THE EFFECT OF THE NEUROPEPTIDE FMRFamide ON APLYSIA CALIFORNICA SIPHON MOTONEURONS INVOLVES MULTIPLE IONIC CURRENTS THAT VARY SEASONALLY

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Accepted 13 May; published on WWW 14 July 1998

Summary

The molluscan neuropeptide FMRFamide has a number of inhibitory actions on the sensory neurons and motoneurons mediating the defensive gill and siphon withdrawal reflex pathway of Aplysia californica. Exogenous application of FMRFamide has a biphasic, dual-polarity effect on the majority of LFS siphon motoneurons, causing a transient depolarization followed by a prolonged hyperpolarization. FMRFamide induces this response in LFS neurons by causing an increase in multiple ionic currents, including a transient Na\textsuperscript{+} current, a slow prolonged Na\textsuperscript{+} current, a 4-aminopyridine (4-AP)-sensitive K\textsuperscript{+} current and a 4-AP-insensitive K\textsuperscript{+} current. We have found that a subset of LFS neurons exhibits an exclusively excitatory, biphasic response to FMRFamide, consisting of a transient depolarization followed by a prolonged depolarization of reduced magnitude. Over a period of 29 months, we consistently observed an increase in the incidence of the exclusively excitatory response during the summer months (June to September). From October to May, we observed an exclusively excitatory response to FMRFamide in 19\% of LFS neurons; yet, in the summer months, 51\% of LFS neurons exhibited this response pattern. We compared the ionic basis of the exclusively excitatory response to FMRFamide with the ionic mechanisms mediating the more frequently observed excitatory/inhibitory response. The exclusively excitatory response involves three of the same ionic components as the more typical excitatory/inhibitory response, including the activation of a transient Na\textsuperscript{+} current, a slow prolonged Na\textsuperscript{+} current and a 4-AP-insensitive K\textsuperscript{+} current. The principal difference between the two response types is that FMRFamide fails to activate a 4-AP-sensitive K\textsuperscript{+} current in those LFS neurons that exhibit an exclusively excitatory response to the peptide. In addition, LFS neurons with an exclusively excitatory response tend to show a coordinated increase in the magnitude of the inward current component of the FMRFamide response. Together, these changes during the summer months may enable this modulatory peptide to bring LFS neurons to suprathreshold levels of activity for eliciting a siphon withdrawal and should substantially alter the neuromodulatory effects of the peptide.

Key words: Aplysia californica, FMRFamide, motor neurone, neuropeptide, Na\textsuperscript{+} current, K\textsuperscript{+} current, seasonal variation.

Introduction

Seasonal variations in behavior, such as hibernation in winter and increased reproductive activity in the spring, are commonplace throughout the animal kingdom. In many instances, annual cycles in behavior are attributable to changes in neuronal circuitry, neuroendocrine output or response. In the case of song learning in canaries, behavioral changes are accompanied by gross alterations in neuroanatomy (Alvarez et al. 1990; Nottebohm et al. 1986). In addition to such changes in neural circuitry, annual cycles in behavior could also result from the alteration of properties of neurons in existing circuits (e.g. Bernard and Ball, 1997; Bishop et al. 1991; Miller et al. 1987; Turek and Van Cauter, 1994; Wayne et al. 1997). However, to date, very few studies have identified seasonal changes either in ionic currents themselves or in the regulation of ionic currents by modulatory transmitters (Bishop et al. 1991; Wayne et al. 1997). In the course of analyzing the response of a population of Aplysia californica siphon motoneurons to a specific neurotransmitter, we identified a discrete change in the response properties of the neurons to the transmitter that is correlated with season.

Throughout most of the year, the molluscan tetrapeptide FMRFamide exerts a biphasic, excitatory/inhibitory effect on the majority of LFS siphon motoneurons of Aplysia californica (Belkin and Abrams, 1993). In a small minority of the LFS...
neurons, FMRFamide has a biphasic, exclusively excitatory effect, rather than an excitatory/inhibitory effect. We observed that the incidence of this exclusively excitatory response has a strong seasonal correlation. Although this response to FMRFamide was observed in only approximately 20% of LFS neurons throughout most of the year, the frequency increased to over 50% during the summer months, between June and September.

We previously characterized the nature of the ionic mechanisms underlying the excitatory/inhibitory response to FMRFamide observed in most LFS neurons and identified four major components of this response (Belkin and Abrams, 1993): (1) an early transient increase in Na+ conductance; (2) a smaller, prolonged increase in Na+ conductance; (3) a prolonged increase in a 4-aminopyridine (4-AP)-sensitive K+ conductance; and (4) a prolonged increase in a 4-AP-insensitive K+ conductance. In combination, these modulatory effects produce a biphase response: a transient depolarization, followed by a prolonged hyperpolarization which suppresses the spontaneous firing of the cell.

