Na⁺-free saline; and in Ca²⁺-free salines were enhanced by 10 mM BaCl₂ (Fig. 1D). In addition, plateaus were prolonged in salines containing K⁺-channel blockers such as TEA (Fig. 1D) and in Cs⁺-loaded cells (not all of these effects are shown).

In an earlier study of the leech heart, Maranto and Calabrese (1984b) found that, although many isolated cells did not spontaneously produce rhythmic bursts, many did so or could be induced to burst with small amounts of depolarizing current. These isolated cells had membrane potentials near -40 mV and oscillated over a 10-mV range. The difference is difficult to explain, although slightly different methods were used to isolate and store heart cells in the two studies.

FMRFamide effects on isolated heart muscle cells

Application of FMRFamide by superfusion (10⁻⁶ M) elicited a strong depolarization of isolated heart cells. This response was associated with a pronounced conductance increase and was initiated after a characteristic 1- to 3-min delay (Fig. 2A). The depolarizing response was also associated with maintained contraction of the muscle cell. Such contractions began abruptly and terminated gradually, paralleling the course of the depolarization.

Recordings of the response to FMRFamide of intact lengths of heart (not treated with enzymes) gave essentially the same result [Fig. 2B; in 10⁻⁴ M curare to block spontaneous excitatory postsynaptic potentials (EPSPs) from axon terminals still attached to the heart]. Isolated lengths of heart, when quiescent, contained cells with resting membrane potentials of ~60 mV, similar to isolated cells. These results indicate that the enzymatic treatment used to isolate heart cells did not significantly hinder the ability of the cells to respond normally to FMRFamide.

Superfusion of FMRFamide (10⁻⁶ M) over heart cells held at rest in voltage clamp induced a net inward current (Fig. 2C). After a characteristic delay of ~2 min, the initial
inward current produced was transient. It was followed by a longer lasting inward current of lower amplitude. The transient current was also exhibited by muscle cells during long applications of peptide, and its amplitude was variable, ranging from <3 to >20 nA; but the sustained low-amplitude current was 0.20 ± 0.01 (SD) nA (n = 37; with FMRFamide at 10^{-6} M). During the sustained response, hyperpolarizing voltage steps resulted in larger current excursions than were elicited before FMRFamide application, indicating that the sustained inward current resulted from a conductance increase (data not shown). The small sustained current was observed to decay to 0 nA several minutes after washout of the FMRFamide. During prolonged applications of FMRFamide (10^{-6} M), the sustained current remained at a constant level (Fig. 2 D). After continuous rinsing with normal saline for >20 min after the peptide was removed (20 min breaks in the record), the current declined to baseline (0 nA).
The effects of FMRFamide application onto isolated cells were repeatable. In sequential trials the amplitude and duration of the responses remained consistent, but the latency of response decreased (Fig. 3A). The latency of the first response in Fig. 3A was 145 s, the second 75 s, the third 65 s, and the fourth 45 s. A common variation in the FMRFamide response, as exemplified by the second trial, was the induction of some oscillatory activity (see also Figs. 4 and 7).

The responses to bath applied FMRFamide long outlasted the duration of the FMRFamide application (cf. Figs. 2 and 3A). In addition, the amplitude of the response failed to diminish during continuous application of the peptide. For example, when 10⁻⁶ M FMRFamide was superfused over a heart cell for 13 min, the depolarization lasted >45 min (Fig. 3H). During the response the depolarization was steadily maintained at close to 0 mV. The maintained depolarization also occurred in the absence of superfusion and thus did not require continual introduction of fresh peptide. This contrasts with the observation that fresh peptide is required for maintained contraction of intact leech longitudinal muscle (Norris and Calabrese 1987).

The FMRFamide response was dose dependent, and a detectable depolarization followed bath application of concentrations of FMRFamide as low as 10⁻¹² M (Fig. 3C). The sensitivity of isolated heart cells to superfused FMRFamide was plotted by measuring the peak amplitude of the FMRFamide depolarization (not including the action potential) over a range of FMRFamide concentrations from below picomolar to above micromolar. The data shown (Fig. 3C) are responses from a single heart muscle cell tested at each concentration of FMRFamide. Four additional heart cells were tested for responsiveness to 10⁻¹² and 10⁻¹³ M FMRFamide. Three of the four depolarized (1-4 mV) in response to 10⁻¹² M solutions of peptide, but none showed detectable depolarizations to the lower concentration of FMRFamide.

