ORIGINAL ARTICLE

MULTIPLE RECEPTOR SITES IN *ACHATINA FULICA FERUSSAC* NEURONS FOR A MOLLUSCAN TETRAPEPTIDE AMIDE (FMRF-AMIDE)

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ABSTRACT: The effects of Phe-Met-Arg-Phe-NH_2 (FMRF-amide) and its analogue Phe-Leu-Arg-Phe-NH_2 (FLRF-amide) on the nervous system were studied on thirty-four identified neurons of the snail *Achatina fulica Ferussac* (locally known as "siput babi"). The results showed that FMRF-amide and FLRF-amide induced three different types of responses on the membrane potential of these neurons. First, FMRF-amide and FLRF-amide induced slow hyperpolarizing responses in TAN, d-LCDN, RAPN, d-LPeCN and V-RPLN. These responses remained unchanged in Cl^--free, Ca^{2+} -free and 20% Na^+ saline. The hyperpolarization was slightly reduced in 200% K^+ and slightly enhanced in 50% K^+ saline. Thus, the slow hyperpolarizing responses induced by FMRF-amide and FLRF-amide in the above neurons were K^+ -dependent.

Second, FMRF-amide and FLRF-amide induced rapid and transient hyperpolarizing responses on RPeNLN, LPeNLN and TAN-2. The effect persisted in 200% K^+, 20% Na^+ and Ca^{2+} -free saline. However, in Cl^--free saline, the effects were transient rapid depolarizing responses. This observation suggested that Cl^- was responsible for the hyperpolarizing responses.

Third, FMRF-amide and FLRF-amide induced depolarizing responses in INN and this effect remained unchanged in Ca^{2+} -free saline. However, in 20% Na^+ saline, the depolarizing responses were abolished. Thus, the involvement of Na^+ was implicated in the observed depolarizing responses.

The observed 3 different responses induced by FMRF-amide and FLRF-amide on *Achatina fulica Ferussac* neurons were comparable to their effects on the neurons of the gastropods *Aplysia* and *Helix*. Thus, FMRF-amide induced its multiple effects via multiple receptor sites, analogous to its action on *Helix* and *Aplysia* neurons. *(JUMMEC 1996: 1(2): 33-38)*

KEYWORDS: FMRF-amide, Neuropeptide, multiple receptor sites

Introduction

Biologically active neuropeptides are frequently used as extracellular chemical mediators in the central nervous system (1). Various neuropeptides have been found in the central nervous system of both vertebrates and invertebrates and many of these substances are widely thought to be potential neurotransmitters (1,2,3,4). Neuropeptides have a variety of distinctive characteristics that distinguish them from the more classical neurotransmitters. For instance, the localization of action of these peptides are often not restricted to synapses. In addition, most neuropeptides are synthesized as part of large precursors in the cell soma, whereas small molecule neurotransmitters are more often synthesized in the nerve terminals (5,6,7). Single neurons have been shown to utilize more than one chemical mediator, often in combination, consisting of a classical neurotransmitter and one or several other neuropeptides (8).

Phe-Met-Arg-Phe-NH_2, a tetrapeptide known as FMRF-amide isolated from the ganglia of the clam *Macrocalista nimbosa* (9), exhibits a potent pharmacological action on cardiac and non-cardiac muscle and on various neurons of several molluscan species (10,11). Electrophysiological investigations on the large neurons of gastropods such as *Aplysia* (12,13,14,15) and *Helix* (16,17) have indicated that FMRF-amide is capable of

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modifying in various ways the electrical properties of the neuronal membrane. However, such observations are not reported in the neurons of *Euhara*, a Japanese domestic snail (18). The responses induced varies depending on the specific neuron investigated. The responses include fast Cl^--dependent hyperpolarizing responses, slow K^+-dependent hyperpolarizing responses and depolarization responses that could be attributed to influx of sodium/calcium ions or suppression of voltage-dependent calcium-activated potassium current (19).

The snail, *Achatina fulica Ferussac*, was used in the present study. On the basis of localization, axonal pathways and resting membrane potential characteristics, Takeuchi *et al* (20) had identified 34 giant neurons in the ganglia of this specie of invertebrates. The large (500 μm) and easily identifiable neurons of this species provide an excellent experimental neural system model for electrophysiological research on the mechanisms of action of neurotransmitters, neuropeptides or neurotoxin.