In the present study, we have investigated the nature of the exclusively excitatory response to FMRFamide and have compared it with the more commonly observed dual-polarity response as characterized by Belkin and Abrams (1993). (We use the term ‘dual-polarity’ in this report because even the exclusively excitatory response was usually biphasic.) We have analyzed the currents modified by FMRFamide in the subset of LFS cells that exhibit an exclusively excitatory response and have found several similarities between the currents activated by the peptide in the two populations of neurons exhibiting the different types of responses. The primary difference between the two response patterns is that the 4-AP-sensitive outward current component of the prolonged inhibitory response is not present in the LFS cells that exhibit an exclusively excitatory response.

Materials and methods

Experiments were conducted on Aplysia californica, weighing 100 to 200 g, collected by Marinus (Long Beach, CA, USA) off the Palos Verde peninsula, Los Angeles county, at a depth of less than 6 m. During two consecutive winter months, animals, collected at the same geographic area and depth, were obtained instead from Alacrity Marine Biological Specimens (Redondo Beach, CA, USA). No differences in LFS neuron responses were observed in animals from these two sources; specifically, among animals from each supplier, there were individuals showing exclusively excitatory responses and individuals showing excitatory/inhibitory responses. Animals were stored in our tanks for up to 3 weeks. Tank temperature was maintained at 11–13°C and did not vary seasonally. The fluorescent light cycle was 13 h of light per day; however, because of a window in the aquarium room, daylight exposure increased to a maximum of 15 h per day during the longest days in June in Philadelphia.

Electrophysiological methods were similar to those described previously (Belkin and Abrams, 1993). Abdominal ganglia were removed from animals that had been anesthetized by injection of isoctane MgCl2 (360 mmol l⁻¹), and the left ventral hemiganglion was desheathed. During experiments, abdominal ganglia were superfused with normal physiological saline containing 460 mmol l⁻¹ NaCl, 10 mmol l⁻¹ KCl, 11 mmol l⁻¹ CaCl₂, 55 mmol l⁻¹ MgCl₂, 10 mmol l⁻¹ sodium Hepes (pH 7.6), 7 mmol l⁻¹ glucose, MEM (minimum essential medium), essential and non-essential amino acids (0.2 × normal concentration, Gibco/Life Technology), and MEM vitamin solution (0.7 × normal concentration, Gibco/Life Technology). In low-[Na+] saline, N-methyl-D-glucamine or Tris substituted for 75% of the Na+; a further reduction in [Na+]o often resulted in non-specific effects and reduced viability in LFS neurons. Saline containing a high concentration of divalent cations (high-divalent saline) (similar to normal saline except that it contained 110 mol l⁻¹ CaCl₂, 110 mol l⁻¹ MgCl₂ and 230 mol l⁻¹ NaCl) (Belkin and Abrams, 1993) was used in a small number of experiments. Tetrodotoxin (TTX, Calbiochem) and 4-aminopyridine (4-AP, Sigma) were dissolved directly into the appropriate extracellular medium. All recordings were conducted in a interior room with no windows at room temperature (20–24°C); small variations in building temperature were not correlated with outside temperature. Bath temperatures were 1–1.5°C below room temperature (presumably due to evaporative cooling). Changing the bath temperature by 2–3°C (by changing the temperature of the superfusion saline within an experiment) had no effect on the polarity of the FMRFamide response.

Intracellular recordings were made using 1.0 mm borosilicate glass microelectrodes containing either 2 mol l⁻¹ KCl or 2 mol l⁻¹ potassium acetate, with resistances of 6–12 MΩ. The great majority of recordings were made with potassium acetate. When the electrolyte was changed, there was no obvious effect on the incidence of exclusively excitatory responses observed. An Axoclamp 2A voltage clamp (Axon Instruments) was used for both current-clamp and single-electrode voltage-clamp recordings. For current-clamp recordings, membrane potential was altered by injection of current via the bridge circuit. For single-electrode voltage-clamp recordings, a single electrode cycled between a voltage-recording mode (70% of the cycle) and a current-passing mode (30% of the cycle) at 4–8 kHz, depending on the time constant of the individual neuron. Data were acquired digitally with a DT 2821 A/D board (Data Translation, Marlboro, MA, USA) using the software program Spike (Hilal Associates, Englewood Cliffs, NJ, USA). Data were analyzed using the programs Spike and Igor (Wavemetrics, Lake Oswego, OR, USA).

