Modulatory actions of the neuropeptide FMRFamide on synaptic transmission in Helisoma neurons

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Iowa State University

1991

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Modulatory actions of the neuropeptide FMRFamide on synaptic transmission in *Helisoma* neurons

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Iowa State University, 1991
Modulatory actions of the neuropeptide FMRFamide on synaptic transmission in *Helisoma* neurons

by

Helen J. Man-Son-Hing

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GENERAL INTRODUCTION

Behavioral and cellular modifications in learning and memory

Organisms must be capable of continually modifying their behavior in response to a changing environment. Changes in the brain, or more specifically, changes in synapses are widely believed to underlie changes in behavior. That is, synaptic plasticity is thought to underlie behavioral plasticity. Synaptic plasticity can be brought about from changes at many levels. Synapses may vary their function, be replaced, increase or decrease in number, and sprout or prune their axonal branching patterns. Synaptic connections are formed and broken continuously throughout life (Cotman and Nieto-Sampedro, 1984). Systems in which to study the synaptic mechanisms underlying the processes of learning and memory following either behavioral or electrophysiological conditioning are available.

In the nervous system, neurons communicate primarily through chemical synapses. A chemical neurotransmitter is released by the presynaptic cell that activates receptors on the postsynaptic neuron. An ion conductance change in the postsynaptic membrane may result in a change in the postsynaptic potential. The efficacy of synaptic transmission can be influenced by the presynaptic neuron's electrophysiological history or by neuromodulators released from nearby nerve terminals. In the early part of the twentieth century, Cajal proposed that learning and memory arise from changes in the strength of synaptic transmission (Cajal, 1911). Short-term changes in the synapse may last for minutes. The presynaptic neuron may vary the amount of neurotransmitter released or the postsynaptic neuron may modify its response to a given amount of transmitter. This may be the cellular correlate of learning and memory. Long-term changes in the synapse may last for hours or days and may underlie memory. Persistent changes in biochemistry, morphology, and gene expression may occur in presynaptic and postsynaptic neurons.

There is a great deal of interest in determining the basic mechanisms of learning but only recently have these mechanisms begun to be studied at the cellular level. In part, this has been made possible by the use of invertebrates as model systems. Some invertebrate preparations are valuable as model systems in that all behavioral functions are controlled by simple nervous systems with ganglia containing relatively small numbers of large cells (Kandel and Schwartz, 1982). These neurons can be consistently identified in virtually all preparations. Once the neuronal architecture is known, it becomes possible to determine systematically which neurons are responsible for the behavior under investigation. Once the neurons exhibiting plasticity are
known, it is possible to identify changes in their structure and function that are responsible for the observed changes in behavior. These studies have identified many processes underlying the net change in synaptic strength including changes in ion conductances, GTP-binding proteins (G proteins), second messengers, protein kinases, and mobilization of synaptic vesicles. One organism that is widely used to study subcellular changes is the invertebrate marine mollusc, *Aplysia* (Kandel and Schwartz, 1982). Another approach is to study the mammalian hippocampus, an area of the brain known to be involved in learning (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). Experiments in both of these systems have produced evidence that there are specific changes at the level of the synapse that possibly underlie the modification of behavior.

Through studies in *Aplysia* during the last few decades, it appears that both learning and memory are due to changes in neural connections (Castellucci et al., 1970; Hawkins et al., 1983; Kandel et al., 1986). Simple forms of learning may have cellular correlates at synapses between specific neurons. A simple reflex, the gill withdrawal reflex, in *Aplysia* can be modified by a form of nonassociative learning, sensitization. This is a form of short-term memory that is a nonspecific enhancement of a response. If a noxious sensitizing stimulus is delivered prior to a weak stimulus, the animal will retract more vigorously than if the weak stimulus is delivered alone.

Critical synaptic changes underlying sensitization have been intensely investigated. Presynaptic facilitation of synaptic transmission at the sensory-motor neuron synapse is involved. Application of the neurotransmitter serotonin mimics the sensitizing stimulus by eliciting presynaptic facilitation, a short-term increase in transmitter release, from the sensory neuron (Brunelli et al., 1976). The modulation of ion conductances by second messengers contributes to this process. In sensory neuron somata, serotonin causes the closure of a novel class of potassium channels called the "S" (serotonin-sensitive) channels (Klein et al., 1982). This action on the potassium channels is mediated by an increase in cAMP levels that then activates cAMP-dependent protein kinase (Klein et al., 1982; Siegelbaum et al., 1982; Shuster et al., 1985). The channels have a decreased probability of opening upon exposure to serotonin or injection of cAMP (Scholz and Byrne, 1988). This reduced potassium conductance leads to enhanced action potential duration in the somata, a greater influx of calcium, and a facilitation of transmitter release. The overall effect of serotonin is thought to underlie sensitization.
The same reflex in *Aplysia* can undergo behavioral inhibition after a different training paradigm (Mackey et al., 1987). Studies to elucidate the underlying synaptic mechanisms indicate that there is a hyperpolarization of the sensory neurons, a decrease in the action potential duration, and a reduction in synaptic transmission from sensory neurons to follower cells. These modifications are mimicked by application of the neuropeptide FMRFamide. This modulator causes a presynaptic inhibition of transmitter release in sensory neurons through lipoxygenase metabolites of arachidonic acid (Piomelli et al., 1987). FMRFamide has dual effects on the "S" channels. It causes an increase in the probability of opening of the channels and reverses the closures of the channels previously modulated by serotonin (Belardetti et al., 1987). FMRFamide has also been found to decrease the magnitude of calcium currents (Colombaioni et al., 1985; Brezina et al., 1987; Kramer et al., 1988). Together, these actions lead to a decrease in action potential duration, decreased influx of calcium, and a reduction in transmitter release.

Modulation of pre-existing synapses has also been observed in the hippocampus. The hippocampus is a region of the brain that exhibits both morphological and functional plasticity. It has previously been proposed to be a site involved in the location of memory sorting based on observations of severe memory impairment in humans after bilateral lesions of the hippocampal region (Scoville and Milner, 1957). Long-term potentiation (LTP) is an enhancement of synaptic strength in the hippocampus following repeated use. To study synaptic changes involved in LTP, the hippocampus is cut into thin slices such that each slice contains many layers of the entire circuit involved. LTP can be elicited by brief (1 second or less) tetanic stimulation of a sufficient intensity to coincidentally activate a critical number of presynaptic fibres. There are two distinct stages that are necessary for LTP to occur, induction and expression (Bliss and Lynch, 1988; Nicoll et al., 1988). Induction is the trigger necessary for the establishment of LTP and expression is the maintenance of enhanced synaptic efficacy.

