Segment-Specific Effects of FMRFamide on Membrane Properties of Heart Interneurons in the Leech

JOACHIM SCHMIDT, SIGLINDE GRAMOLL, AND RONALD L. CALABRESE
Department of Biology, Emory University, Atlanta, Georgia 30322

SUMMARY AND CONCLUSIONS

1. The effects of Phe-Met-Arg-Phe (FMRF)amide (10⁻⁶ M) on membrane properties of heart interneurons in the third, fourth, and fifth segmental ganglia [HN(3), HN(4), and HN(5) cells, respectively] of the leech were studied using discontinuous current-clamp and single-electrode voltage-clamp techniques. FMRFamide was focally applied onto the soma of the cell under investigation.

2. Application of FMRFamide depolarized HN(3) and HN(4) cells by evoking an inward current. These responses were subject to pronounced desensitization. The inward currents evoked by application of FMRFamide were associated with an increase in membrane conductance and appeared to be voltage dependent. Currents were enhanced at more depolarized potentials.

3. The responsiveness of the HN(3) and HN(4) cells was not affected when the Ca²⁺ concentration in the bath saline was reduced from normal (1.8 mM) to 0.1 mM. The depolarizing response on application of FMRFamide was blocked when Co²⁺ was substituted for Ca²⁺.

4. HN(3) and HN(4) cells did not respond to FMRFamide application in Na⁺-free solution. Inward currents were largely reduced when bath saline with 30% of the normal Na⁺ concentration was used. When Li⁺ was substituted for Na⁺ in the saline, application of FMRFamide still evoked depolarizing responses in HN(3) and HN(4) cells.

5. We conclude that focal application of FMRFamide onto the somata of HN(3) and HN(4) cells evokes a voltage-dependent inward current, carried largely by Na⁺.

6. Focal application of FMRFamide onto somata of HN(5) cells hyperpolarized these cells by activating a voltage-dependent outward current.

7. HN(5) cells were loaded with Cl⁻ until inhibitory postsynaptic potentials carried by Cl⁻ reversed. Cl⁻-loaded cells still responded with a hyperpolarization when FMRFamide was applied onto their somata. Therefore the outward current evoked by FMRFamide appears to be mediated by a K⁺ conductance increase.

8. Application of FMRFamide onto the somata of HN(5) cells enhanced outward currents that were evoked by depolarizing voltage steps from a holding potential of −45 mV.

9. We conclude that the hyperpolarizing response of HN(5) cells to focal application of FMRFamide onto their somata is the result of an up-regulation of a voltage-dependent K⁺ current.

INTRODUCTION

Blood flow in the circulatory system of the leech is controlled by rhythmic constrictions of two muscular lateral “heart tubes” (Boroffka and Hamp 1969; Kralh and Zerbst-Boroffka 1983). The period of the constriction rhythm is ~10 s. Constrictions of both heart tubes are coordinated such that one heart tube constricts in a rear to front peristalsis whereas the other constricts synchronously. This pattern switches every 20–40 heartbeat cycles. After a switch the formerly peristaltic heart constricts synchronously and vice versa (Calabrese and Peterson 1983; Kralh and Zerbst-Boroffka 1983; Thompson and Stent 1976a). Heart muscle activity is a result of rhythmic excitatory input from heart motor neurons (HE cells) that are found in the 3rd through the 18th segmental ganglia (Thompson and Stent 1976a). These motor neurons receive rhythmic inhibitory input from heart interneurons (HN cells) (Fig. 1A; Calabrese 1979). Pairs of HN cells are found in the first [HN(1) cells] to the seventh [HN(7) cells] segmental ganglia. These HN cells appear to be homologous because of the identical positions of their somata in each ganglion and several characteristic morphological features (Shafer and Calabrese 1981). HN cells form a network of inhibitory connections, thereby establishing the heartbeat central pattern generator (Fig. 1B; Calabrese 1977; Thompson and Stent 1976b,c).

HN cells in the first four ganglia form a timing oscillator. HN(1) and HN(2) cell pairs synchronize the activity of HN(3) and HN(4) cells (Peterson 1983b). HN(3) and HN(4) cells, respectively, form reciprocal inhibitory synapses, thereby establishing half-center oscillators that appear to pace the timing of the rhythm (Fig. 1; Calabrese and DeSchutter 1992; Peterson 1983; Thompson and Stent 1976c). HN(3) and HN(4) cells also make synaptic connections with ipsilateral motor neurons and the ipsilateral HN(5) cell (Fig. 1).