Isolated heart cells reliably depolarized to FMRFamide during the 2 yr of our studies, except for both summers (i.e., late June through mid-August). In mid-June, some cells exhibited a partial response to superfused FMRFamide. Superfusion of FMRFamide (at 10⁻⁶ M) failed to produce depolarization of heart cells then, but it dramatically enhanced plateaus (Fig. 3D, n = 6). During the summertime when heart cells were insensitive to superfused FMRFamide, they did not depolarize or produce altered plateaus, suggesting the possibility of seasonal variation in responsiveness. New batches of leeches, collected by European suppliers, were received at the beginning of each summer, but the leeches did not have light cycle cues once they were in our laboratory (see METHODS).

Effects of FMRFamide on myogenic activity

Fewer than 1% of isolated heart cells exhibited a spontaneous depolarization rhythm (as in Fig. 1A). These cells (a total of 5 out of ~600 recorded) showed slow oscillations in membrane potential over an ~35-mV range, from just above −30 to just below −60 mV. The depolarizing phase of the oscillation often began steeply with a single action po-
potential, and then the prolonged plateau potential decayed more slowly. Oscillations have been observed in which action potentials did not occur, or in which only small-amplitude spikes were present, suggesting that oscillations are not dependent on spikes or on reaching membrane potentials above $-30\,\text{mV}$. The troughs of the oscillations reached $-60$ to $-65\,\text{mV}$.

In some cells that were initially quiescent, bath application of FMRFamide elicited an episode of oscillatory activity (Fig. 4A). This rhythmic response showed a similar delay and duration to the more typical FMRFamide responses (steady depolarization). However, the oscillations were pronounced, riding on a sustained depolarization of only $10\,\text{mV}$. The oscillatory responses were remarkably similar in form to those recorded from spontaneously oscillating isolated heart cells and occur with a similar periodicity ($8-15\,\text{s}$). In $\approx5\%$ of isolated heart cells did FMRFamide application induce oscillatory activity. When FMRFamide ($10^{-6}\,\text{M}$) was applied to isolated heart cells that were already exhibiting spontaneous oscillations in normal saline.
FMRFAMIDE EFFECTS ON LEECH HEART CELLS

285

16% FMRFamide

10⁻⁶ M FMRFamide

FIG. 4. Effects of FMRFamide on rhythmic activity of heart cells. A: induction of a polarization rhythm in a quiescent isolated heart cell by a pulse of superfused FMRFamide (10⁻⁶ M, at the bar). Effects of superfusion of a pulse of FMRFamide (10⁻⁶ M) on the spontaneous polarization rhythm of an isolated heart cell (B) and on a cell oscillating while still in the heart tube (C, in 10⁻⁴ M curare).

(n = 2), the cell displayed a sustained depolarization obliterating the oscillations (Fig. 4B). The same effect, i.e., depolarization overriding the oscillations in membrane potential, was recorded when FMRFamide was applied to spontaneously oscillating leech hearts that had not been enzymatically treated (Fig. 4C, n = 12). In this experiment, the initial effect of the FMRFamide application was an acceleration of the myogenic rhythm, suggesting that at lower concentrations of peptide (which would be present as the bath is equilibrating during superfusion) FMRFamide could increase the frequency of the polarization rhythm. Measurements of heart rate from previous tension recordings in the intact heart have demonstrated a heart rate increase in response to bath-applied FMRFamide (10⁻⁷–10⁻⁶ M; Kuhlman et al. 1985b).