The neurotransmitter released from the presynaptic terminals is thought to be glutamate. After diffusing across the synaptic cleft, glutamate binds to several types of receptors present in the postsynaptic membrane. A subclass of glutamate receptors, the NMDA receptors, has an unusual property. At resting membrane potentials, the ion channel associated with the receptor is blocked by magnesium ions. If the postsynaptic membrane is depolarized in the presence of glutamate, the magnesium block is removed (Nowak et al., 1984). Glutamate binding to the NMDA receptors now allows the flow of calcium ions into the postsynaptic cytoplasm (Mayer et al., 1987). This entry of calcium is a crucial event in the induction of
LTP. Several lines of evidence indicate that protein kinase C and calcium/calmodulin-dependent protein kinase are involved in the induction process (Malenka et al., 1986; Hu et al., 1987; Malenka et al., 1989; Malinow et al., 1989). A third protein that is thought to be involved in the induction process is the calcium-activated protease, calpain I. It has been hypothesized that the protease may hydrolyze enzymes such as the kinases mentioned above, rendering them constitutively active (Siman and Noszek, 1988). In addition to enzyme involvement in the induction of LTP, evidence suggests that a pertussis toxin-sensitive G protein that is not located in the postsynaptic neuron is another required component. LTP could not be induced in hippocampus slices that had been pretreated with pertussis toxin. This G protein appears to be upstream of protein kinase C in that activation of the kinase could still induce LTP even though the G proteins had been previously inactivated.

Once LTP has been successfully induced, enhanced synaptic transmission is maintained on a long-term basis, on the order of hours and weeks. Evidence suggests that it is the presynaptic terminal that is involved in the maintenance phase by releasing greater quantities of glutamate. Two recent studies have used new techniques of patch clamping the presynaptic neurons in hippocampal slices. With these techniques, LTP could be recorded with an improved signal-to-noise ratio that allowed for a quantal analysis to be performed (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). This type of statistical analysis demonstrated that the probability of transmitter release is increased during LTP pointing to a presynaptic locus of expression. Although this type of analysis provided evidence of a change occurring in the presynaptic neuron, an increase in postsynaptic responsiveness could not be ruled out in these studies.

**Morphological modifications**

Using electron microscopy, studies of morphological changes in the hippocampus following learning have recently begun. In the brain, synapses occur on spines that project from the dendrites rather than on dendritic shafts. Using high speed freeze-fixing techniques, the size of spines has been shown to increase when their synaptic contacts are activated. This may cause a decrease in the electrical resistance between the spine and its parent dendrite (Gray, 1987). Two theories have been proposed to account for the spine swelling: a contractile actin cytoskeleton within the spine cytoplasm remodels the spine; and/or osmotically active substances build up in the spine during stimulation that then produces an intake of water from the extracellular compartment, thus swelling the spine. In another study, Lee et al.
(1980) found that there were 30% more synaptic contacts found on dendritic shafts of potentiated neurons after induction of LTP.

Morphological correlates have been observed to develop at the level of the synapse in *Aplysia* (Bailey and Chen, 1988a). The connections between sensory and motor neurons are a critical locus for memory storage. The modulation of synapse number may be important in the maintenance of long-term memory. In studies examining presynaptic terminals, Bailey and Chen (1988a) found that the total number of presynaptic varicosities had decreased in animals that had undergone behavioral inhibition and had increased in sensitized animals. In addition, there were enlarged neuropil arbors observed in sensitized animals (Bailey and Chen, 1988a). In further studies, Bailey and Chen (1988b) determined that long-term sensitization also alters the structure of the postsynaptic motor neuron by increasing the surface area of the motor neuron that was occupied by synaptic contacts.

**Subcellular mechanisms**

There are many cases of neurotransmitters exerting their actions through G proteins. Accordingly, the role of G proteins in the actions of serotonin and FMRFamide on the K⁺ channels was determined in *Aplysia* (Volterra and Siegelbaum, 1988). The effect of FMRFamide on the K⁺ channels was mediated through a pertussis toxin-sensitive G protein. This G protein may be involved in an early stage of arachidonic acid metabolism that is coupled to channel modulation. There is another G protein, most likely related to Gₛ, that couples serotonin to stimulation of adenylate cyclase.

Neuronal calcium channels serve many functions (reviewed in Miller, 1987). Accordingly, there are multiple types of calcium channels and their subcellular localization may optimize their effectiveness. The types of calcium channels with distinct electrophysiological and pharmacological profiles that are found in the plasma membrane vary from cell to cell depending on the particular functions that calcium plays in that cell. Calcium functions as a second messenger in the cell and events that are controlled by calcium entry through voltage-gated calcium channels include enzyme activation, metabolism, gene expression, neurite outgrowth, and transmitter release. Dorsal root ganglion neurons contain three main channel types based on their electrophysiological and pharmacological profiles (Nowicky et al., 1985). The L-type channels are thought to cause the release of substance P from dorsal root ganglion neurons (Perney et al., 1986; Rane et al., 1987). However, in other systems it appears that the N-type current is responsible for triggering transmitter release (Perney et al., 1986; Hirning et
al., 1988). Modulation of the calcium current involved in release is one means of causing presynaptic facilitation or depression of transmitter release.

There are many neurotransmitters that modulate calcium currents and many act through a G protein either through its direct action on calcium channels or through second messenger systems. The involvement of a G protein has been demonstrated with the use of guanine nucleotide analogues, antibodies, and pertussis toxin.

Noradrenaline was the first transmitter found to modulate calcium channels in cultured cardiac cells (Cachelin et al., 1983). Through β-adrenergic receptors, noradrenaline is coupled to Gs that activates adenylate cyclase. The increased levels of cAMP then activate cAMP-dependent protein kinase that phosphorylates channels. The gating kinetics of the channels are changed such that there is an increase in the macroscopic calcium current. Other neurotransmitters act through G proteins to activate phospholipase C and phospholipase A2. Some studies have shown that G proteins directly modulate channel activity (for example, Cantau et al., 1980; Yatani et al., 1987; Volterra and Siegelbaum, 1988; Imoto et al., 1988; Kim et al., 1989; reviewed in Dolphin, 1990). In addition to noradrenaline modulating a calcium current, a number of other neurotransmitters have modulatory effects on calcium currents through G proteins in a variety of systems. Several studies have shown that a pertussis toxin-sensitive G protein is able to couple inhibitory neurotransmitters to calcium channels in chick and rat dorsal root ganglion neurons (Holz et al., 1986; Ewald et al., 1988; Ewald et al., 1989), neuroblastoma X glioma hybrid cells (Hescheler et al., 1987), AtT-20 pituitary cells (Lewis et al., 1986), rat sensory and sympathetic neurons (Dolphin and Scott, 1987; Wanke et al., 1987), and Helix neurons (Harris-Warrick et al., 1988).