To deliver FMRFamide, puff pipettes (8–10 μm tip diameter), filled with 2 μmol l⁻¹ FMRFamide, were positioned approximately one cell body width to the side of and approximately 50 μm above the soma of the neuron being monitored; approximately 100 kPa of pressure was applied, for a duration of 2, 5 or 10 s. To reduce desensitization of the
FMRFamide response (Belkin and Abrams, 1993). FMRFamide was applied at 2 min intervals. When studying the early component of the FMRFamide response, which desensitizes more rapidly than the late component, we used 2 s puffs. Under voltage-clamp, cells were held at their resting potential, typically −60 mV. The reversal potential of the late phase of the response was measured by giving 10 s puffs of FMRFamide at various holding potentials and observing the polarity of the response at the end of the puff; the holding potential was varied in 2 mV increments within the range in which the response reversed. Membrane conductance was measured by voltage-clamping the cell and recording the change in current produced by a 300 ms step of −15 mV.

A single LFS neuron was analyzed in each abdominal ganglion. The LFS motoneurons were identified by their morphological and physiological properties. LFS neurons are located on the left ventral surface of the ganglion, near the point of entry of the siphon nerve into the ganglion, in the cluster of small (40–60 μm diameter) neurons that also contains the LE siphon sensory neurons. The axons of both LFS and LE neurons are activated directly by siphon nerve stimulation. These two neuronal populations can be distinguished by their unique electrophysiological characteristics. The LFS neurons fire repetitively at rest and exhibit a characteristic response to the removal of hyperpolarizing current in which there is a dip or inflection in the membrane potential as the neuron depolarizes; this inflection has the appearance of the transient hyperpolarization that results from a transient activation of an A-type K⁺ current (Connor and Stevens, 1971). In contrast, the LE neurons do not fire spontaneously and exhibit rapid and dramatic spike accommodation, generally firing action potentials only at the onset of a prolonged depolarizing step. LFS neurons were further distinguished from other neighboring motoneurons by an absence of interneuron II excitation and a generally low level of synaptic input.

LFS neurons can be subdivided into LFS-A and LFS-B neurons, according to the direction of siphon movement elicited; there are several of each type per abdominal ganglion (Fang and Clark, 1996; Frost et al. 1988; Hickie and Walters, 1995). Our analysis of the ionic currents contributing to the FMRFamide response was conducted on isolated abdominal ganglia, and we were therefore unable to characterize the siphon movements produced by individual LFS neurons. Instead, in most experiments, we classified LFS neurons into two subcategories on the basis of the magnitude of their adaptation during maintained branchial nerve stimulation (at 10 Hz for 5 s) (Belkin and Abrams, 1993). Of the LFS neurons whose responses to branchial nerve stimulation were characterized, approximately 50% adapted completely, ceasing firing within 1–2 s after the onset of nerve stimulation, whereas approximately 50% were more modestly adapting. Fang and Clark (1996) have found that, on average, the populations of LFS-A neurons and LFS-B neurons differ in the magnitude of their adaptation during maintained branchial nerve stimulation. Although the pattern of adaptation during branchial nerve stimulation does not correlate strictly with the previously described categorization of LFS neurons as A and B subtypes, LFS neurons that adapt completely during maintained branchial nerve stimulation are almost always LFS-B cells (X. Fang and G. A. Clark, personal communication) (see Fang and Clark, 1996; their Fig. 5).

Statistical analyses were performed using a two-tailed t-test, unless otherwise noted. Seasonal effects were analyzed with a χ²-test and analysis of variance (ANOVA) for binary data. Whenever possible, statistical comparisons were of within-preparation differences (i.e. within-preparation effects produced by the treatment), analyzed with a paired t-test. The criterion for statistical significance was P<0.05. All averaged data are expressed as mean ± standard error of the mean (S.E.M.).

The data presented here on LFS motoneurons exhibiting an exclusively excitatory response to FMRFamide were obtained during the same period as the results reported by Belkin and Abrams (1993) on LFS neurons with excitatory/inhibitory responses (August 1989 to February 1992). LFS neurons with exclusively excitatory responses were initially considered anomalous and were not originally studied in detail; after the circannual variation in the incidence of exclusively excitatory responses became apparent, the mechanisms underlying exclusively excitatory responses were analyzed in detail during the summer months of 1991. Data included, for comparison, on excitatory/inhibitory responses were obtained in the course of the study of Belkin and Abrams (1993).

**Results**

When FMRFamide was puffed onto the cell bodies of the majority of LFS neurons, the peptide typically caused a transient increase in firing rate, followed by a prolonged membrane hyperpolarization and suppression of the spontaneous firing of the cell (Fig. 1Ai). If, prior to FMRFamide application, the spontaneous firing was blocked, either by perfusing the ganglion with high-divalent saline or by hyperpolarizing the cell slightly below firing threshold, then a transient depolarization in response to FMRFamide was observed, followed by a prolonged hyperpolarization that persisted for many seconds after the cessation of the puff (Fig. 1Aii) (Belkin and Abrams, 1993).