Ion substitution experiments

The depolarization due to FMRFamide (10⁻⁶ M) was greatest at hyperpolarized levels and was reduced gradually but not reversed when heart cells were held at various membrane potentials from -120 to +10 mV (in 2-electrode current clamp) (Fig. 5). This result suggested that the depolarization of resting heart cells by FMRFamide was at least partially due to a conductance increase to ions such as Na⁺ or Ca²⁺ with positive reversal potentials. To test for the involvement of Na⁺ or Ca²⁺ currents in the FMRFamide mediated depolarization, we applied FMRFamide (10⁻⁶ M) to heart cells in experimental salines with Na⁺ replaced by N-methyl-D-glucamine (Fig. 6A); with Ca²⁺ removed and Co²⁺, Cd²⁺, or Mn²⁺ added (Fig. 6B); or with Ba²⁺ (Fig. 6C). Unfortunately, the results of these experiments were in some cases difficult to interpret. In many experiments (n = 13 of 16), replacement of external Na⁺ caused the heart cell membrane potential to hyperpolarize initially, and then over the next several minutes the cells slowly depolarized to near 0 mV. These cells became leaky and blistered in appearance and could not produce plateaus. This effect was not reversible. A more successful strategy for replacing Na⁺ was to reduce the Na⁺ concentration gradually (Fig. 6A). In normal saline a sustained FMRFamide response was initially produced. When the Na⁺ concentration was reduced to 75%, the response was oscillatory, and at 25% the oscillations were severely reduced in amplitude. The response was blocked with Na⁺ completely replaced by N-methyl-D-glucamine. This was followed by a weak sustained depolarization to FMRFamide on return to normal saline. It is well known that extracellular Na⁺ can have profound effects on membrane properties apart from any possible action on peptide-mediated currents. These include Na⁺-coupled or -dependent processes, such as the Na⁺-K⁺ exchange (Thomas 1972), the Na⁺-Ca²⁺ exchange (Requena et al. 1977) or Na⁺-H⁺ transport (Thomas 1977). However, complete replacement of Na⁺ by Li⁺ (superfusion for 4 h) did not diminish the ability of FMRFamide to induce a strong depolarization, indicating that the FMRFamide response was unlikely to be mediated by the Na⁺-Ca²⁺ exchanger. The heart cells also fared better when the Na⁺ was replaced with divalent cations and/or THA (see experiments below).

In experiments with Ca²⁺ channel blockers, control FMRFamide depolarizations were first recorded in normal saline. The heart cells were then equilibrated for up to 2 h in

![Graph showing response amplitude vs. holding potential](image...

FIG. 5. Effect of membrane potential of isolated heart cells on the amplitude of the response to FMRFamide application (10⁻⁶ M). Each curve on the graph represents the peak depolarizing response of an individual cell held at the several membrane potentials indicated. Family of curves appears nonlinear and to extrapolate to a reversal potential >0 mV.)
50 μM Cd$^{2+}$, 5 mM Mn$^{2+}$, or 10 mM Co$^{2+}$. When FMRFamide was added to the superfusate, a depolarizing response still occurred in the majority of experiments ($n = 26$ of 29). When FMRFamide was added a third or additional times, either in test saline or after return to normal saline, the response was blocked and it did not recover, despite prolonged rinsing and repeated tests (Fig. 6B). Thus the typical effect of Ca$^{2+}$ channel blockers was an irreversible block of the FMRFamide depolarization after a single failure to block.

Replacement of Ca$^{2+}$ with 10 mM Ba$^{2+}$ enhanced the response to FMRFamide ($n = 56$; Fig. 6C). Ba$^{2+}$ ions are thought to be more efficiently translocated through many Ca$^{2+}$ channel types than Ca$^{2+}$ itself (Hille 1986). In addition, Ba$^{2+}$ ions are K$^+$-channel blockers, so reduction of K$^+$ currents could also contribute to the enhancement observed in Ba$^{2+}$ salines. In experiments not shown ($n = 3$), elevation of external Ca$^{2+}$ concentration to 10 mM (normal is 1.8 mM) blocked, immediately and irreversibly, the depolarization elicited by FMRFamide. Blocking of K$^+$ channels by external TEA, internal Cs$^+$, and Ba$^{2+}$ served to enhance the depolarization elicited by FMRFamide (see also voltage-clamp records, Figs. 8 and 9).