**Dissertation experiments**

The present studies have used a model system of cultured somatic synapses from the freshwater pond snail *Helisoma trivolvis* to investigate subcellular changes underlying synaptic plasticity. This organism was initially chosen for neurobiological studies because of its definable behavior and accessible nervous system. As in *Aplysia*, *Helisoma* ganglia contain a relatively small number of large neurons within its nervous system. Individual neurons can be visually identified for all members of the species based on the cells' relative position within the ganglia, pigmentation, size, and axonal geometry. Experiments performed in culture conditions have the advantage of allowing the investigator full control over the intracellular and extracellular environments of the neurons. The cell bodies of the neurons comprise the
periphery of the ganglia. This configuration aids in the removal of specific neurons from the ganglionic environment to be transferred into a culture environment.

In general, previous studies of "classic" synapses have been hampered by the small size of and the lack of electrophysiological access to the presynaptic terminal. To overcome this shortcoming, a system of "giant" synaptic terminals has been developed to investigate mechanisms underlying synapse formation and synaptic plasticity (Haydon, 1988). When cultured in the presence of Helisoma hemolymph, the neurons do not extend neurites, retain a spherical geometry, and readily adhere to one another. The large size (60 µM diameter) and spherical shape of the neurons allow for direct electrophysiological access to both the presynaptic and postsynaptic cells. When the cell bodies of identified buccal neurons 5 and 19 (B5 and B19) are in contact, a unidirectional cholinergic synapse forms between the two somata with neuron B5 assuming the role of the presynaptic terminal.

With this unique system, synaptic plasticity and the subcellular mechanisms underlying this process can be studied at an increased resolution. During regulated secretion from neurons, vesicles line up adjacent to the plasma membrane. When the appropriate signal (an action potential) reaches the presynaptic terminal, there is an increase in the level of intracellular calcium due to the opening of voltage-sensitive calcium channels. The calcium channels are probably situated 10 to 20 nm away from the vesicles (Almers, 1990). In a short period of time, several events following the action potential must occur in the presynaptic neuron to promote neurotransmitter release: the calcium channels must open to admit calcium into the nerve terminal, the level of calcium in the region surrounding the vesicles must increase, calcium must bind to a receptor, the receptor must change conformation, and membrane fusion must occur to release the neurotransmitter (Almers, 1990). Many of these events involve the secretory machinery (or secretory apparatus). The components composing the secretory machinery are not well identified but are assumed to be made up of vesicles containing neurotransmitter, the calcium receptor, and a family of synaptic proteins associated with the vesicles and/or the plasma membrane.

Using the "giant" Helisoma synapses, the calcium currents of secretory membranes can be analyzed with voltage clamp techniques. Additionally, using a photolabile calcium chelator to promote secretion from the presynaptic neuron, the secretory apparatus can be studied independently of the voltage-sensitive calcium channels. In this manner, changes in ion conductances and secretory machinery during synaptic plasticity can be investigated separately.
Finally, macromolecules can be dialyzed or injected into the somata to test for the involvement of G proteins and second messengers.

FMRFamide is a molluscan neuropeptide (Greenberg and Price, 1983) that has been reported to reduce calcium currents (Colombaioni et al., 1985; Brezina et al., 1987; Kramer et al., 1988) and increase potassium currents (Colombaioni et al., 1985; Belardetti et al., 1987). FMRFamide and two analogues, FLRFamide and GDPFLRFamide, are abundant in Helisoma nervous tissue (Bulloch et al., 1988). FMRFamide causes inhibition of synaptic transmission (Man-Son-Hing et al., 1989) by an inhibitory action solely in the presynaptic terminal (Zoran et al., 1989). FMRFamide has dual actions within the presynaptic neuron. My studies have focussed on: 1) the reduction of the secretory machinery's sensitivity to calcium and the macroscopic calcium current modulation by FMRFamide and 2) the involvement of a pertussis toxin-sensitive G protein in the reduction of the calcium current and the effect of G protein injection on the calcium current.

Explanation of dissertation format

This dissertation is composed in the alternate format. Each of the sections is a complete manuscript modified to conform to the specifications of the Iowa State University Thesis Office. Each section has its own introduction, materials and methods, results, discussion, and references. A general summary follows Section 2 and discusses the entire body of work.
SECTION 1. FMRFamide ACTS PRESYNAPTICALLY TO INHIBIT SYNAPTIC TRANSMISSION: MODULATION OF A CALCIUM CURRENT AND SECRETORY MACHINERY
INTRODUCTION

In neurons, vesicles collect in the active zone, a region adjacent to the presynaptic plasma membrane. An action potential causes an influx of calcium through voltage-sensitive calcium channels that may also be clustered in the active zone. The influx of calcium across the plasma membrane results in a localized increase in the level of intracellular free calcium (Smith and Augustine, 1988). This rise in calcium is sufficient to trigger exocytosis of neurotransmitter (Katz, 1969).

Neuromodulators use multiple mechanisms to modulate synaptic transmission. Targets of neuromodulators include neurotransmitter receptors in the postsynaptic neuron and voltage-sensitive ion channels and the secretory apparatus of the presynaptic terminal. For example, quantal analysis of LTP in the Schaffer collateral pathway in the hippocampus indicated that the expression of LTP is manifested by a change in quantal size that is consistent with an alteration in the sensitivity of postsynaptic receptors (Bliss, 1990). Additionally, Davies et al. (1989) demonstrated that postsynaptic sensitivity of CA1 neurons to exogenously-applied neurotransmitter increased following induction of LTP. Neuromodulators can also target the presynaptic neuron. A presynaptic locus was found during the initial half hour following LTP induction (Davies et al., 1989). In separate studies, quantal analysis of LTP in the CA1 region of the hippocampus pointed to a presynaptic locus of LTP expression (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). Modulation of potassium and calcium channels in the presynaptic terminal alters the duration of the action potential, changes the amount of calcium entering the cytoplasm and thus, modulates the amount of neurotransmitter released. For example, during the cellular correlate of sensitization in Aplysia, there is an increase in the amount of transmitter released from the presynaptic terminal after the application of serotonin. This effect is partly due to a decrease in a potassium conductance that is mediated by an increase in cAMP levels (Klein et al., 1982; Siegelbaum et al., 1982; Shuster et al., 1985). The longer duration of the action potential, that results from the reduced potassium current, allows a greater influx of calcium into the cytoplasm that, in turn, causes a facilitation of transmitter release. In contrast, FMRFamide causes a decrease in action potential duration, decreased influx of calcium, and a reduction in transmitter release. FMRFamide has dual effects on the serotonin-sensitive potassium channels and calcium channels (Colombaioni et al., 1985; Belardetti et al., 1987; Brezina et al., 1987; Kramer et al., 1988). It causes an increase in the probability of opening of the potassium channels and reverses the closures of
the channels previously modulated by serotonin. In frog sympathetic neurons, catecholamines reduce the amount of neurotransmitter released by modulating a specific type of calcium current, the N-type current (Lipscombe et al., 1989).