HN(5) cells do not form reciprocal inhibitory synapses, but they do make direct inhibitory synapses with the HN(6) and HN(7) cells on both sides. The two HN(5) cells alternately switch between a rhythmically active and an inactive state (see Fig. 10A), thereby alternatively distributing the oscillatory activity pattern of HN(3) and HN(4) cells to the HN(6) and HN(7) cells. Ultimately this leads to the peristaltic and synchronous constriction pattern of the heart tubes (Calabrese and Peterson 1983).

Rhythm generation in HN cells is independent of sensory input. However, the period of the rhythmic activity is modulated by the activity of certain sensory neurons or when swimming is initiated by neuronal stimulation (Arbas and Calabrese 1984). The motor program for swimming can be elicited by depolarizing with injected current a particular cell, interneuron 204 (Weeks and Kristan 1978). Stimulation of cell 204 leads to an acceleration of rhythmic activity in HN cells (Arbas and Calabrese 1984). The swim interneuron 204 contains Phe-Met-Arg-Phe (FMRF)amide-like immunoreactivity (Kuhlman et al. 1985a). A family of five RFamide peptides, including FMRFamide, has been isolated for the leech CNS (Evans et al. 1991). Superoxidation of a third or fourth segmental ganglion with FMRFamide (10⁻⁸–10⁻⁶ M)
and a hyperpolarizing outward current in HN (5) cells. In-
of FMRFamide onto the respective somata. a depolarizing inward current in HN (3) and HN (4) cells
FMRFamide in the switch interneurons HN (5). We describe
rons HN (3) and HN(4) with responses evoked by
responses evoked by FMRFamide in the oscillator interneu-
chains of midbody ganglia three to six, because oscillatory activity
of HN cells tends to be less regular in isolated ganglia. When
were anesthetized in cold saline and ganglia were removed for
experiments on HN (5) cells we removed
an identical circuit diagram showing connections
from HN to HE cells. B: synaptic connections among all identified HN
cells. Neurons with identical input and output connections are lumped to-
gether. Large open circles: neurons (each identified by the number of its
ganglion). Lines: major neurites or axons. Small filled circles: inhibitory
chemical synapses (after Calabrese et al. 1989).

M)-containing saline leads to an acceleration of the heart-
beats rhythm, thus mimicking the effects of stimulation of
cell 204 (Kuhlman et al. 1985b). Higher concentrations of
FMRFamide (>10⁻⁷ M) tend to disrupt the rhythmic activity
of HN cells. Hyperpolarizing shifts in steady-state activation
and inactivation of the K⁺ current, I_K (Simon et al. 1992), and presynaptic modulation of spike-mediated synaptic
transmission between HN cells (Simon et al. 1994), have
been reported and may account, at least partially, for the
described effects of FMRFamide. In contrast to HN (3) and
HN (4) cells, nothing is known about effects of FMRFamide
on the switch interneurons HN (5).

Our goal here was twofold. First, we wanted to gain more
insight into the effects of FMRFamide on HN (3) and HN (4)
cells by using a faster form of application of FMRFamide
than used in previous studies. Second, we wanted to compare
responses evoked by FMRFamide in the oscillator interneu-
rorns HN (3) and HN (4) with responses evoked by
FMRFamide in the switch interneurons HN (5). We describe
a depolarizing inward current in HN(3) and HN(4) cells
and a hyperpolarizing outward current in HN (5) cells. In-
ward and outward current were elicited by focal application
of FMRFamide onto the respective somata.

METHODS

Leeches (Hirudo medicinalis) were obtained from Leeches USA
(Westbury, NY), Biopharm (Charleston, SC), or from the breeding
colonies of Dr. Eduardo Macagno (Columbia University, New
York, NY) and maintained in artificial pond water at 15°C. Animals
were anesthetized in cold saline and ganglia were removed for
study. For experiments on HN (3) and HN (4) cells we removed individual ganglia. For experiments on HN (5) cells we removed
chains of midbody ganglia three to six, because oscillatory activity
of HN (5) cells tends to be less regular in isolated ganglia. When
regular oscillation was not required we used short chains of ganglia
two to six or isolated ganglion five. Ganglia were pinned ventral
side up in Sylgard (Dow-Corning)-lined petri dishes with a bath
volume of 0.5 ml. The connective tissue sheath overlying the nerve
cell bodies was removed immediately before the experiments.
Ganglia were continuously superfused (1.5–2 ml/min) with normal
leech saline (Nicholls and Baylor 1968) containing (in mM) 115 NaCl, 4 KCl, 1.8 CaCl₂, 10 glucose, and 10 tris (hydroxymethyl)aminomethane (Tris) buffer, pH adjusted to 7.4 with HCl.
In some experiments saline with reduced Na⁺ content or "Na⁺-
free saline" was used. In these cases NaN⁺ was substituted by an
equimolar concentration of N-methyl-D-glucamine. Na⁺-free saline
must be used to block action potentials in HN cells because
tetrodotoxin, saxitoxin, and other fast Na⁺ channel blockers have
little effect on action potentials in HN cells (Opdyke and Calabrese
1994) or many other leech neurons (Johansen and Kleinhaus
1987). Some experiments were performed in Na⁺-free Co⁺²-sa-
line, in which 1.8 M Co⁺² substituted for Ca⁺². Occasionally we
added bicuculline methiodide (10⁻⁴ M, Sigma) to the saline to
block synaptic transmission between HN cells.