**FMRFamide effects on ionic currents**

Under voltage clamp in normal saline, isolated quiescent heart cells exhibited very low leak currents corresponding to a measured input resistance of > 100 MΩ. In response to depolarizing voltage ramps (or steps) in normal saline, they displayed only pronounced outward rectification (see Fig. 7A). No region of negative slope conductance, which is characteristic of other types of oscillatory cells (see Adams and Benson 1985), was revealed. The recorded current-voltage (I-V) relation is consistent with observations in unclamped cells that isolated muscle cells were normally not spontaneously active.

Superfusion of FMRFamide (at $10^{-6}$ M) induced an inward current with two components when the heart cell was held at rest, a transient larger current of variable amplitude ($n = 14$ of 16) and a smaller sustained current ($n = 16$ of 16) (Fig. 2C). The initial large current may be an ionic current, or it could result from damage associated with the contraction at the beginning of the response to FMRFamide. At the gain in the voltage-clamp device required to observe the small sustained current, the large component would often set the voltage clamp into oscillations at which damaging amounts of compensating current were injected into the cells. For this reason, we chose not to study the transient current further. However, the low-amplitude inward current was persistent in the continued presence of FMRFamide. The stability of the current in the continued presence of
At higher depolarized levels, the net outward current exhibited steep voltage dependence \((n = 16\) with FMRFamide \(10^{-9}\) M). The unexpected enhancement of outward currents proved to be exceedingly large and difficult to clamp at depolarized levels when the peptide was applied at the customary \(10^{-6}\) M \((n = 35;\) see results with K⁺-channel blocking agents below). Along with the results from ion-substitution experiments, these results suggest that the effects of FMRFamide are complex, involving more than a single conductance change.

To focus on the inward current(s) affected by FMRFamide, we penetrated heart cells with Cs⁺-filled microelectrodes. Cs⁺-loaded heart cells have substantially reduced K⁺ currents, which permitted the voltage ramps to be extended to more depolarized levels, for example, from -90 to +10 mV (Fig. 8). Subtraction of the currents in normal saline from the currents in FMRFamide-containing saline yielded the difference current, which, under these conditions, was inward over the entire range of the voltage ramp \((n = 18,\) Fig. 8). The inward current (Fig. 8B) gradually declined from a level of -0.2 nA at -90 mV to slightly more than -0.1 nA at -30 mV, demonstrating a linear component. A voltage-dependent increase in inward current was seen above -20 mV. The inward current peaked at -0.8 nA at +5 mV.

**FIG. 7.** Current-voltage \((I-V)\) relation of an isolated heart cell in normal saline and in saline containing FMRFamide at a low concentration \((10^{-9}\) M) \((A)\) and difference current due to FMRFamide \((B)\). \(A:\) current was recorded during voltage ramps over the range of -90 to -20 mV lasting 2 min in normal saline (trace labeled Normal, N). Another identical ramp was delivered to this cell after the response to \(10^{-9}\) M FMRFamide had stabilized in the continued presence of peptide (FMRFamide trace, F). \(B:\) current dependent on FMRFamide was obtained by subtraction of the \(I-V\) curve in normal saline from the \(I-V\) curve in the presence of FMRFamide (Difference). Difference relation shows both net inward and outward components.

FMRFamide expedited further measurements of the \(I-V\) relation of the peptide response from isolated heart cells. The steady-state \(I-V\) relation was measured by the use of slow voltage ramps (i.e., 10 mV/15–20 s). To obtain the FMRFamide-dependent difference current, isolated cells were first penetrated with current and voltage electrodes, and a control voltage ramp was delivered. Next, the cell was taken out of voltage-clamp mode (without removing the 2 microelectrodes) and the FMRFamide response was elicited. In the continued presence of FMRFamide, the cell was returned to voltage clamp for another ramp. This procedure avoided the necessity of clamping the transient current during the initial contraction of the cell.