In addition to affecting ion channels, there are other means of modulating secretion. For example, in nonneural cells, G proteins and second messengers can modulate the sensitivity of the secretory apparatus to the level of internal free calcium (reviewed in Gomperts, 1990). The physiology of exocytosis has been studied extensively in mast cells (for example, Bennett et al., 1981; Gomperts et al., 1983; Fernandez et al., 1984; Howell et al., 1987; Howell and Gomperts, 1987; Sorimachi et al., 1987; Sorimachi et al., 1988). Both calcium and guanine nucleotides are essential for the process of degranulation to occur (Howell et al., 1987). Together, they are sufficient to cause a maximal exocytotic event (Howell et al., 1987). This process can be modulated. In the presence of ATP, the concentrations of calcium and guanine nucleotide required to induce exocytosis are significantly reduced (Howell et al., 1987). Additionally, the calcium receptor's affinity for calcium and the G protein's (termed Gq) affinity for guanine nucleotide are controlled by phosphorylation by protein kinase C (Howell et al., 1988).

Other non-neural systems have been studied with respect to the modulation of secretion. In platelets, calcium alone can trigger exocytosis but the presence of GTP analogues enhances the affinity for calcium such that exocytosis of amines occurs at calcium concentrations below physiological resting levels (Haslam and Davidson, 1984). At very high levels of GTP analogues, calcium-independent secretion can occur. Individually, calcium and guanine nucleotides stimulate secretion from neutrophils (Barrowman et al., 1986). Together, their effects on secretion are additive. In HL-60 cells, exocytosis will occur with any two of calcium, guanine nucleotides, and phorbol ester (Snutchfield and Cockcroft, 1988).

In neural cells, it has been suggested that the secretory machinery can be regulated. In studies performed by Dale and Kandel (1990), serotonin increased and FMRFamide decreased the frequency of spontaneous miniature excitatory postsynaptic potentials from sensory neurons. This modulation by both neurotransmitters occurred in external solutions that lacked calcium and contained calcium-chelating agents. Additionally, modulation occurred in solutions containing cadmium, a calcium channel blocker. Finally, modulation occurred when BAPTA, a calcium chelator, was injected into the presynaptic terminal. These results suggest that modulation of spontaneous release does not require an elevation of intracellular calcium and is due to the effects on the secretory machinery. In the squid giant synapse, the state of
phosphorylation of a protein associated with secretory vesicles, synapsin I, regulates the magnitude of the postsynaptic potential in response to a constant calcium current (Llinas et al., 1985). From these studies in neurons and in non-neural cells, it appears that both ion channels and secretory apparatus can be targets of modulation.

FMRFamide is a peptide endogenous to many molluscs (Greenberg and Price, 1983) and has widespread effects in molluscan systems. For example, FMRFamide has inhibitory effects on feeding behavior (Murphy et al., 1985) and digestive tract motility (Lehman and Greenberg, 1987), and cardioexcitatory effects (Lehman and Greenberg, 1987). On the cellular level, FMRFamide modulates ion currents and spontaneous release (Colombaioni et al., 1985; Belardetti et al., 1987; Brezina et al., 1987; Kramer et al., 1988; Dale and Kandel, 1990). The goal of the present studies was to use the increased resolution made possible with giant synapses from Helisoma to examine the effects of FMRFamide on synaptic transmission. Specifically, the effects of FMRFamide on the calcium currents and the secretory machinery in the presynaptic neuron were investigated.
MATERIALS AND METHODS

Cell culture

**Neuron isolation**  All experiments were performed on adult specimens of the OR1 strain of the pond snail *Helisoma trivolvis*. The animals were maintained in laboratory aquaria at 22 to 25°C and fed trout chow and lettuce. The snails were deshelled by cutting the shell perpendicular to the whorl. The animals were placed into a solution of 25% Listerine in antibiotic saline (ABS; Table 1; Wong et al., 1981) for 10 minutes.

During the dissection, the snails were bathed in ABS and pinned ventral side down in a silicon rubber (Sylgard 182 silicon; Dow Corning, Midland, MI) coated petri dish. An incision was made on the dorsal side from the mantle to the level of the tentacles. The sides of the animal were pinned to expose the underlying nervous system. The paired buccal ganglia with a piece of the esophagus were isolated and placed into ABS. The ganglia were treated with a 0.2% solution of trypsin (Sigma Chemical Company; Type III) in defined medium (DM; Table 1; Wong et al., 1981) for 20 to 25 minutes.

The enzymatically-treated ganglia were pinned in a dissecting dish. A small cut was made in the connective sheath overlying the neuron of interest with an electrolytically-sharpened tungsten microknife. The neuron was removed from the ganglion with suction from a fire-polished glass pipette (100 μM tip diameter). The isolated neurons were temporarily held in a 35 mm petri dish (#1008 Falcon dish) containing 2 ml of a 1% solution of *Helisoma* hemolymph in DM (hemolymph extraction is discussed below). Placement of the cells in this solution prevents adherence to the substratum (Haydon, 1988).

**Culture dishes**  The cells were plated into 50 μl culture wells containing 10% unfiltered snail hemolymph (see below). The well was made by drilling a hole (5 mm diameter) in the bottom of a Falcon #1008 petri dish. A glass coverslip was attached to the bottom of the dish using a silicon seal (Weller silicone rubber adhesive sealant). The rubber was allowed to harden for 24 hours. The dishes were then soaked in distilled water for 48 hours to remove acetic acid. The culture wells were sterilized by soaking in 70% ethanol until the alcohol evaporated.
Hemolymph extraction Neurite extension was prevented by culturing the neurons in a nonadhesive environment. Unfiltered *Helisoma* hemolymph prevented adhesion and subsequent neurite extension; thus, neurons maintained a spherical geometry (Haydon, 1988).