FMRFamide (Bachem, Peninsula Laboratories, and Sigma) was
dissolved in distilled water at a concentration of 5 mg/ml stored
frozen, and dissolved in saline used for superfusion immediately
before use. FMRFamide was applied focally to receptors on the
somata of HN cells with a picospritzer (General Valve, Picospritzer
II). Pipettes for application (borosilicate glass, 1 mm OD, 0.75
mm ID) were pulled on a picotte puller (Flaming Brown Micropi-
pette Puller, model P-80/PC, Sutter Instruments). The dye Fast
Green was added to the picotte solution to visualize the distribution
of the ejected substance. Fast Green did not itself influence the
activity of HN cells. Because HN cells are located on the posterior
edge of the ganglion, it was possible to orient the ganglion into
the saline current such that FMRFamide ejected from the picotte
did not reach cells other than HN cells.

Cell bodies of HN cells and HE cells were penetrated with
microelectrodes made of the same glass as described above and
were filled with 4 M KAc/20 mM KC₁ solution. Electrode resist-
ances were between 30 and 40 MO. An Axoclamp 2A amplifier
(Axon Instruments) was used in BRIDGE mode for recording or in
discontinuous current-clamp mode for current-clamp experiments.
Sample rates in discontinuous current-clamp mode typically were
in the range of 2–3 kHz. In discontinuous single-electrode voltage-
clamp (dSEVC) mode sample rates of 4–5 kHz were used. Higher
sample rates were made possible by reducing the electrode capaci-
tance by coating the electrode tip with Sylgard (Dow-Corning)
or dimethylpolysiloxane (Sigma). The voltage gain was typically set
at ~25 nA/mV. Output bandwidth was set at 0.3 kHz.
In some experiments we loaded HN (5) cells with Cl⁻. In these
experiments recordings were made with electrodes filled with 4 M
KCl and Cl⁻ was allowed to diffuse into the cells.

Data were recorded on a VHS video cassette recorder modified
for frequency modulation recording (Vetter, model 240) for later
playback on paper chart recorders (Gould). Episodic voltage-
clamp protocols were generated by and associated clamp currents
were digitized on-line and stored on a personal computer using a
TL-1 interface (Axon Instruments) and pClamp (Axon Instruments)
software. Digitized data were analyzed and in some cases
leak subtracted (voltage steps equal but opposite to the depolarizing
voltage steps were used to estimate leak currents) using pClamp
(Axon Instruments) or KaleidaGraph (Abelbeck) software and
displayed on a laser printer (Hewlett Packard, LaserJet III).

Unless indicated otherwise, all experiments were carried out at
least three times.

RESULTS

FMRFamide depolarizes HN (3) and HN (4) cells

Previous experiments for the purpose of studying the effects
of FMRFamide on HN cell activity were performed by
adding FMRFamide to the superfusate (Simon et al. 1992, 1994). This technique has disadvantages. For a period after switching from normal saline to FMRFamide-containing saline, a solution of FMRFamide with changing concentration is superfused, because of dilution by remaining normal saline in the system. This effect may prevent the detection of changes that are subject to rapid inactivation or desensitization. Another drawback of superfusion of FMRFamide may be that indirect effects in the cell under observation caused activity of presynaptic cells that are directly affected by FMRFamide.

To avoid these problems, FMRFamide was applied focally onto the somata of HN cells. Such an experiment is shown in Fig. 2, where FMRFamide (10^{-6} M) was applied for 5 s onto the soma of an HN(3) cell. The recording shows all the features of a typical response to fast FMRFamide application: the cell depolarized with a peak ~4 s after stimulus onset. The depolarization declined, even while the stimulus was on (see also Fig. 4). The bursting pattern was disrupted for ~10 s after stimulus offset. The cell then developed its typical bursting pattern, but was transiently accelerated (note period—distance between lines—before and after application) before settling back to its original period. In Fig. 2B the shortening of period caused by focal FMRFamide application on a single HN neuron is shown in more detail: for each of seven experiments, the mean period of 5 cycles of the HN cell activity before FMRFamide application was calculated. This mean was used to normalize the period of HN activity for each experiment. The first column shows mean period of 5 cycles before FMRFamide application in 7 experiments. Bars: SD. Other columns show mean periods of cycles 1–9 after FMRFamide application of 5 s (N = 7). Cycles were counted after the disruption of rhythmic activity due to FMRFamide application (as indicated in A). Asterisks: the 3rd period after the disruption showed the minimum average period and was significantly shorter than mean of periods before FMRFamide application (Student’s t-test, P < 0.01). In cell names R and L refer to the right or left body side.
FMRFamide (10^-6 M)

FIG. 3. Depolarizing response of HN cells to focal application of FMRFamide (10^-6 M) was subject to pronounced desensitization. The responsiveness of the HN(L,4) cell declined dramatically after the 1st focal application of FMRFamide onto the soma.

ization caused by FMRFamide has not been observed before, and the effect seems to depend on fast application.