In contrast, a minority of the LFS neurons exhibited an exclusively excitatory response to FMRFamide (Fig. 1B). In these LFS neurons, FMRFamide caused a prolonged increase, rather than a decrease, in firing rate (Fig. 1Bi). When these LFS neurons were hyperpolarized below firing threshold, or their responses were recorded in high-divalent saline, it was possible to observe more clearly this excitatory response to FMRFamide. Because, during FMRFamide exposures of more than a few seconds, responses typically began to decay from a large transient depolarization to a lower plateau depolarization which persisted after the end of the puff (Fig. 1Bii), we considered these exclusively excitatory responses to be biphasic. However, in the absence of the change in polarity that
occurs in the excitatory/inhibitory response, the separation between the two phases was often not distinct. These exclusively excitatory responses persisted in TTX (see below) and also in high-divalent saline, suggesting that they were mediated directly, rather than via activation of interneurons.

In principle, the difference among neurons in the polarity of the prolonged component (i.e. inhibitory rather than excitatory) could be explained by desensitization. This is unlikely, however, because the early excitatory component of the response tended to desensitize faster than the prolonged component, and the early depolarizing component was usually present in the neurons displaying an exclusively excitatory response. Furthermore, within individual LFS neurons, a change was almost never observed in the polarity of the prolonged component of the response (see below for exceptional cases).

It is possible that the polarity of the prolonged phase of the FMRFamide response correlates with a specific subtype of LFS neuron. LFS neurons have previously been divided into two subtypes, LFS-A and LFS-B, according to the different siphon movements they elicit (Fang and Clark, 1996; Frost et al. 1988; Hickie and Walters, 1995). In the present study conducted on isolated abdominal ganglia, we divided LFS neurons into two subcategories on the basis of the magnitude of their adaptation during maintained branchial nerve stimulation, a property characterized previously by X. Fang and G. A. Clark (personal communication). In total, we categorized the adaptation responses of 63 LFS neurons; 33% of the completely adapting neurons (N=33) and 37% of the more modestly adapting neurons (N=30) exhibited an exclusively excitatory response to FMRFamide. Thus, the two groups did not differ in the proportion of cells that showed the exclusively excitatory response. Since almost all of the completely adapting cells are LFS-B neurons (see Materials and methods), these neurons must be able to express either the excitatory/inhibitory or the exclusively excitatory response to FMRFamide. The population of LFS-A neurons, which constitutes the majority of the modestly adapting LFS neurons, is also likely to show both types of FMRFamide response. Since there was no difference in the incidence of the exclusively excitatory response between the two groups, and since we could not detect any other differences in the FMRFamide response between cells showing either complete or modest adaptation during maintained branchial nerve stimulation, we have pooled the data from these two subpopulations of LFS neurons. It is worth noting that, in two individual neurons out of 238 LFS neurons studied, we observed a switch in response type from excitatory/inhibitory to exclusively excitatory during the course of an experiment. This rare change in response type also indicates that the polarity of the prolonged phase is not a characteristic of the different subtypes of LFS neurons that differ in the direction of the siphon movement they produce.

The incidence of excitatory response is seasonally dependent

When we began studying the response of LFS neurons to FMRFamide in the month of August, all LFS neurons that we tested exhibited a biphasic exclusively excitatory response to FMRFamide. The next autumn, we observed a puzzling change in the nature of the FMRFamide response from exclusively excitatory to predominantly excitatory/inhibitory. If we subsequently observed that the incidence of the excitatory response increased during the summer months each year. We examined the data acquired during a 31 month period, from August 1989 to February 1992, and have found that, indeed, the proportion of LFS neurons with a prolonged excitatory response to FMRFamide increased significantly from June to September compared with the rest of the year (P<0.01, χ²-test) (Fig. 2). Of 166 LFS neurons studied from October to May,
only 34 displayed exclusively excitatory responses; whereas, of 72 LFS neurons studied from June to September, 37 displayed exclusively excitatory responses. We analyzed the distribution of the two response types among three 4 month periods, June–September, October–January and February–May, using an ANOVA for binary data (Agresti, 1984). The effect of season was highly significant ($F_{2,231}=11.45, P<0.001$), whereas the effects of the year and of the year $\times$ season interactions were not significant ($F_{2,231}=0.29$ and $F_{2,231}=1.67$, respectively).

Although recordings were conducted at room temperature, the occurrence of exclusively excitatory responses did not correlate with experimental temperature. There were no changes in response type during small shifts in bath temperature of as much as 3 °C within individual experiments. Moreover, the small changes in laboratory temperature were not correlated with outside temperature. An analysis of the currents activated by FMRFamide in LFS neurons exhibiting the exclusively excitatory response is presented below. Almost all of these cells with exclusively excitatory responses that were analyzed were studied in the summer months.