The hemolymph was extracted on each day of culture. Intact snails were subjected to a series of antiseptic washes. First, the snails were soaked in ABS for 15 minutes. This was followed by a 15 minute incubation in a 1:3 Listerine:ABS solution. Finally, the snails were returned to ABS for 15 minutes. A small piece of the shell was removed to expose the foot and mucus was wiped off with a sterile cotton ball. The foot was pierced with the tips of a pair of forceps (Dumoxel #5) and the hemolymph, that flowed out of the foot, was collected in sterile capillary tubes. The hemolymph was centrifuged (International Hematocrit Centrifuge) for 5 minutes to remove noncellular debris. The culture medium contained a 10% hemolymph solution made up of 8 µl hemolymph and 72 µl DM. Individual neurons were plated into the culture wells. If single neurons were required for calcium current experiments, the neurons were plated at a distance from each other in the well. If synapses were to be studied, buccal neurons 5 and 19 were made to contact in the well. The cells adhered to one another for the duration of the culture period but not to the substratum. The synaptic pairs do not extend neurites and physical contact is made directly between the somata (Haydon, 1988). The neurons were transferred daily into new culture wells containing fresh medium and hemolymph.

Electrophysiology

The neurons were immobilized for electrophysiological recordings by plating them into a Falcon #1008 petri dish or a 12 mm culture well with a poly-L-lysine coated coverslip. The poly-L-lysine solution was made using 1 mg poly-L-lysine hydrobromide (Type VI; Sigma) in 1 ml of 0.15 M Tris, pH 8.4. This solution was pipetted into each well and allowed to incubate for several hours. The wells were then rinsed twice with distilled water and air dried.

Synapses The culture wells were filled with DM or 1 µM FMRFamide in DM during electrophysiological recordings from synapses. Throughout the study of synapses, postsynaptic neurons were voltage clamped using Dagan Corporation 8900 patch amplifiers. Postsynaptic pipettes were made using the standard two-step pulling technique with a
Narashige PP-83 patch pipette puller. The pipettes contained (in mM): 50 KCl, 5 MgCl₂, 5 EGTA, 5 K⁺HEPES, pH 7.3 and typically had resistances of 4 MΩ.

Action potentials were evoked in the presynaptic neuron using microelectrodes with resistances of 10 to 40 MΩ when filled with 1.5 M KCl. The microelectrodes were connected to a Getting Model 5A amplifier. To quantify the effect of FMRFamide on action potential-evoked release, the experiment was divided into three periods: before the addition of FMRFamide, in the presence of FMRFamide, and following washout of FMRFamide. Before addition of FMRFamide, the amplitude of the three evoked postsynaptic currents (PSCs) were averaged and normalized to a value of 1.0. The amplitude of the three PSCs in the presence of FMRFamide, and the three PSCs following washout of FMRFamide were averaged and compared to the control value of 1.0.

Since spontaneous miniature postsynaptic currents (MPSCs) were the quantal events underlying action potential-evoked PSCs, asynchronous release of MPSCs was studied to determine the locus of FMRFamide action. Patch pipettes were used to voltage clamp the presynaptic neuron. Pipettes contained a solution similar to the postsynaptic solution that contained 35 mM CsCl instead of KCl plus 5 mM ATP and 1 mM GTP and had resistances of approximately 2 MΩ. A set of three depolarizing steps was delivered at 30 second intervals in each of three conditions: before the addition of FMRFamide, in its presence, and following washout. The number of MPSCs was counted in each of the depolarizing periods. For all synapses, the number of MPSCs in the period before addition of FMRFamide was averaged and normalized to a value of 1.0. The numbers of MPSCs counted in the presence of FMRFamide and after washout were also averaged and compared to the control value of 1.0.

Nitr-5 Photolytically-released calcium was used to trigger secretion to examine the effects of FMRFamide on the secretory apparatus. The presynaptic patch pipette contained: 20 mM nitr-5, 18 mM CaCl₂, 35 mM KCl, 5 mM MgCl₂, 5 mM ATP, 1 mM GTP, 400 µM fura-2, 5 mM K⁺HEPES, pH 7.3. Fura-2 is a reliable means of monitoring the level of internal free calcium. The background intensity of the cells was measured before loading with fura-2 and was subtracted from all subsequent measurements. The fluorescent emission from fura-2 was collected at 510 nm with a photomultiplier. The emission intensities at 340 nm and 380 nm excitation were recorded using the UMANS system (Chester M. Regen Ph.D., Urbana, IL).

A concentration of 20 mM nitr-5 (Calbiochem; Adams et al., 1988) was loaded to 90% capacity with 18 mM calcium. Adequate dialysis of the presynaptic neuron was judged as an
increase in the background rate of transmitter release. At this point, photolysis was performed
to raise the level of intracellular free calcium.

Nitr-5 is synthesized by the introduction of a photolabile group into the chelator BAPTA
(Adams et al., 1988). Photolysis of nitr-5 yields compounds with affinities for calcium that are
lower than the precursor molecule. Exposure to ultraviolet light causes the conversion of a 2-
nitrobenzyl moiety to a 2-nitroso structure. A water molecule is ejected and a ketone residue
replaces the original alcohol group. Preceeding ultraviolet illumination, nitr-5 has a Kd for
calcium of 0.145 \( \mu \)M and following photolysis, the Kd changes to 6.3 \( \mu \)M resulting in an
irreversible release of calcium into the cytoplasm.

A Nikon diaphot inverted microscope with a 40X oil immersion objective was used to view
the synaptic pairs. After the presynaptic neuron was dialyzed with the nitr-5-containing
solution for 1 to 5 minutes, the rate of release of ACh increased. The calcium was then
released from its cage by light provided by a 75 watt Xenon arc lamp and passed through a 340
nm band pass filter and a 400 nm dichroic mirror. The ultraviolet light was focussed onto the
neuron through the 40X oil immersion objective. In most experiments, the intensity of
illumination was decreased by a 17% transmittance neutral density filter.

The rate of transmitter release was determined by measuring the reciprocal of the interval
between each postsynaptic event. The variability in graphing the instantaneous release rate was
reduced by averaging each interval with the moving bin method (Rahamimoff and Yaari,
1973). The value from each interval was averaged with the values from the intervals
immediately before and after. The rate of postsynaptic currents was also calculated by
measuring the average rate over a 3 second interval.