The \(I-V\) relation of the FMRFamide-dependent currents in normal saline contains both inward and outward components (Fig. 7). Over the range of -90 to -20 mV, when FMRFamide was superfused at a low concentration \((10^{-9}\) M), the FMRFamide-dependent current consisted of a small inward current that reversed near -50 mV. More depolarized than -50 mV, the current became net outward. The current-voltage \((I-V)\) relation of an isolated heart cell in normal saline and in saline containing FMRFamide at a low concentration \((10^{-9}\) M) \((A)\) and difference current due to FMRFamide \((B)\). \(A:\) current was recorded during voltage ramps over the range of -90 to -20 mV lasting 2 min in normal saline (trace labeled Normal, N). Another identical ramp was delivered to this cell after the response to \(10^{-9}\) M FMRFamide had stabilized in the continued presence of peptide (FMRFamide trace, F). \(B:\) current dependent on FMRFamide was obtained by subtraction of the \(I-V\) curve in normal saline from the \(I-V\) curve in the presence of FMRFamide (Difference). Difference relation shows both net inward and outward components.

**FIG. 8.** \(I-V\) relation of a heart cell loaded with internal Cs⁺ and effect on the response to FMRFamide \((10^{-9}\) M). Internal Cs⁺ reduced outward currents, further isolating the net inward current induced by FMRFamide. \(A:\) current in response to voltage ramps lasting 3 min in heart cell loaded with Cs⁺ and measured in normal saline before (Normal) and during the stabilized response to continuous superfusion of FMRFamide. \(B:\) difference current obtained by subtracting the Normal curve from the FMRFamide curve. Current is inward over the voltage range of -90 to +10 mV.
With \( \text{Na}^+ \) replaced by \( \text{Ba}^{2+} \) (20 mM) and TEA (75 mM), the small inward current present at membrane potentials below -40 to -50 mV was absent, and the modulation of the voltage-dependent inward current was revealed (Fig. 9, A and B). In these experiments \((n = 28)\), heart cells were clamped with voltage ramps to still greater levels of depolarization, from -90 to +50 mV. The subtracted current elicited by FMRFamide was inward above -40 to -50 mV. This inward current displayed voltage dependence, peaking between 0 and +5 mV. \( \text{Ba}^{2+} \) currents are also elicited by depolarizing steps in isolated heart cells (activation curve shown in Fig. 9D). The inward current demonstrated in these records may represent the voltage-dependent \( \text{Ca}^{2+} \) current present in heart cells (see Fig. 8). These results suggest that FMRFamide application modulated the voltage-dependent \( \text{Ca}^{2+} \) current normally present in heart cells. It appears to do so, not by altering the peak current but by reducing the rate of inactivation, as seen by comparing \( \text{Ba}^{2+} \) currents activated by voltage steps with and without FMRFamide (10^{-6} M) in Fig. 9C.

The use of \( \text{Cs}^+ \) acetate electrodes and an experimental saline containing 10 mM \( \text{Co}^{2+} \), 25 mM TEA, and 0 mM \( \text{Ca}^{2+} \) served to separate the \( \text{Na}^+ \) component of the inward current induced by FMRFamide (Fig. 10). The experimental saline blocked the large voltage-dependent increase in inward current seen in Figs. 8 and 9, leaving a nearly linear current that reversed near +6.5 mV. Figure 10, inset, shows result from one cell. The current present with \( \text{Ca}^{2+} \) and \( \text{K}^+ \) currents substantially blocked showed little voltage dependence; the current appeared to resemble a primarily \( \text{Na}^+ \)-selective leak. The reversal potential, however, suggests that, although this current may be dependent primarily on \( \text{Na}^+ \) ions, it is not entirely selective to \( \text{Na}^+ \) and may also...
involve K⁺ ions or be similar to a nonspecific cation current.

Thus the voltage-clamp experiments revealed at least three effects of FMRFamide application. At rest, FMRFamide induced a slow, partially Na⁺-dependent current. In addition, FMRFamide modulated both voltage-sensitive Ca²⁺ currents and voltage-dependent outward currents.