The action of FMRF-amide and its naturally occurring analogue peptide FLRF-amide (Phe-Leu-Arg-Phe-NH_2) were investigated on these 34 identified *Achatina* neurons. To tentatively identify the ionic species and channels responsible for the responses, the experiments were repeated following changes in the ionic composition of the bathing medium.

Materials and Methods

The snails, *Achatina fulica Ferussac*, were collected and kept at room temperature between 22-25 °C until required. The snail neurons were prepared according to the method of Sun and Takeuchi (21). Briefly, the snail ganglia were exposed and the outer layers of connective tissue protecting the ganglia were removed manually until the last layer. The tissues enclosing the neurons were then softened with 0.7% trypsin (Type III, Sigma Chemicals, U.S.A.) at room temperature (25° ± 1°C) for 15 minutes. The ganglia were pinned onto a sylgard layer in a 0.2 ml experimental chamber and the remaining tissues were carefully removed with sharp tweezers to expose the neuron soma.

Conventional electrophysiological recording techniques were used to record the intracellular membrane potentials. Recording glass microelectrode were filled with filtered 3M potassium acetate (adjusted to pH 6.8). Electrode resistance measured ranged between 8-30 MΩ. Thirty-four identified neurons of *Achatina fulica Ferussac* (Figure 1A & 1B) were tested.

Saline flow to the tissue chamber was maintained at a rate of 4 ml per minute. Prior to the application of the FMRF-amide or FLRF-amide (Sigma Chemicals, U.S.A) the perfusion was stopped and 0.6 ml of saline were applied into the experimental chamber as control (no changes in the neuronal membrane potential). Later 0.6 ml of FMRF-amide or FLRF-amide dissolved in the appropriate solution to 10^{-4} M were directly applied into the chamber at a speed of 0.6 ml / 5-8 s. Any changes in the properties of the neuron membrane potential were detected via a two-channel pulse code modulation analogue to digital (PCM A/D) adapter (Medical System, U.S.A) and recorded by a video recorder. A hard copy was obtained through the play back facility of the MacLab System (Macintosh) via the PCM A/D adapter.

Normal saline had the following composition : NaCl 65.6 mM, KCl 3.3 mM, $CaCl_2$ 10.7 mM, $MgCl_2$ 13.0 mM, Tris HCl 9.0 mM and Tris Base 1.0 mM with pH adjusted to 7.5 (22). The extracellular K^+ and Ca^{2+} were augmented by simple addition of KCl or $CaCl_2$. The reduction of the extracellular Na^+, K^+, Cl^-, Ca^{2+} were made by replacing the ions with Tris. Ca^{2+}-free solution was made by replacing Ca^{2+} with Co^{2+}. Ca^{2+}-free solution enriched (3x) with Mg^{2+} was used to reduce trans-synaptic events.

Results

Neuronal electrical membrane responses to FMRF-amide and FLRF-amide on thirty-four identified Achatina *fulica Ferussac* neurons (Figure 1A and 1B) could be characterised into three main classes:

1. *Slow hyperpolarizing responses caused by both FMRF-amide and FLRF-amide.* These responses were exhibited by five identified neurons - TAN, d-LCDN, RAPN, d-LPeCN and V-RPLN. This class of responses is characterized by a slow onset (few seconds) and long lasting hyperpolarization. Examples of the slow hyperpolarizing response on TAN to FMRF-amide (Figure 2A) and FLRF-amide (Figure 3B) are shown. The responses were attenuated in 200% K^+ (FMRF-amide: Figure 2B; FLRF-amide: Figure 3B), and slightly enhanced in 50% K^+ saline (FMRF-amide: Figure 2C; FLRF-amide: Figure 3C). In Ca^{2+}-free saline enriched with 3 times Mg^{2+} and 20% Na^+ saline, the hyperpolarizing responses to FMRF-amide and FLRF-amide remained unchanged.