**Calcium currents** The calcium currents of secretory neurons were studied using standard
voltage clamp techniques. The neurons were transferred to a Falcon #1008 culture dish to
immobilize them for electrophysiological recordings. Calcium currents were
pharmacologically and ionically isolated for study with a standard external saline consisting of
(in mM): 0 NaCl, 4.1 CaCl2, 1.5 MgCl2, 1.7 KCl, 30 tetraethylammonium Br, 10 4-
aminopyridine, 30 sucrose, and 10 HEPES, pH 7.3. Presynaptic pipettes had resistances of 2
M\( \Omega \) when filled with (in mM): 35 CsCl, 5 MgCl2, 5 EGTA, 5 ATP, 1 GTP, 5 K+HEPES,
pH 7.3.

Neurons were voltage clamped using a Dagan 8900 patch clamp amplifier and signals were
filtered with a corner frequency of 1 kHz. In all figures, leakage and capacitative currents have
been digitally subtracted using appropriately scaled hyperpolarizing pulses. Series resistance compensation was employed. Voltage clamp experiments and analyses were performed using pClamp software (Axon Instruments, CA).

When the effect of FMRFamide (Bachem) on the calcium current was studied, a standard stimulus paradigm was used. Neurons were repetitively depolarized at 30 second intervals for nine episodes from a holding potential of -60 mV to a command potential that evoked the maximum current in an individual cell. The current magnitude was monitored for three depolarizations in standard external saline to ensure the stability of the current. If the calcium current decreased by > 5% in this time period, analysis of the cell was terminated. If the current remained stable, the average magnitude of the control HVA current evoked by the three depolarizations was assigned a value of 1.0. The mean peak calcium current from three depolarizations in 1 μM FMRFamide, and the mean peak current from three depolarizations following washout of the peptide, were calculated and normalized to the control value of 1.0. Significant differences were tested using 1- and 2-tailed student's t-tests. The values expressed are the mean ± s.e.m. unless otherwise stated. Typically, 1 μM FMRFamide was used throughout the experiments.

The micropipettes and patch pipettes were manipulated using Narishige micromanipulators. The membrane currents were displayed on a Tektronix 5111 storage oscilloscope and on a Gould 220 chart recorder.
<table>
<thead>
<tr>
<th></th>
<th>ABS (in mM)</th>
<th>DM(^a) (in mM)</th>
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</thead>
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<tr>
<td>NaCl</td>
<td>51.3</td>
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<tr>
<td>CaCl(_2)</td>
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<tr>
<td>MgCl(_2)</td>
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<td>1.5</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Gentamycin Sulfate (Sigma)</td>
<td>150 µg/ml</td>
<td>50 µg/ml</td>
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<tr>
<td>L-Glutamine (Gibco)</td>
<td></td>
<td>0.15 mg/ml</td>
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<tr>
<td>L-15 (Leibovitz) (see Table 2)</td>
<td></td>
<td>50 %</td>
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\(^a\) DM is filtered through a 0.45 µm filter unit (Nalgene).
**Table 2. Contents of L-15 Medium**

**L-15 (Leibovitz) Medium**

<table>
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<tr>
<td>D (+) Galactose</td>
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<tr>
<td>Phenol red</td>
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<td>Sodium pyruvate</td>
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**Amino acids:**

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**VITAMINS:**

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<td>Nicotinamide</td>
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Table 2 (Continued)

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<tr>
<td>Riboflavin-5'-phosphate, sodium</td>
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</tr>
<tr>
<td>Thiamine monophosphate</td>
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RESULTS

FMRFamide reduces the magnitude of the PSC in response to action potentials

Over the course of two days in culture, a unidirectional chemical synapse forms between neuronal somata of neurons B5 and B19. The presence of a chemical connection can be detected with electrophysiological techniques. The voltage of the presynaptic neuron was monitored with a microelectrode and a patch pipette was used to record current from the postsynaptic neuron. Action potentials in the presynaptic neuron evoke the release of neurotransmitter that causes chloride-dependent postsynaptic currents (PSCs) in the postsynaptic cell. At a holding potential of -80 mV and with dialysis with 50 mM KCl from the postsynaptic pipette, these PSCs become inward currents. Neurotransmitter is released in a calcium-dependent manner from somatic synapses since calcium-deficient bathing medium depresses synaptic transmission (Haydon, 1988).

The effect of the neuropeptide FMRFamide was tested on synaptic transmission. Action potentials were evoked in B5 at 1 minute intervals. Figure 1A shows the effect of application of 1 μM FMRFamide, the standard concentration of the peptide used throughout this study. The pooled data from five synapses are shown in the histogram in Figure 1B. In the presence of the peptide, the magnitude of the action potential-evoked PSC is reduced to 0.29 ± 0.10 (mean ± s.e.m.) of the control period. Following washout of FMRFamide, there was little recovery of the magnitude of the PSC. In the washout period, the PSC had a value of 0.37 ± 0.15. The incomplete recovery of the magnitude of the PSC may be due to incomplete washout of the agonist or perhaps, long-lasting effects of FMRFamide. Nonetheless, FMRFamide has a pronounced inhibitory effect on synaptic transmission between neurons B5 and B19.

FMRFamide has a presynaptic locus of action

Although it is apparent that FMRFamide reduces the magnitude of the PSC, its locus of action is unknown. Potential targets of the effects of FMRFamide are the presynaptic neuron, the postsynaptic neuron, or both sites. The locus of its action can be determined by examining its effects on desynchronized release. A presynaptic locus of action would be manifested by a decrease in the frequency of MPSCs. A postsynaptic locus would be detected as a reduction in the amplitude of each event although the frequency of events would remain constant. A
Figure 1. FMRFamide reduces the action potential-evoked PSC magnitude

(A) The presynaptic neuron was impaled with a microelectrode to record and evoke action potentials. The postsynaptic neuron was voltage clamped at a holding potential of -80 mV with a patch pipette. Action potentials were evoked in the presynaptic neuron at 1 minute intervals. The current in the postsynaptic neuron in response to presynaptic action potentials is shown. FMRFamide (1μM) reduces the magnitude of the PSC. (B) Histogram showing the data from 5 synapses. FMRFamide reduces the magnitude of the PSC. The bars represent the mean ± s.e.m.
A

Control

FMRFamide

Wash

B

PSC Magnitude (normalized)