Usually the depolarizing response of HN cells to locally applied FMRFamide was subject to rapid desensitization. Not only did the response decline during application (Figs. 2A and 4), but also the responsiveness to repetitive application of FMRFamide decreased dramatically (Fig. 3). In 22 of 43 experiments a second application of FMRFamide did not elicit a detectable response. In another 10 of the 43 experiments no response was detectable after the second application of FMRFamide. Even waiting periods of 20 min between applications were not sufficient for removal of the desensitizing effect. A lower concentration of FMRFamide (10^-7 M) had a similar desensitizing effect. However, it should be noted that in two of four batches of leeches from three different suppliers, some animals were found in which HN cells showed less desensitization. These few animals were used for experiments where repetitive FMRFamide applications were necessary (e.g., Fig. 7). Even in these preparations the response was completely desensitized within four to five applications of FMRFamide. Consequently, experiments involving more than one application of FMRFamide often had to be performed without a complete set of controls to avoid desensitization.

FMRFamide induces an inward current in HN(3) and HN(4) cells that is associated with an increase in membrane conductance

Using dSEVC, the inward current responsible for the depolarizing response elicited by focal application of FMRFamide could be demonstrated (Fig. 4). To test whether this membrane current is associated with a membrane conductance increase or decrease, small repetitive negative voltage steps (-15 mV, 500 ms) were applied on a steady holding potential (-40 mV). During these experiments bicuculline methiodide (10^-4 M) was added to the superfuse. Bicuculline prevents bursting by blocking inhibitory interactions between HN(3) and HN(4) cells, thus providing a stable baseline of activity (Schmidt and Calabrese 1992). The amplitude of membrane currents associated with the voltage steps increased during the application of FMRFamide (Fig. 4). This current increase indicates a membrane conductance increase associated with the application of FMRFamide.

It should be noted here that in 7 of all 149 experiments performed, focal application of FMRFamide evoked a biphasic response. The depolarization was followed by a hyperpolarization that was stable with repeated application of FMRFamide (data not shown), whereas the depolarizing response desensitized normally.

FMRFamide-induced inward current in HN(3) and HN(4) cells is voltage dependent

To characterize voltage dependence of the FMRFamide-induced inward current, voltage ramps from -25 to -105 mV were applied to the cell (n = 7). These ramps were given before FMRFamide application and at the peak of a response (Fig. 5A). The difference between the currents recorded for each ramp revealed the voltage dependence of the inward current induced by FMRFamide. Difference currents were enhanced at more depolarized potentials with a maximum of ~300 pA around -30 mV (Fig. 5B, bottom panel). The difference current-voltage (I-V) curves were relatively flat between -105 and -65 mV and became steep between 65 and 35 mV. Around 30 mV the curves appeared to flatten out again. Unfortunately it was not possible to characterize the I-V relationship at more depolarized potentials than -25 mV because recordings were totally obscured by spike activity, which could not be blocked without blocking the FMRFamide-induced inward current (see METHODS).

It was not possible to determine the reversal potential of the FMRFamide current for the same reason.

Ionic dependence of the FMRFamide-induced current in HN(3) and HN(4) cells

To characterize further the inward current evoked by focal application of FMRFamide, we determined its ionic depen-
EFFECTS OF FMRFamide ON HEART INTERNEURONS IN THE LEECH

FIG. 4. Focal application of FMRFamide (10^-6 M) onto the soma of an HN(R,3) cell triggers an inward current that is associated with an increase of membrane conductance. HN cell was voltage clamped. Repetitive voltage steps (−15 mV, 500 ms) superimposed on a steady holding potential (V_h) (−40 mV) were applied. Oscillation was disrupted by bath application of bicuculline methiodide (10^-4 M), which blocks inhibitory transmission between HN cells in the 3rd and 4th segmental ganglia (HN(3) and HN(4) cells) (Schmidt and Calabrese 1992). Current trace is obscured by action potentials.