2. *Fast and rapid hyperpolarizing responses.* This class of induced responses by FMRF-amide and FLRF-amide lasted only a few seconds and were only observed in three identified neurons- RPeNLN, LPeNLN and TAN-2. Examples of the fast and rapid hyperpolarizing responses induced by FMRF-amide and FLRF-amide on LPeNLN are shown in tracing 4A and 4B. In Cl^--free saline, FMRF-amide (Figure 4C) and FLRF-amide (Figure not shown) induced a fast depolarization (compare the response in normal saline). In Ca^{2+}-free saline enriched

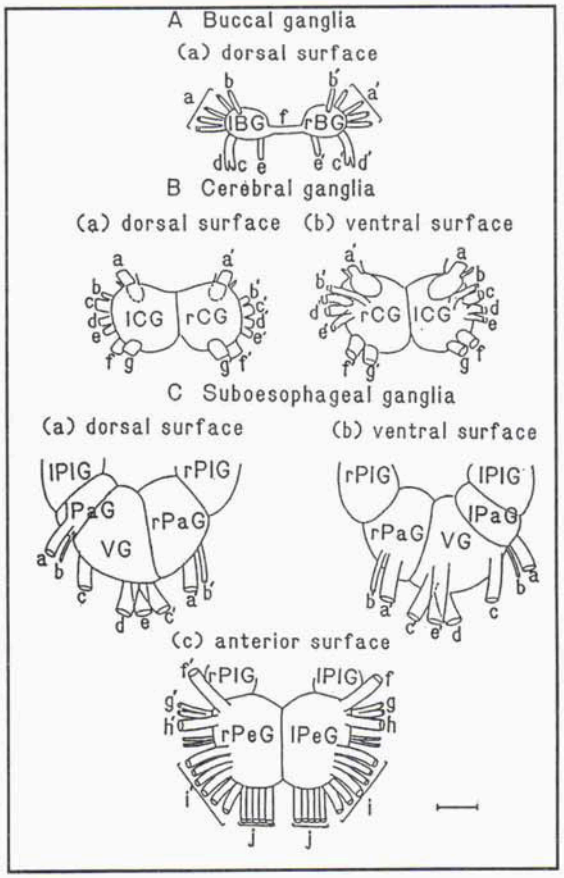

Figure 1A

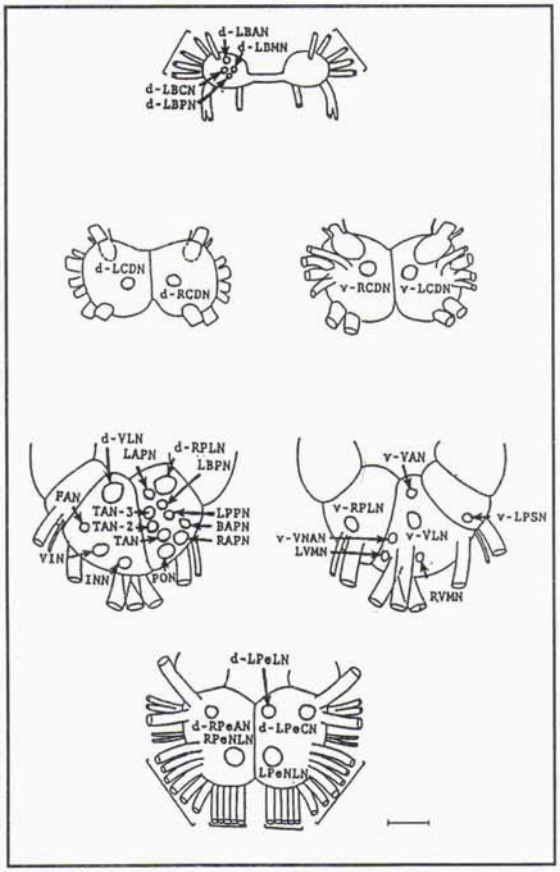

Figure 1B

Figure 1A:

Schematic drawing of the three ganglia of Achatina fulica Ferussac. A. buccal ganglia (dorsal surface). lBG. left buccal ganglion: rBG right buccal ganglion. B. cerebral ganglia. (a). dorsal surface; (b) ventral surface. lCG. left cerebral ganglion; rCG. right cerebral ganglion. C. suboesophageal ganglia. (a). dorsal surface; (b). ventral surface; Cc). anterior surface. 1PlG. left pleural ganglion; lPaG. left parietal ganglion; VG. visceral ganglion; rPaG. right parietal ganglion; rPlG right pleural ganglion; rPeG. right pedal ganglion; lPeG left pedal ganglion. Horizontal bar: scale (500 μm).

Figure 1B:

Identification of giant neurons.