**The early transient phase of the response involves an increase in Na$^+$ conductance**

We were interested in determining the extent to which the currents activated by FMRFamide in LFS neurons exhibiting an excitatory/inhibitory response were also activated in neurons exhibiting a biphasic exclusively excitatory response. We began our analysis by determining the ionic basis of the early transient inward current. Brief (2 s) puffs of FMRFamide were presented in order to prevent any desensitization of the transient component of the response over the course of the experiment (Belkin and Abrams, 1993). FMRFamide caused an increase in conductance of 1.8±0.6 nS ($N=37$) during the early component of the response (measured 1.8 s after puff onset). The mean magnitude of the early inward current was $-232±117$ pA in LFS neurons with an exclusively excitatory response ($N=3$; measured 1.8–2 s after puff onset), which was significantly greater ($P<0.01$) than the transient inward current of $-47±8$ pA ($N=8$) observed by Belkin and Abrams (1993) in neurons with an excitatory/inhibitory response to FMRFamide.

To examine the Na$^+$-dependence of the early inward current component of the exclusively excitatory FMRFamide response, we superfused the ganglion with a low-[Na$^+$] saline in which 75% of the Na$^+$ was substituted with either N-methyl-D-glucamine or Tris. Reducing the extracellular Na$^+$ concentration caused a substantial decrease in the magnitude of the early inward current component of the FMRFamide response (Fig. 3A). In some cases, the early inward current disappeared or even reversed to an outward current in the presence of low-[Na$^+$] saline. To quantify this reduction in inward current, we specifically compared the magnitudes of the responses 2 s after the onset of a 2 s puff, which is typically near the peak of the transient inward current. A reduction in extracellular [Na$^+$] resulted in a change in the early current component of the FMRFamide response from a mean value of $-232±117$ pA (net inward) in normal saline to $+7±14$ pA (net outward) in 25% normal [Na$^+$] ($N=3$). The early phase of the FMRFamide response was not affected by TTX (data not shown) and thus is mediated by TTX-insensitive current(s), much as described by Belkin and Abrams (1993).

**The late component also involves an increase in Na$^+$ conductance**

To study the ionic mechanisms underlying the prolonged phase of the FMRFamide response, we presented longer (10 s) puffs of the peptide. The late inward current component of the response to FMRFamide also involved an increase in membrane conductance, averaging 1.8±0.9 nS, measured at the end of the puff ($N=8$). Thus, during the prolonged phase of the response, membrane conductance did not decrease relative to the transient component of the response (measured 1.8 s after...
puff onset), although the inward current was somewhat greater during the early transient component; this anomalous result is discussed below. A 75% reduction in external [Na+] caused a substantial reduction in the magnitude of the late inward current component of the FMRFamide response (Fig. 3B), resulting in a reversal of the late phase in some neurons. The magnitude of the late inward current (measured at the end of the 10 s puff of FMRFamide) was significantly reduced from $-194\pm68$ pA in normal saline to $-6\pm17$ pA in 25% normal [Na+] ($P<0.05$, $N=9$). Similarly, perfusion with low-[Na+] saline caused a reduction in the magnitude of the late depolarizing component of the FMRFamide response in eight additional preparations studied in current-clamp mode. Substitution of Na+ with either N-methyl-D-glucamine or Tris produced similar reductions in the size of the late component of the response, suggesting that this effect was due to the change in the extracellular Na+ concentration rather than to a non-specific effect of these compounds on a FMRFamide receptor. These results indicate that both the early and the prolonged components of the excitatory response to FMRFamide involve an increase in Na+ conductance. However, the reduction in the late inward current in 25% normal [Na+] was substantially greater than the approximately 27–29% decrease in inward current that would be predicted for an exclusively Na+-dependent response (assuming an intracellular Na+ concentration of 45 mmol l$^{-1}$, a 75% reduction in extracellular [Na+] should result in a shift in $E_{\text{Na}}$ from +59 mV to +25 mV). This discrepancy suggests that the late phase of the exclusively excitatory response to FMRFamide also involves the activation of an outward current. The prolonged increase in Na+ conductance was TTX-insensitive; the magnitude of the response was $-56\pm27$ pA in normal saline and $-65\pm30$ pA in 100 $\mu$mol l$^{-1}$ TTX ($N=3$). The average reduction in the late inward current in low-[Na+] saline in these cells with exclusively excitatory responses (169$\pm72$ pA, $N=9$) was greater than the reduction due to low [Na+] recorded in neurons with an excitatory/inhibitory response ($42\pm9.2$ pA, $N=7$, measured in the presence of 4-AP).