**DISCUSSION**

In this study, FMRFamide has been found to modify the membrane properties of heart cells in several respects. This peptide depolarized heart cells and induced polarization rhythms in some cases. It did so by modulating ionic conductances over the entire physiological voltage range in which the cells are active. Significantly, these effects included activation of a voltage-independent current that served to depolarize heart cells from rest and allowed them also to experience the additional modulation of voltage-dependent Ca²⁺ and K⁺ currents. Thus the data from ion-substitution and voltage-clamp experiments suggest that FMRFamide influences at least three separate currents in heart cells. First, it activates a small persistent inward current that shows Na⁺ selectivity but probably has a K⁺ component. Features in common with the slow Na⁺ current found in other studies are persistence in Ca²⁺-saturated, voltage independence (including availability at hyperpolarized levels), absence of effect of K⁺-channel blockers, and block by high external Ca²⁺ concentration (Ichinose and MacAdoo 1988). This current is also activated by FMRFamide in a number of other systems (Ichinose and MacAdoo 1988; Ruben et al. 1986). Second, FMRFamide application modulated voltage-dependent outward currents that were blocked by K⁺-channel blockers. Third, FMRFamide modulated voltage-dependent Ca²⁺ currents.

In other types of cardiac cells and oscillating neurons, a pacemaking current, such as If (DiFrancesco 1981) or Ifs (Adams and Benson 1985); a persistent leakage current with a depolarized reversal potential; or Ca²⁺-activated cation current, IfCa(Reversal potential about −20 mV; Kass et al. 1978; Krammer and Zucker 1985) is present. In these cells such currents function to allow neurons and muscle cells to spontaneously depolarize after a period of quiescence either by being activated by the hyperpolarization or by being persistently active. In leech heart cells we have no evidence for such a current other than the small persistent current gated by FMRFamide. The observation that application of low concentrations of FMRFamide leads to depolarization or an episode of myogenic oscillations suggests that the Na⁺-dependent current gated by FMRFamide may provide the depolarizing drive enabling the production of spontaneous myogenic activity. Furthermore, the heart accessory and heart excitatory (HA and HE) neurons that supply the heart are continuously active and, presumably, tonically releasing small amounts of FMRFamide or a FMRFamide-like peptide. Thus one possibility is that the FMRFamide-gated current is essential to drive the polarization rhythm of heart cells. Conversely, currents may be present in the intact heart that are lost on isolation. We have tried to minimize this possibility by using gentle methods for isolating heart cells and by cross-comparing the effects of FMRFamide on isolated cells with the effects on lengths of heart, and in these cases we have observed no differences. The enhancement of Ca²⁺ and K⁺ currents by FMRFamide may also be necessary for myogenicity, because simple depolarization did not induce the normal myogenic polarization rhythm in heart cells in the present study (but see Maranto and Calabrese 1984b).

Isolated leech heart cells were exquisitely sensitive to the peptide, depolarizing at picomolar concentrations. This observation contrasts with other studies in mollusks and leech, in which responses require 10⁻¹⁰⁻¹⁰⁻⁶ M (Norris and Calabrese 1987; Stone and Mayeri 1981). More sensitivity is seen in clam heart and whelk radula muscle (10⁻⁸–10⁻⁶ M; Doble and Greenberg 1982; Price and Greenberg 1980). It is possible that intact muscles are less sensitive because of the presence of protease systems for breaking down peptides, systems that could be lost in isolated cells (see Norris and Calabrese 1990). The broadness of the dose-response curve for leech heart cells is probably due to the fact that three different currents are activated, two of which are voltage sensitive. We did not investigate the basis of the heart cell's failure to respond normally to FMRFamide application in the summer months, but seasonal variation in responsiveness to peptides has been observed in other systems (e.g., Bishop et al. 1991).