A. *Buccal ganglia (dorsal surface). d-LBAN (dorsal-left buccal anterior neuron) d-LBMN (dorsal-left buccal medial neuron) d-LBCN (dorsal-left buccal central neuron) : d-LBPN (dorsal-left buccal posterior neuron).*
B. *Cerebral ganglia. (a). dorsal surface. d-LCDN (dorsal-left cerebral distinct neuron) d-RCDN (dorsal-right cerebral distinct neuron). (b). ventral surface. v-LCDN (ventral-left cerebral distinct neuron) v-RCDN (ventral-right cerebral distinct neuron).*
C. *Suboesophageal ganglia. (a). dorsal surface. d-VLN (dorsal visceral large neuron) FAN (frequently autoactive neuron) VIN (visceral intermittently autoactive neuron) INN (intestinal nerve neuron) PON (Periodically oscillating neuron) TAN, TAN-2; TAN-3; (tonically autoactive neuron) RAPN (right anterior pallial nerve neuron) d-RPLN (dorsal-right parietal large neuron) LAPN (left anterior pallial nerve neuron) LBPN (left bifurcate pallial nerve neuron) LPPN (left posterior pallial nerve neuron) : BAPN (bilateral anterior pallial nerve neuron). (b). ventral surface. v- LPSN (ventral- left parietal silent neuron) VLN (ventral - visceral large neuron) v-VAN (ventral -visceral anterior neuron. old name: v-l-VOrN) LVMN (left visceral multiple spike neuron. old name: l-VMN) RVMN (right visceral multiple spike neuron. old name: r-VMN); v-VNAN (ventral-visceral noisy autoactive neuron) : v-fRPLN (ventral right parietal large neuron). (c). Anterior surface. d-LPeLN (dorsal-left pedal large neuron) LPeCN (dorsal-left pedal constantly firing neuron) LPeNLN (left pedal nerve large neuron) : d-RPeAN (dorsal right pedal autoactive neurone) PeNLN (right pedal nerve large neuron). Horizontal bar scale (500 μm).*

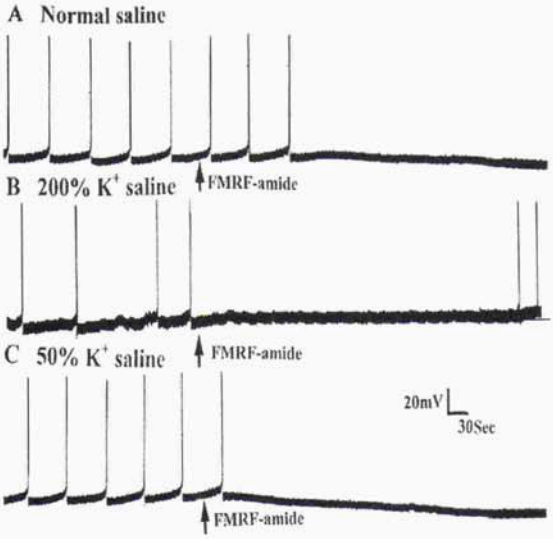

Figure 2. *The effect of FMRF-amide inducing a slow hyperpolarizing response on TAN (Figure 2A). The responses were slightly reduced (shortened) in 200% K^+ (Figure 2B) and slightly enhanced in 50% K^+ saline (Figure 2C).*

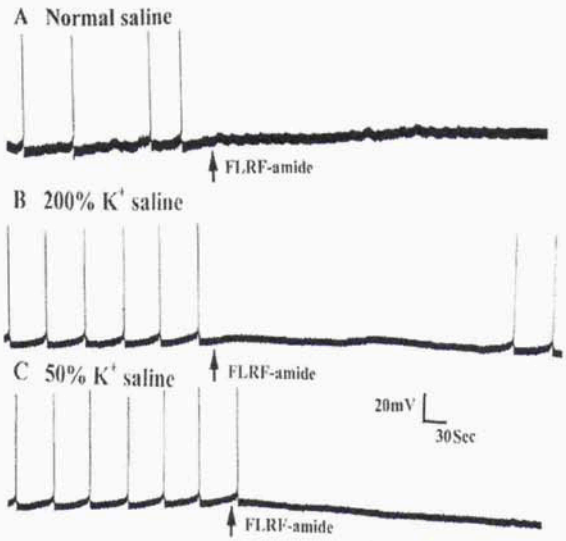

Figure 3. *The effect of FLRF-amide inducing a slow hyperpolarizing response on TAN (Figure 3A). The responses were slightly reduced (shortened) in 200% K^+ (Figure 3B) and slightly enhanced in 50% K^+ saline (Figure 3C).*