Fig. 3. Effect of reducing external [Na+] on the early and late components of the FMRFamide response in LFS neurons with an exclusively excitatory response to the peptide. In Aii and Bii, 75% of external Na+ was replaced with N-methyl-D-glucamine. (A) Effect of reducing external [Na+] on the early component of the response. The currents activated by FMRFamide were measured with the membrane potential clamped at $-60$ mV. Puff durations were 2 s. In this LFS neuron with this brief puff duration, there was little activation of the late component of the FMRFamide response. (Ai) FMRFamide induced an inward current response in normal saline. (Aii) In low-[Na+] saline, the early inward current component of the response was reduced and an outward current component of the response was unmasked. Similar results were observed in three additional preparations. (B) Effect of reducing external [Na+] on the late component of the response. Membrane potential was clamped at $-50$ mV. Puff durations were 10 s. (Bi) FMRFamide induced an inward current response in normal saline. Note that the early component of the FMRFamide response had substantially desensitized in response to these longer puffs. (Bii) In low-[Na+] saline, the inward current response was reduced and a late outward current component of the response was unmasked. Dotted lines indicate baseline holding current level prior to the FMRFamide puff.

Fig. 4. The exclusively excitatory (E/E) response to FMRFamide is insensitive to 4-aminopyridine (4-AP). The histogram compares the 4-AP sensitivity of the exclusively excitatory (E/E) and excitatory/inhibitory (E/I) FMRFamide responses. The vertical axis indicates the reduction in outward current observed in 5 mmol l$^{-1}$ 4-AP. Current was measured at the end of a 10 s puff. Data for excitatory/inhibitory responses are from the earlier study by Belkin and Abrams (1993). Values are means + s.e.m.
by Belkin and Abrams, 1993); however, this difference was not significant.

**FMRFamide also activates a prolonged outward current**

In 70% of cells with an exclusively excitatory response, a reduction in the external Na\(^+\) concentration to 25% of the normal value unmasked a late inhibitory response to FMRFamide (Fig. 3Bii); thus, at least in the majority of the LFS neurons with an exclusively excitatory response to FMRFamide, the prolonged phase of the response also involved an outward current. The activation of a prolonged outward current would explain why there was no net change in conductance between the early and late phases of the response, despite the decrease in net inward current. The reversal potential of the prolonged outward current unmasked in low external [Na\(^+\)] ranged from −45 to −85 mV. The variability in reversal potentials suggests that the relative contribution of the outward and inward currents to the response varies substantially among individual neurons.

We wished to determine whether the prolonged outward current activated by FMRFamide in these cells involved the same two K\(^+\) conductances activated by FMRFamide in LFS neurons with an excitatory/inhibitory response. A major component of the more typical prolonged hyperpolarizing response of LFS neurons to FMRFamide is a 4-AP-sensitive outward current (Fig. 1 in Belkin and Abrams, 1993). At 5 mmol\(\cdot\)l\(^{-1}\), 4-AP is maximally effective in blocking this current (Belkin and Abrams, 1993). In contrast to neurons with a prolonged inhibitory response, the neurons with a prolonged excitatory response showed very little sensitivity to 5 mmol\(\cdot\)l\(^{-1}\) 4-AP (N=7). We studied the effect of 5 mmol\(\cdot\)l\(^{-1}\) 4-AP on the response to FMRFamide in low-[Na\(^+\)] saline; there was no significant effect of 4-AP on the outward current during the late phase of the response (the net current at the end of the 10 s puff was −34±31 pA in 25% normal [Na\(^+\)], compared with −50±37 pA in low-[Na\(^+\)] with 5 mmol\(\cdot\)l\(^{-1}\) 4-AP; P=0.1, one-tailed t-test, N=5). Moreover, this 16±10 pA (N=5) decrease in outward current produced by 5 mmol\(\cdot\)l\(^{-1}\) 4-AP is significantly less than the 4-AP-induced decrease in outward current of 99±8 pA (N=7) previously observed by Belkin and Abrams (1993) in LFS neurons with an inhibitory response (P<0.01) (Fig. 4). These results indicate that the outward current component of the FMRFamide response that was unmasked by the reduction of external [Na\(^+\)] (Fig. 3Bii) is primarily due to the activation of a 4-AP-insensitive outward current. Thus, FMRFamide activates a 4-AP-insensitive outward current in both types of LFS neurons: those with prolonged excitatory and those with prolonged inhibitory responses (see Discussion). We observed no significant difference in the magnitude of the 4-AP-insensitive current, recorded in the presence of 25% normal [Na\(^+\)], in the two cell types.

**Discussion**

In the majority of LFS motoneurons that we studied, FMRFamide induced a biphasic, dual-polarity response consisting of a large transient depolarization followed by a prolonged hyperpolarization, which persisted throughout the duration of the puff and decreased gradually (Belkin and Abrams, 1993). In contrast, a subset of LFS neurons displayed an exclusively excitatory response to FMRFamide. This excitatory response was also biphasic and followed a time course similar to that of the more frequently observed excitatory/inhibitory response, except that both the early and prolonged components were excitatory.