To test for Ca^{2+} as a charge carrier, the normal 1.8 M Ca^{2+} concentration in the saline was reduced to 0.1 M Ca^{2+}. Reduced Ca^{2+} did not reduce the amplitude of the depolarizing responses of the cells (Fig. 6). These experiments indicate that Ca^{2+} is not the main charge carrier for the current. When 1.8 M Co^{2+} was added to 0.1-M-Ca^{2+} saline, the response to focal application of FMRFamide was blocked (n = 4, Fig. 7). A second application of FMRFamide in normal saline evoked a small depolarizing response.

Two sets of experiments were performed to determine the role of Na^+ in the FMRFamide-induced inward current. In the first set of experiments, Na^+ in the bath saline was reduced to 30% of its normal concentration and cells were voltage clamped at −35 mV for maximum response. Under these conditions the inward current elicited by focal application of FMRFamide onto the soma of an HN cell is much smaller (Fig. 8). The responses from the cell shown in this figure were not subject to strong desensitization; thus another response could be elicited. After evoking a response in 30%-Na^+ saline, a second, larger response could be evoked after 70 s of superfusion with normal saline. In experiments where sodium-free saline was used for superfusion, no response could be elicited by FMRFamide application. These experiments indicate that Na^+ is a prominent charge carrier of the FMRFamide-induced current.

The second set of experiments supports these results. When Li^+ was substituted for Na^+ in the saline, focal application of FMRFamide still evoked depolarizing responses (Fig. 9). The preparation was superfused with Li^+-containing saline for 3 min. Continuous superfusion with Li^+ disrupts rhythmic activity. FMRFamide was focally applied shortly before the disruption. Known Na^+ channels are equally permeable to Li^+ and Na^+ (Hille 1992). Because the permeability of Ca^{2+} channels for Li^+ is poor, it seems to be most likely that Li^+ entered the cells through Na^+ channels on focal application of FMRFamide. Therefore these experiments also indicate the Na^+ dependence of the FMRFamide-induced inward current.

FMRFamide hyperpolarizes HN(5) cells

An HN(5) cell switches spontaneously and continuously every several hundred seconds from its rhythmically active state to its inactive state and back (Fig. 10A). The switch from the active to the inactive state is associated with a membrane conductance increase. The conductance is not voltage dependent. It reverses around −60 mV (Gramoll et al. 1994). The two activity states differ in the amplitude of membrane potential oscillation (Calabrese 1977; Gramoll et al. 1994). In the inactive state an HN(5) cell produces few or no action potentials.

In contrast to HN(3) and HN(4) cells, focal application of FMRFamide onto the somata of a HN(5) cells in the active or inactive state hyperpolarizes the cells. Figure 10B shows an example where FMRFamide (10^-6 M) was focally applied onto the soma of an active HN(5) cell. The cell hyperpolariz-
FMRFamide-induced outward current in HN(5) cells is voltage dependent

To determine voltage dependence of the FMRFamide-induced outward current in HN(5) cells, we voltage clamped the cells at different holding potentials (total range: -60 to -15 mV) and focally applied FMRFamide (10^{-6} M) onto the somata. Bicuculline methiodide (10^{-4} M) was added to saline to obtain a stable baseline. Cells were held for 2 s at a given holding potential before FMRFamide was applied. The outward current induced by FMRFamide increased with increasing holding potential (Fig. 12A). The peak current measured in up to six such experiments was plotted against holding potential (Fig. 12B: open circles indicate values from individual experiments; solid circles indicate average currents). The FMRFamide-induced outward current activated around -40 mV. The nonlinearity in the I-V curve indicates that the outward current is voltage dependent. Similar results have been obtained in Ca^{2+}-free, 1.8-M-Co^{2+}-containing saline (n = 4, data not shown). Small responses sometimes measured below -40 mV were probably due to movement artifacts associated with pressure ejection of FMRFamide. The artifactual response did not change ampli-
EFFECTS OF FMRFamide ON HEART INTERNEURONS IN THE LEECH

FIG. 7. A: when Co\(^{2+}\) is substituted for Ca\(^{2+}\), the depolarization on application of FMRFamide is blocked. The rhythmic activity in HN cells was interrupted in Co\(^{2+}\)-containing saline, because Ca\(^{2+}\) blocks synaptic transmission between HN cells. B: same cell HN(L,3) shows a small depolarization on a 2nd application of FMRFamide after wash with normal Co\(^{2+}\)-free saline.