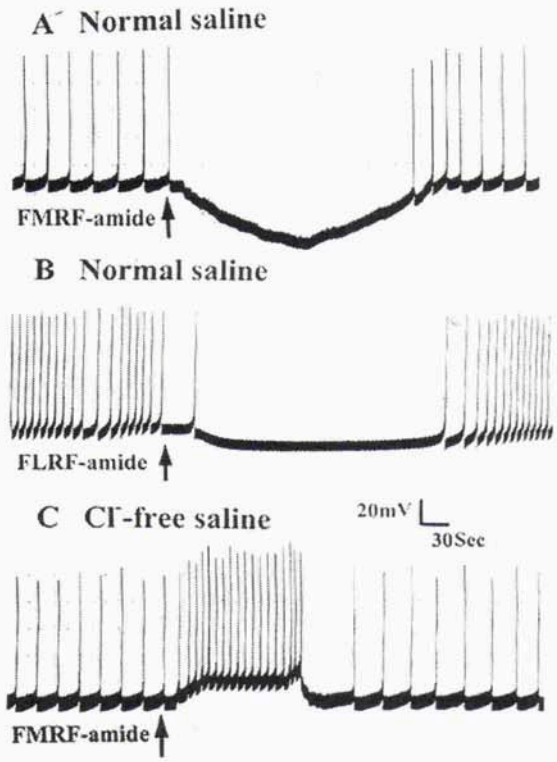

Figure 4. *The fast hyperpolarizing response to FMRF-amide (Figure 4A) in LPeNLN . A similar effect was obtained with FLRF-amide. (Figure 4B). In Cl^--free saline, FMRF-amide caused depolarization (Figure 4C; contrast with the response with normal saline).*

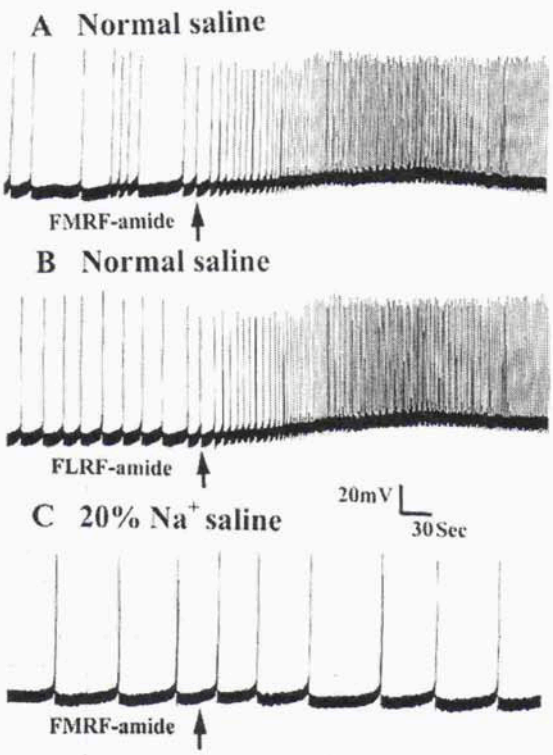

Figure 5. *The fast and rapid depolarizing response to FMRF-amide on INN(Figure 5A). FLRF-amide when given directly into INN induced a rapid depolarizing response similar to FMRF-amide (Figure 5B). In 20% Na^+ saline, the excitatory effects of FMRF-amide was abolished (Figure 5C). A similar response was obtained with FLRF-amide.*

with 3 times Mg^{2+}, 20% Na^+ saline, 200% K^+ and 50% K^+ saline, the responses to FMRF-amide and FLRF-amide remained unchanged.

3. *Fast and rapid depolarizing responses induced by FMRF-amide and FLRF-amide were characterized by fast onset and long-lasting excitatory effect.* This type of response could only be observed in INN (Figure 5 and 5B). These responses remained unchanged in Ca^{2+}-free saline enriched with 3 times Mg^{2+} or Cl^--free saline, 200% K^+ saline or 50% K^+ saline. However, in 20% Na^+ saline, the FMRF-amide fast depolarization was abolished (Figure 5C). A similar result was seen with FLRF-amide.