Of the four components of the FMRFamide response of LFS neurons with a typical excitatory/inhibitory response, three components are also displayed by LFS neurons with an exclusively excitatory response: (1) an early transient increase in Na\(^+\) conductance; (2) a prolonged increase in Na\(^+\) conductance, and (3) activation of a prolonged 4-AP-insensitive outward current. The principal difference between the two subpopulations of LFS neurons is that the neurons exhibiting a prolonged excitatory response lacked the fourth component, a 4-AP-sensitive outward current activated during the prolonged phase of the response. The failure of FMRFamide to activate this 4-AP-sensitive outward current in the LFS neurons with exclusively excitatory responses appears to account for most of the change in the polarity of the late phase of the response. In addition, the magnitude of the early transient inward current was greater in the LFS neurons with an exclusively excitatory response than in the neurons with an excitatory/inhibitory response; moreover, the Na\(^+\) component of the late phase of the response was more than fourfold greater in neurons with an exclusively excitatory response, although this difference was not significant because of the large variability in the magnitude of this late inward current. These results demonstrate that the multiple components of the FMRFamide response can be differentially regulated in a coordinated manner and illustrate how a change in the relative contribution of the different currents can qualitatively alter the nature of the response of these cells to FMRFamide.

The incidence of the exclusively excitatory response to FMRFamide in LFS neurons varied seasonally, occurring more frequently during the summer months. This change in incidence is unlikely to be the result of variables that affect the neurons at the time of experiments. For example, experimental temperature showed little variation throughout the year, and comparable shifts in bath temperature did not alter the polarity of the response. It is also unlikely that the increase in the numbers of LFS neurons with an exclusively excitatory response in the summer is due to a seasonal increase in the number of neurons with this response type through neurogenesis, rather than to alterations in the properties of existing neurons. Except for the bag cells, which continue to be produced into adulthood (McAllister et al. 1983), there is little overall neurogenesis in the central nervous system as a whole, and in the abdominal ganglion in particular (Cash and Carew, 1989). Alternatively, the increase in the incidence of the exclusively excitatory response pattern in the summer months may well represent a developmental change in existing neurons. Since peak egg laying occurs during the summer, and
An increase both in the synthesis of ELH (Berry, 1984) and in water temperature of experimental animals in captivity causes reproductive hormones. It is currently believed that a rise in change we observed results from direct or indirect actions of increase during the summer months. There are several possible loci in the cellular cascade that mediates the response to FMRFamide where alterations could underlie the selective reduction in the late hyperpolarizing component of the response that was observed during the summer months. The different components of the FMRFamide response in LFS neurons involve separate ionic currents. There could be a loss of a specific K+ current or a change in its voltage-dependence. Arachidonic acid application mimics the prolonged hyperpolarization, but not the transient depolarization, of the typical FMRFamide response (Belkin and Abrams, 1993), suggesting that the individual components of the response are mediated by separate receptor-to-channel coupling systems (e.g. different second messenger systems), which could be selectively altered. Recently, Wayne et al. (1997) observed a seasonal change in the contribution of second messenger cascades in initiating a neurosecretory response in Aplysia californica bag cells. Finally, it is possible that FMRFamide activates different components of the response via multiple receptor subtypes, as has been described for Helix aspersa neurons (Cottrell and Davies, 1987). A seasonal change in a receptor subtype, a second messenger system or an ion channel could account for the change in response that we have observed.

It is unclear which environmental cues are responsible for inducing this seasonal physiological change. The physiological signal responsible for triggering seasonal or maturational changes in the FMRFamide response is also unknown. Since reproductive activity (Audesirk, 1979; Berry, 1982) and release of egg-laying hormone (ELH) from bag cell neurons (Berry, 1982; Loh and Gainer, 1975; Schwartz et al. 1971; Strumwasser et al. 1969; Stuart and Strumwasser, 1980) both increase during the summer months, it is possible that the change we observed results from direct or indirect actions of reproductive hormones. It is currently believed that a rise in temperature accounts for the increase in egg-laying activity of Aplysia californica during the summer because increasing the water temperature of experimental animals in captivity causes an increase both in the synthesis of ELH (Berry, 1984) and in egg-laying behavior (Pinsker and Parsons, 1985). Moreover, the effect of α-bag cell peptide on the bag cells, which themselves release the peptide, switches from inhibitory to excitatory when recording chamber temperature is increased (Redman and Berry, 1991, 1993). Our experimental subjects were captured in the wild and stored in our artificial seawater system for up to 3 weeks (with no observable difference in physiology related to length of time in the tank). Neither tank temperature nor recording temperature varied seasonally, and light cycle varied only slightly (see Materials and methods).

Therefore, it is likely that the physiological change we observed was long-lived, persisting at least a few weeks. As discussed above, it is also possible that the increase in the incidence of the exclusively excitatory response during the summer resulted from a change in the developmental state of the animal, rather than being triggered by an environmental cue; however, because changes in maturational state (e.g. reproductive condition) can be synchronized by environmental cues, it may be difficult to differentiate between developmental and seasonal changes.