Ionic dependence of the FMRFamide outward current in HN(5) cells

The membrane conductance increase elicited by focal application of FMRFamide indicates K\(^+\) or Cl\(^-\) ions as charge carriers of the outward current. To determine the responsible ion for the current, we loaded HN(5) cells with Cl\(^-\) by impaling them with 4-M-KCl microelectrodes. About 7 min after impalement, inhibitory postsynaptic potentials recorded in the cells reversed, indicating that the intracellular Cl\(^-\) concentration had been elevated so that the Cl\(^-\) reversal potential was more positive than the membrane potential excursions of the cell. When FMRFamide (10\(^{-6}\) M) was applied focally onto the soma of a Cl\(^-\)-loaded cell, the response was still hyperpolarizing (Fig. 13A). As a control, carbachol was applied onto the same cell. Carbachol is an analogue of acetylcholine, the inhibitory transmitter of the presynaptic HN cells (Schmidt and Calabrese 1992). As expected, carbachol led to a depolarization mediated by a conductance increase to Cl\(^-\). These results suggest that K\(^+\) is the main charge carrier of the FMRFamide-evoked outward current.

FMRFamide modulates a slow potassium current

Simon et al. (1992) showed that FMRFamide modulates an outward current, I\(_K\), in HN(3) and HN(4) cells by hyperpolarizing shifts in steady-state activation and inactivation. The FMRFamide-induced outward current in HN(5) cells could be based on similar action of FMRFamide. Figure 14B

FIG. 8. Reduction of extracellular Na\(^+\) concentration to 30% reduces the inward current elicited by focal application of FMRFamide (10\(^{-6}\) M). A HN(L,3) cell was voltage clamped at a V\(_h\) of -35 mV. In normal saline the current trace is obscured by action potentials.
FIG. 9. Focal application of FMRFamide (10^-6 M) elicits depolarizing responses when extracellular Li^+ is substituted for Na^+. Application was given after 3 min of superfusion with Li^-containing saline, before Li^+ induced disruption of burst activity.

FIG. 10. A: continuous intracellular recording of the activity of an HN(L,5) cell shows a switch from the inactive state to the active state and back. B: focal application of FMRFamide (10^-6 M) onto the soma of an HN(L,5) cell evokes a hyperpolarization of the cell.
EFFECTS OF FMRFAMIDE ON HEART INTERNEURONS IN THE LEECH

**Fig. 11.** Focal application of FMRFamide (10^{-6} M) onto the soma of an HN(L,5) cell evokes an outward current that is associated with an increase of membrane conductance. The HN(L,5) cell was voltage clamped. Repetitive voltage steps (5 mV, 500 ms) superimposed on a steady $V_h$ (−35 mV) were applied. Oscillation was disrupted by bath application of bicuculline methiodide (10^{-4} M), which blocks inhibitory transmission between HN cells in the 5th segmental ganglion HN(5) cells and HN(3) and HN(4) cells, respectively (Schmidt and Calabrese 1992). The current trace is obscured by action potentials.

shows leak-subtracted outward currents elicited by stepping the membrane potential of an HN(5) cell for 22 s from a holding potential of −45 mV to −35, −25, and −15 mV. After some initial inactivation, the outward currents remained steady for the duration of the voltage steps. These outward currents can be identified as $I_K$ because of their relatively slow inactivation kinetics. [No attempt to separate a noninactivating component, $I_{K1}$, and a noninactivating component, $I_{K2}$, of $I_K$ was made (Simon et al. 1992).] Moreover, $I_K$ (the fast transient outward current) can be assumed to be inactivated by ≈80% in these experiments, because cells were held for some seconds at 45 mV before application of the depolarizing voltage steps (Simon et al. 1992). For Fig. 14C the same step protocol was used as for Fig. 14B, but this time the membrane potential was stepped 10 s after onset of a persistent application of FMRFamide. Peak and plateau phase of outward currents elicited during application of FMRFamide were larger than in control experiments. The difference between currents elicited during application of FMRFamide and currents elicited in control experiments gives the portion of the currents induced by FMRFamide (Fig. 14D).

**Discussion**

In this study we demonstrate segment-specific effects of FMRFamide, when it is focally applied onto HN somata, on membrane properties of HN cells. HN(3) and HN(4) cells were depolarized by application of FMRFamide (10^{-6} M). In 5% of all experiments performed on HN(3) or HN(4) cells, we observed a biphasic response, that is, the depolarization was followed by a hyperpolarizing response of the cells. In contrast, HN(5) cells exhibited only a hyperpolarizing response when FMRFamide was focally applied onto their somata.