Discussion

The molluscan neuropetide Phe-Met-Arg-Phe-NH_2 (FMRF-amide) was discovered in the ganglia of the clam *Macrocallista nimbosa* by Price and Greenberg (9). FMRF-amide was initially found to be a cardioexcitatory neuropeptide, but it was later discovered to have a potent pharmacological action on a range of cardiac and non-cardiac muscles as well as neurons of different species of mollusc (10,11). Extensive studies on the action of FMRF-amide had been carried out on *Aplysia* and *Helix* neurons. Multiple actions of FMRF-amide were reported on *Helix* neurons (17) leading to the identification of multiple receptor sites for FMRF-amide on Helix neurons (19). Multiple actions of FMRF-amide on *Aplysia* neurons were published by Stone and Mayeri (12) and FMRF-amide was believed to act as a neurotransmitter, neuropeptide or neurohormone in *Aplysia* (23). Price (11) discovered two other naturally occurring analogues of FMRF-amide (FLRF-amide and pQDPFLRF-amide) in the same clam.

The data from these experiments support the hypothesis that FMRF-amide has multiple actions and acts on spatially different receptor sites. At least three different classes of actions were identified in *Achatina* neurons. First, slow inhibitory responses induced in five neurons - TAN, d-LCDN, RAPN, d-LPeCN and v-RPLN (see Figure 1a and 1b for location and identity of neurons). Second, rapid and transient inhibition on three neurons- RPeNLN, LPeNLN and TAN-2. Third, fast and rapid depolarization observed only in INN. Similar, responses in the same identified neurons were observed when another naturally occurring analogue of FMRF-amide, FLRF-amide, was used. There is a strong possibility that both FMRF-amide and its analogue, FLRF-amide, mediate their responses via the same receptors. However, the receptor species vary with different identified neurons leading to different classes of neuronal membrane characteristics. Tentative conclusions relating to the specie of ions and type of ionic channel involved could be derived from experiments where different ionic concentrations of different ionic species were changed in the bathing mediums. The results of these types of experiments suggest that the neuropeptide induces at least three different ionic mechanism on *Achatina fulica Ferussac* neurons. Based on their induced responses, FMRF-amide and its analogue FLRF-amide acted either as inhibitory or excitatory neurotransmitter or neuropeptide in the *Achatina* neural system. On the basis of the population of the responses, the amides are believed to serve mainly as inhibitory rather than excitatory neurotransmitters or neuropeptide. The results clearly show that FMRF-amide and FLRF-amide receptors are specifically localized, since the induced responses were only found on certain neurons. These neurons may be equipped with specific ionic mechanisms which were not abolished in Ca^{2+}-free Mg^{2+} enriched (3x) saline. This observation clearly indicates that the responses observed could not be attributed to trans-synaptic input.

Both FMRF-amide and FLRF-amide induced slow hyperpolarization in five neurons of *Achatina fulica Ferussac*. The onset of the response occurred a few seconds after introduction of both amides and could last for a few minutes. These responses were enhanced in 50% K^+ saline indicating enhanced K^+ efflux out of cell along the K^+ concentration gradient. When extracellular concentration of K^+ was increased to 200%, responses to both amides also decreased. Changing extracellular composition of the bathing milieu to Cl^--free, Ca^{2+}-free and 20% Na^+ salines did not affect the responses to the amides. Thus, the slow inhibitory responses in the presence of FMRF-amide and FLRF-amide could tentatively be attributed to the opening of K^+ channels. FMRF-amide induced slow hyperpolarization responses in association with K^+ was also reported in *Aplysia* (12) and *Helix* neurons (17).

Fast and rapid hyperpolarizing responses to both amides in three identified *Achatina* neurons could be attributed to the opening of Cl^- channels. Influx of the Cl^- ions into the cell via these receptors could be responsible for the observed hyperpolarization. The hypothesis is augmented with the observation that the initial rapid hyperpolarizing responses could be reversed, showing rapid depolarization when extracellular of Cl^- was removed. Chloride channels opened by FMRF-amide and FLRF-amide in this instance would cause an efflux of the Cl^- ions and thus depolarization of membrane potential. Similar results were also obtained in *Helix* neurons (17).

Finally, only one identified neuron of *Achatina fulica Ferussac* responded with depolarization in the presence of FMRF and FLRF-amide. This response was sensitive only to reduction in Na^+ ions. The ionic basis of these depolarizing responses is in concurrence with *Helix* neurons (17) and *Aplysia* neurons (12).

In conclusion, FMRF-amide is capable of inducing three different kind of receptor responses on *Achatina fulica*

Ferussac neurons, possibly with involvement of spatially distinctive receptor sites and species.

Acknowledgements

This work was supported by Malaysia Toray Science Foundation research grant 1992 & 1994, CMB 3099 and Vote-F from the University of Malaya.

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