Tension measurements were used in previous studies to analyze the effects of FMRFamide on the leech heart. The effects of FMRFamide on tension included tonic contraction, increased beat tension, and induction or acceleration of myogenic rate (Calabrese and Maranto 1984, Kuhlman et al. 1984b). In other, unrelated studies it has been shown that changes in the contractile properties of muscle are not necessarily ascribable to changes in ionic currents. For example, the well-documented effects of proctolin on insect muscle occur without any change in muscle membrane potential or input resistance (Adams and O'Shea 1983). On the other hand, Ca²⁺ conductance is greatly increased in vertebrate heart cells after application of adrenaline (Brown et al. 1979; Reuter 1967). In leech heart cells it appears that FMRFamide exerts its effects on tension by modifying electrical properties. Each of the previously observed effects of FMRFamide on tension is consistent with effects on ionic currents found in the present study. The tonic contraction would be expected with the tonic depolarization induced by FMRFamide superfusion. The enhancement of strength of rhythmic contractions (beat tension) would be a predicted result of the modulation of voltage-dependent Ca²⁺ currents by FMRFamide. Parallel to FMRFamide effects on activating myogenic contractions, activation of a polarization rhythm took place after FMRFamide application in some isolated heart cells. The electrical oscillations were associated with myogenic contractions. Although the evidence from this study is less direct in reference to augmentation of heart rate by FMRFamide, Maranto and Calabrese (1984b) showed that depolarization increases the myogenic rate of isolated heart cells, and the tonic depolarization by FMRFamide was amply demonstrated by the cells in this study.

The role of Ca²⁺ ions in the FMRFamide response warrants special consideration. The results of this study
suggest that Ca\(^{2+}\) may also be involved at some level in a second-messenger system mediating FMRFamide action. Thus the persistent inward current could be a consequence of Ca\(^{2+}\), as a second messenger, turning on a slow Na\(^{+}\)-dependent current, or Ca\(^{2+}\), internally, acting to directly activate the slow Na\(^{+}\)-dependent current. The problems we encountered in attempts to block the FMRFamide response with Mn\(^{2+}\), Co\(^{2+}\), and other Ca\(^{2+}\)-channel blockers were peculiar in that the blocking effect took place long after voltage-dependent Ca\(^{2+}\) currents were blocked, but the block was irreversible. This suggested to us that FMRFamide may have been acting through a second-messenger system such as inositol triphosphate that led to release of Ca\(^{2+}\) from internal stores. Thus the first application of FMRFamide might still activate release of internal stores of Ca\(^{2+}\), but when the stores were refilled they would be empty or poisoned by the Co\(^{2+}\). We tried to deactivate inositol triphosphate mechanisms with external Li\(^{+}\), completely replacing Na\(^{+}\). Li\(^{+}\) has been shown in other systems to block inositol triphosphate mechanisms with external Li\(^{+}\), completely replacing Na\(^{+}\). However, the evidence from this and other studies suggests that the multitude of FMRFamide actions are through a second-messenger system. The response is slow and delayed in onset, it is persistent and slow to wash out, and it appears "pruned" by repeated applications of peptide. Together these features suggest the involvement of one or more second-messenger systems (see Kaczmarek and Levitan 1987).

The modulation of motor systems by peptides and other substances is an area of intense interest in neurobiology (Kaczmarek and Levitan 1987). One general finding from such studies is that motor systems appear to be modulated at every level, from neuronal excitability to strength of synaptic interactions to the function of the peripheral effector organs (muscle) (e.g., Harris-Warrick and Flamm 1986; Meyrand and Moulins 1986; O’Shea 1985). In the leech, there are in addition to modulation of heart muscle a variety of identified sensory and motor pathways that modulate the period of the central pattern generator (Arbas and Calabrese 1984; Calabrese and Arbas 1985). Thus the leech heartbeat system comprises two oscillators, one central and one peripheral; modulation occurs at both levels. The flexibility of motor systems, including the superimposed levels of modulation, is thought to permit the animal to cope with the changing demands of its internal and external environment.

The authors thank Dr. James D. Angstadt for helpful discussions during the course of this project and Dr. Joseph E. Freschi for comments on the manuscript. The work was supported by National Institute of Neurological Disorders and Stroke Grant NS-24072 to R. L. Calabrese. Address for reprint requests: K. J. Thompson, Dept. of Biology, Emory University, 1510 Clifton Rd., Atlanta, GA 30322.

Received 2 May 1991; 4 October 1991.

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