**Depolarizing response in HN(3) and HN(4) cells**

The FMRFamide-induced depolarization in HN(3) and HN(4) cells appears to be caused by a slow voltage-dependent inward current that is mainly carried by $Na^+$. We observed maximum current flow around −25 mV. Unfortunately, we could not determine the $I$-$V$ relationship at values more positive than −25 mV because spike activity prevented sufficient voltage control. Currents with similar $I$-$V$ characteristics, induced by FMRFamide and other neuromodulators or transmitters, that are mainly carried by $Na^+$ have been shown in a variety of studies: FMRFamide in the gastropod *Aplysia californica* (Ichinose and McAdoo 1988), serotonin in the gastropod *Heliosoma* (Price and Goldberg 1993), and proctolin in the crab *Cancer borealis* (Golowasch and Marder 1992). There is evidence that induction of these currents involves a second messenger system. The $Na^+$ current in *Heliosoma* evoked by serotonin is adenosine 3',5'-cyclic monophosphate (cAMP) dependent (Price and Goldberg 1993). Intracellular injection of cAMP evokes a slow inward current mainly carried by $Na^+$ in some neurons in *Aplysia* (Kehoe 1990) and *Pleurobranchaea* (Gillette and Green 1987) and other marine gastropods (Connor and Hockberger 1984).

In the leech the slow and delayed onset of the inward current in HN(3) and HN(4) cells evoked by FMRFamide also suggests mediation by a second messenger. In HN cells reduction of extracellular $Ca^{2+}$ from 1.8 to 0.1 M did not affect the responsiveness of the cells. This result suggests that external $Ca^{2+}$ entering the cell does not act as a second
messenger. In other systems inward currents mainly mediated by Na⁺ were even blocked when the extracellular Ca²⁺ concentration was elevated (Gillette and Green 1987; Golowasch and Marder 1992; Ichinose and McAdoo 1988; Kehoe 1990). Gillette and Green (1987) could show that blocking intracellular activity of Ca²⁺ by injecting ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid into the cells did not prevent the cAMP-mediated inward current; in fact, the current amplitude was augmented.

The blocking effect of extracellular Ca²⁺ could be mimicked by some other divalent ions, which are more or less effective depending on the system. In HN(3) and HN(4) cells, Co²⁺ effectively blocks the FMRFamide-elicted depolarization. Co²⁺ also effectively blocks the inward current elicited by proctolin application in crab neurons (Golowasch and Marder 1992) and has some blocking effect on the cAMP-induced inward current in snail neurons (Gillette and Green 1987). Co²⁺ may bind to extracellular Ca²⁺ binding sites when leading to a block of inward currents. However, that is not the case in Aplysia neuron R14, where elevated Ca²⁺ blocks the FMRFamide-induced inward current, but Co⁴⁺ substituted for Ca²⁺ had no effect (Ichinose and McAdoo 1988). Here it is interesting to note that Co²⁺ had an irreversible blocking effect on a FMRFamide-induced inward current in leech heart muscle cells that has an Na⁺ component (Thompson and Calabrese 1992). In leech heart muscle cells, the blocking effect took place after repetitive FMRFamide application, long after a voltage-dependent Ca²⁺ component of the current was already blocked. This result indicates Ca²⁺ may act as a second messenger released from internal stores. After depletion of the internal stores, the Co²⁺ would prevent a Ca²⁺ refill. This mechanism seems unlikely for HN cells because Co²⁺ blocked the inward current immediately and the blocking effect was reversible.

The striking similarity between the FMRFamide-induced inward current described here and a persistent inward current, \( I_p \), reported in HN(3) and HN(4) cells by Opdyke and Calabrese (1994) provides an alternative explanation for the ability of Co²⁺ to block the FMRFamide-evoked depolarization. \( I_p \) is a persistent voltage dependent inward current predominantly carried by Na⁺ and activated near -60 mV. The I-V characteristics of \( I_p \) are similar to those of the FMRFamide-induced current. \( I_p \) is present at normal extracellular Ca²⁺ levels, but \( I_p \) is augmented in Ca²⁺-free Co²⁺ saline (Opdyke and Calabrese 1994). If the FMRFamide-evoked current and \( I_p \) are the same, Co²⁺ saline may prevent up regulation of the current by FMRFamide.
The similarity between the FMRFamide-evoked inward current and $I_p$ is striking and suggests a similar function, that is, providing a source of excitatory drive to support burst generation. At this point, however, it is important to consider the artificial way in which FMRFamide was applied in our experiments. The focal application of FMRFamide only reaches somatic receptors, and we do not know whether somatic FMRFamide receptors have similar properties to dendritic receptors. Sargent et al. (1977), for example, noted profound pharmacological differences between extra- and subsynaptic acetylcholine receptors on leech neurons.

Moreover, it seems unlikely that neuronal release of FMRFamide only affects one of the two HN(3) or HN(4) cells, respectively. So far we do not know how synchronous depolarization of both HN cells by FMRFamide alters the oscillatory activity of the network.