An interesting aspect of the response of LFS neurons to FMRFamide is the activation of multiple ionic currents that have opposing effects on membrane potential and neuronal excitability. Since the description of a biphasic response of neuron L7 in Aplysia californica to acetylcholine (Blankenship et al. 1971; Wachtel and Kandel, 1967), there have been numerous other examples of dual-polarity transmitter actions. FMRFamide has been demonstrated to evoke multicomponent, dual-polarity responses in several molluscan neurons besides LFS cells, including identified neurons F2 and C1 of Helix aspersa (Cottrell et al. 1984) and neurons L4 and L6 of Aplysia californica (Ruben et al. 1986), increasing both inward and outward currents in all of these neurons. FMRFamide also elicits multiple responses in Aplysia californica mechanosensory neurons, which have opposite physiological consequences, including increases in several K+ conductances, a decrease in the Ca2+-dependent K+ current (Critz et al. 1991) and a decrease in a Ca2+ current (Edmonds et al. 1990).

The neurons providing FMRFamidergic input to the LFS neurons have not yet been characterized, and the functional role of these biphasic inputs is not known. However, our observations suggest that the differential activation of multiple ionic currents with opposing actions provides a mechanism by which the polarity of the FMRFamide response can be modulated in a seasonal manner. We observed that the early inward current component of the response increased during the summer months in the same animals in which the 4-AP-sensitive outward current component of the response was absent. The finding that these two components of the FMRFamide response change seasonally in a coordinated manner suggests that the loss of the inhibitory portion of the FMRFamide response may be of functional importance.

It is difficult to speculate on the specific behavioral consequences of this shift in response of LFS neurons to FMRFamide, because FMRFamidergic neurons that synapse onto the LFS cells have not yet been identified. It is also not
clear whether a very brief release of FMRFamide would evoke both the components of the response that we observed or only the early excitatory phase. However, it is clear from what is known about LFS activity that a change in the polarity of the FMRFamide response of LFS motoneurons would have a significant effect on the modulation of the siphon withdrawal reflex by FMRFamide.

FMRFamide affects both the membrane potential and the tonic firing rate of LFS neurons. As is true of all examples of postsynaptic modulation, any change in membrane potential will alter the response of the neuron to subsequent stimulation. The change in the tonic firing rate of LFS neurons will also have functional significance for the output of the circuit. A single action potential in an LFS neuron is not sufficient to cause a muscle contraction; in order for a single LFS motoneuron to elicit a siphon movement, the firing frequency must reach a threshold value of 3 Hz (Frost et al. 1988). The exogenous puffs of FMRFamide that we presented were effective in producing a suprathreshold firing rate in those LFS neurons exhibiting a purely excitatory response. In contrast, neurons with an excitatory/inhibitory response to FMRFamide rarely reached a firing frequency of 3 Hz during the early phase of the response. If LFS neurons respond similarly to release of endogenous FMRFamide, then LFS neurons with an exclusively excitatory response would produce a siphon movement in response to an FMRFamidergic input; in this case, the neuropeptide would, by itself, initiate a behavioral response and would therefore be acting as more than a neuromodulator. This is not the only mechanism by which the effects of FMRFamide on LFS neurons can alter the behavioral output of the circuit, since it has been demonstrated that a subthreshold increase in background firing rate will increase the magnitude of siphon movements in response to a given suprathreshold stimulus (Frost et al. 1988). Similarly, a decrease in the background firing rate would have a modulatory effect in the opposite direction. Therefore, either the transient increase or the prolonged decrease in firing rate that occurs in the majority of LFS neurons in response to FMRFamide might be sufficient to modulate the siphon withdrawal initiated by these motoneurons. It is worth noting that, whereas during the summer months there is an enhancement of the effect of FMRFamide that increases the output of these siphon motoneurons, β-bag cell peptide, which is released during egg laying (which takes place primarily during the summer), has been found to inhibit the synaptic connections within the circuit for the defensive tail withdrawal reflex (Goldsmith and Byrne, 1993).

The actions of modulatory transmitters provide a means of altering neuronal and synaptic properties and thus help account for the wide range of behaviors that the nervous system is capable of producing. In characterizing the modulatory effects of the neuropeptide FMRFamide on LFS motoneurons, we have found that this modulation can, in turn, be altered in a seasonal manner. Such shifts in the responses of the nervous system to modulatory factors may play an important role in seasonal changes in behavior.

We thank Drs Gregory Clark, Marc Dichter, Sol Erulkar Bruce Goldsmith and Marc Klein for helpful discussions and for critically reading and commenting on earlier versions of this manuscript. These studies were conducted with support from National Institutes of Health grants NS 25788 and MH 55880 to T.W.A.

References