Control

FMRFamide

Wash

0

1

500 pA

25 ms
decrease in the sensitivity of postsynaptic receptors results in a smaller response in the postsynaptic neuron to a given quantity of transmitter. Both presynaptic and postsynaptic neurons were voltage clamped with patch pipettes. The presynaptic neuron was held at -60 mV and depolarized for 4 seconds to potentials that caused reliable release of neurotransmitter. Depolarization of B5 for several seconds causes a desynchronized release of neurotransmitter. A series of MPSCs can be seen throughout the 4 second voltage step (Figure 2A). The amount of transmitter released in response to a long-duration stimulus varies with the command potential. During stronger depolarizations, a larger population of voltage-sensitive calcium channels opens which then triggers the release of more transmitter as shown in Figure 2A. This desynchronized release is due to the calcium-dependent release of ACh (Zucker and Haydon, 1988). As shown in Figure 2B, the number of MPSCs is reduced when the synapses were bathed in a solution that contained no added calcium. Release of neurotransmitter is not fully blocked in nominally calcium-free saline because there is still a substantial electrical gradient for calcium that can result in elevated internal calcium levels (Mulkey and Zucker, 1990).

The effect of FMRFamide was tested on desynchronized release. As shown in Figure 3, FMRFamide reduced the number of MPSCs during a constant voltage step to 0.62 ± 0.07 of the control period. The reduction in the frequency of MPSCs was not due to depression of the synapse since it subsequently increased to 0.85 ± 0.08 following washout (Figure 3B; P<0.025; 2-tailed student's t-test). The reduction in the frequency of MPSCs in the presence of FMRFamide is consistent with a presynaptic locus of action.

These experiments do not exclude additional modulatory effects on the postsynaptic neuron. The somatic synapse system allows for a detailed examination of presynaptic mechanisms underlying synaptic plasticity. However, to confirm that FMRFamide is not exerting additional effects on the postsynaptic cell, ACh was applied to postsynaptic neurons. In the presence of FMRFamide, the magnitude of the PSC in response to ACh remained constant (Zoran et al., 1989).

HVA calcium current

Neuromodulators can modulate action potential-evoked release of neurotransmitter by causing changes in ion conductances. Thus, the effect of FMRFamide was tested on the calcium currents of presynaptic neuron B5. When the cell body of neuron B5 is plated into a nonadhesive culture environment for two days, it retains a spherical geometry, is devoid of
Figure 2. Desynchronized release is dependent on voltage and calcium

(A) Presynaptic and postsynaptic neurons were voltage clamped with patch pipettes and held at -60 mV. A 4-second voltage step was delivered to the presynaptic neuron to evoke the desynchronized release of transmitter. This is shown in the postsynaptic current records as downward deflections. Each deflection represents a MPSC. Presynaptic command potentials are indicated next to each postsynaptic record. Steps to greater depolarized potentials evoke the release of more transmitter. (B) The presynaptic neuron was depolarized to -10 mV from a holding potential of -60 mV. Bathing synaptic pairs in a low-calcium medium reduces the release of neurotransmitter. Addition of a calcium-containing medium restores transmitter release.
A B

\[ V_{\text{pre}} \]

-20 mV

-10 mV

0 mV

4.1 mM calcium

low calcium saline

4.1 mM calcium

\[ V_{\text{pre}} \]

-10 mV

I_{\text{post}}

20 pA

400 ms
Figure 3. FMRFamide causes a presynaptic inhibition of secretion

(A) Presynaptic and postsynaptic neurons were voltage clamped in the whole-cell configuration. The presynaptic neuron was depolarized from a holding potential of -60 mV to -15 mV. This causes a desynchronized release of neurotransmitter which was seen as downward deflections in the current recordings from the postsynaptic cell (I_post). Each deflection represents a MPSC. Application of 1 μM FMRFamide reduces the number of desynchronized MPSCs. In the example shown, FMRFamide reduced the number of MPSCs from 16 to 1. (B) Histogram showing the reversible reduction in the frequency of MPSCs caused by FMRFamide (n=9). The mean ± s.e.m. are shown.
A

Control

FMRFamide

Wash

B

Number of PSCs (normalized)

- Control
- FMRFamide
- Wash
neurites, and gains the ability to release neurotransmitter in response to action potentials. Neurosecretory somata contain a High-Voltage-Activated (HVA) calcium current that is activated by depolarizations from a holding potential of -60 mV to about -20 mV or greater. This current is sustained during the 200 ms voltage pulse.

Bath application of 1 μM FMRFamide reversibly reduced this net inward current (Figure 4A; Man-Son-Hing et al., 1989). The effect of FMRFamide on the HVA current was quantified. Addition of 1 μM FMRFamide caused a reduction of the calcium current to 0.87 ± 0.02 (mean ± s.e.m.) of the control current magnitude value of 1.00 (Figure 4B; n=27 cells). The effect of FMRFamide was reversible as the calcium current increased to a significantly greater value of 0.96 ± 0.02 following washout of the peptide (Figure 4B; n=27; P<0.001; 2-tailed student's t-test).

I have demonstrated that the HVA calcium current was specifically blocked by FMRFamide because: 1) as shown in the current/voltage relationship in Figure 5A, FMRFamide decreased the HVA current at all command potentials; 2) the extrapolated reversal potential (the point where the curve intercepts the X axis) of the HVA current remained unchanged at +60 mV during the application of FMRFamide (Figure 5A); 3) when the magnitude of the calcium current was reduced with Cd²⁺, a calcium channel blocker, the extent of FMRFamide's reduction of the calcium current was diminished but the percent reduction remained constant (Figure 5B). In Figure 5B, FMRFamide caused a 0.75 nA (19%) reduction of the calcium current. Addition of 1 μM FMRFamide in the presence of 50 μM Cd²⁺ caused a 0.30 nA (18%) reduction in the current magnitude. This effect was seen consistently in 12 cells tested. Taken together, these three lines of evidence demonstrate that the HVA calcium current is reversibly reduced by FMRFamide.

FMRFamide reduced the calcium current in a dose-dependent fashion (Figure 5C). FMRFamide caused a maximal reduction at 10⁻⁵ M and had an estimated apparent Kᵣ of about 10⁻⁶ M. Neurons retained the ability to respond to FMRFamide for the duration of the whole-cell recording. Repeated applications of FMRFamide reliably reduced the calcium current throughout the 30 minute recording period.