The FMRFamide-evoked inward current in HN(3) and HN(4) cells was subject to pronounced desensitization. The voltage-dependent, slow inward current induced by FMRFamide in Apysia neuron R14 is also subject to pronounced desensitization (Ichinose and McAdoo 1988), but not as rapidly as reported here. Usually only one or two applications of FMRFamide were sufficient to completely desensitize the response, making well-controlled experiments designed to elucidate the mechanisms of this current difficult. However, in two of four batches of leeches we found some animals in which desensitization was less pronounced. The responsiveness of the cells may depend on the physiological state of the animal, which in turn could be determined by the methods that our various suppliers used in rearing and handling the leeches. Thompson and Calabrese (1992) noted that the responsiveness of heart muscle cells of the leech to FMRFamide might be seasonal. Desensitization of the modulatory effect of the putative FMRFamidergic neuron, cell 204, on the HN(3) and HN(4) cells has not been investigated (Arbas and Calabrese 1984; Kuhlman et al. 1985a; Weeks and Kristen 1978), but such investigation could provide a means of determining whether the desensitization observed here is behaviorally significant.

The inward current evoked by focal application of FMRFamide onto somata of HN(3) and HN(4) cells appears to be specific for these cells, because focal application of FMRFamide onto somata of HN(5) cells always led to hyperpolarizing responses. One might expect to see hyperpolarizing responses in HN(3) and HN(4) cells regularly, because Simon et al. (1992) showed that bath application of FMRFamide shifts steady-state activation and inactivation of $I_K$ to more hyperpolarized levels. This effect of FMRFamide should cause hyperpolarizing responses when FMRFamide is focally applied onto somata. Such responses were only observed in 5% of all experiments, indicating that the respective receptors may not be present on somata of HN(3) and HN(4) cells.

**Hyperpolarizing response in HN(5) cells**

The hyperpolarization elicited in HN(5) cells when FMRFamide was focally applied onto their somata appears to be carried mainly by potassium. Our data indicate modulation of a potassium current, $I_K$, as the mechanism leading to the increase in outward current. A hyperpolarizing shift of steady-state activation of $I_K$ would most easily explain an
enhanced outward current. A similar action of FMRFamide has been shown by Simon et al. (1992) for the inactivating component, \( I_{k1} \), and the noninactivating component, \( I_{k2} \), of \( I_k \) in HN(3) and HN(4) cells. In our experiments the fast inactivating portion and the plateau phase of the outward currents elicited by depolarizing voltage steps were enhanced by local application of FMRFamide onto HN(5) somata.

An increase in \( K^+ \) conductance induced by application of FMRFamide has been observed in mollusc neurons (Belkin and Abrams 1993; Brezina et al. 1987; Cottrell et al. 1984; Fisher et al. 1993; Ruben et al. 1986). In Aplysia bag cells, application of FMRFamide activates an outward current, presumably carried by \( K^+ \) and \( Cl^- \) (Fisher et al. 1993). These authors show that steady-state activation of an outward current in the range of \(-70 \text{ to } -20 \text{ mV} \) is shifted to more hyperpolarized potentials in the presence of FMRFamide (Fisher et al. 1993, Fig. 5). For \( I_k \), Simon et al. (1992) discuss shifts of activation and inactivation by modulatory substances as a common mechanism of modulation of this voltage-gated conductance (e.g., Duchatelle-Gourdon et al. 1989; Giles et al. 1989; Tsien et al. 1972).

Outward current evoked by FMRFamide is not the ‘switch current’

HN(5) cells control intersegmental coordination of HE cells in more posterior ganglia by switching between a rhythmically active and an inactive state. Each activity state lasts for several seconds. Gramoll et al. (1994) found that the inactive state of HN(5) cells is caused by an outward current that is not voltage sensitive. Because the outward current evoked by FMRFamide is voltage dependent, it can be ruled out that the inactive state of HN(5) cells is caused by release of FMRFamide.

Modulation of the IIN(3) and IIN(4) cells by FMRFamide leads to an acceleration of their rhythmic activity. The mechanisms of action of FMRFamide on these cells to bring about this modulation are diverse. FMRFamide modulates \( I_k \) (Simon et al. 1992), it modulates synaptic transmission (Simon et al. 1994), and it evokes the inward current described here. In HN(5) cells, on the other hand, modulation by FMRFamide leads to a general suppression of activity, which appears to be mediated by an effect on voltage-dependent outward currents. The functional implications of this difference are unclear, but it calls into question the homology between these neurons. The HN cells are indeed a functionally very diverse group, but their strong morphological similarities and segmental iteration argue that they are homologues (Shafer and Calabrese 1981).

We thank Dr. E. Magano for a gift of leeches.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-24072 to R. L. Calabrese.


Address reprint requests to R. L. Calabrese.

Received 9 January 1995; accepted in final form 6 June 1995.

REFERENCES


KUHLMAN, J. R., LI, C., AND CALABRESE, R. L. FMRF-amide-like sub-
EFFECTS OF FMRFAMIDE ON HEART INTERNEURONS IN THE LEECH