Selective action on the HVA calcium current

Many neuronal cell types contain multiple types of calcium currents (for review, see Miller, 1987). When neuron B5 is acutely isolated from the nervous system, it contains two distinct calcium currents (day 0 in culture), a Low-Voltage-Activated (LVA) calcium current and a
Figure 4. FMRFamide reversibly reduces the magnitude of a HVA calcium current in neurosecretory somata

(A) A GTP-containing patch pipette was used to voltage clamp a secretory somata. The neuron was repetitively depolarized to +10 mV from a holding potential of -60 mV. Superimposed current traces show that addition of 1 µM FMRFamide reversibly reduces the magnitude of the inward current. The current magnitude increases following washout of FMRFamide. (B) Histogram showing that 1 µM FMRFamide reversibly reduces the magnitude of the calcium current (n=27). The magnitude of the current is represented by the bars and is expressed as mean ± s.e.m.
calcium current magnitude
(normalized)
Figure 5. FMRFamide selectively exerts its effects on a calcium current

(A) A current/voltage relationship of the HVA current is plotted. The peak magnitude of the current was measured in the absence (solid circles) and presence (open circles) of FMRFamide. The difference current (solid squares), obtained by subtracting the current present in FMRFamide from the control current, has a positive extrapolated reversal potential indicating that the calcium current is sensitive to FMRFamide. (B) B5 was depolarized to +20 mV. Addition of FMRFamide reduces the magnitude of the calcium current. 50 μM Cd²⁺, a calcium channel blocker, was added to the bath to reduce the magnitude of the calcium current. The extent of the effect of FMRFamide is reduced in the presence of Cd²⁺. (C) The effects of FMRFamide on the HVA calcium current are concentration-dependent. Values are means ± s.e.m. from 5 to 8 cells.
Figure A: Graph showing command potential (mV) vs. $I_{Ca^{2+}}$ (nA) with different treatments.

- Control
- FMRFamide
- Difference current

Figure B: Graph showing current traces with different treatments.

- Cd$^{2+}$ + FMRFamide
- Cd$^{2+}$
- FMRFamide
- Control

Figure C: Graph showing the percentage inhibition with FMRFamide vs. FMRFamide concentration in µM.
electrophysiological properties. In acutely-isolated cells, the LVA current is selectively activated by depolarizations to -20 mV and decays during the 200 msec pulse (Figure 6A). The HVA current is activated by more positive potentials and is only partially decaying (Figure 6A). Figure 6B shows a current/voltage (I/V) relationship from an acutely-isolated neuron. The presence of two currents with different activation thresholds results in a plateau at negative command potentials. The LVA and HVA calcium currents are also distinct with respect to their pharmacological properties. 10 μM Cd²⁺, a calcium channel blocker, reliably reduces the HVA current while having little effect on the LVA current (Haydon and Man-Son-Hing, 1988). 50 to 100 μM Cd²⁺ is required to affect the LVA calcium current.

To test if FMRFamide also exerts its effects on the LVA current, acutely-isolated neurons were repetitively depolarized to -20 mV to selectively activate LVA. Addition of 1 μM FMRFamide did not cause a significant reduction in the LVA current (Figure 7A). In the presence of 1 μM FMRFamide, the LVA current was 0.98 ± 0.04 of the control current magnitude (n=8). The effect of FMRFamide on the HVA current in such acutely isolated cells was also tested. A prepulse to -20 mV was delivered to inactivate LVA so that a depolarization step to +20 mV selectively activated the HVA current. While the LVA calcium current was unaffected, the HVA current in the same cell was reduced by FMRFamide to 0.80 ± 0.04 of the control current (n=8; Figure 7A). Thus, the effect of FMRFamide on the macroscopic calcium current is selective.

Secretory apparatus

In nonneural cells, G proteins and second messengers directly control the secretory machinery by affecting its sensitivity to the internal level of free calcium (Haslam and Davidson, 1984). To determine if FMRFamide has effects on the secretory machinery of neuron B5, in addition to and separate from its effects on the calcium current, the secretory machinery had to be experimentally separated from voltage-dependent ion conductances. For this purpose, the photolabile calcium cage, nitr-5 (see Materials and Methods; Adams et al., 1988), was used to stimulate secretion independently of all voltage-sensitive ion channels.

The presynaptic and postsynaptic neurons were voltage clamped with patch pipettes and held at -80 mV. Nitr-5 was included in the pipette of the presynaptic cell to dialyze into the cytoplasm. After allowing 1 to 5 minutes for dialysis, the neurons were exposed to 340 nm light to photolytically release calcium from nitr-5. In this manner, the level of intracellular free calcium was raised to promote the secretion of neurotransmitter from the presynaptic neuron.
Figure 6. Acutely-isolated neurons contain 2 types of calcium currents

(A) An acutely-isolated neuron was whole-cell voltage clamped and held at -60 mV. The LVA calcium current is activated selectively by depolarizations to -20 mV and is a decaying current. At +20 mV, the HVA and LVA calcium currents are both activated. The HVA current is more sustained than the LVA current. (B) The neuron was depolarized in 10 mV increments from a holding potential of -60 mV to construct a current/voltage relationship. The plateau in the relationship at negative potentials indicates the presence of 2 types of calcium currents as labelled.
Figure 7. FMRFamide acts selectively on the HVA calcium current

(A) An acutely-isolated neuron containing LVA and HVA calcium currents was whole cell voltage clamped. The neuron was depolarized from a holding potential of -60 mV to -20 mV to selectively activate the LVA calcium current. Application of 1 μM FMRFamide has no effect on the magnitude of this current. (B) The same neuron was depolarized from a holding potential of -20 mV to +20 mV to selectively activate the HVA calcium current. FMRFamide has a marked inhibitory effect on the magnitude of the HVA current.
A  LVA calcium current

Control, FMRFamide

0.25 nA

40 ms

B  HVA calcium current

FMRFamide

Control

2 nA

40 ms
Photolysis caused a sustained increase in the frequency of MPSCs (Figure 8A). As shown in Figure 8B, synaptic transmission under such calcium-clamped conditions is reduced by application of the cholinergic antagonist, tubocurarine. Synapses that had been dialyzed with nitr-5 containing no calcium were exposed to ultraviolet light. There was no increase in the rate of release indicating that the elevation of MPSC frequency observed after ultraviolet illumination of nitr-5/calcium was due to a calcium-dependent release of transmitter rather than to photoproducts of nitr-5 or the ultraviolet light itself (Figure 8C; n=4).

To test the effect of FMRFamide on secretory machinery, nitr-5 was photolyzed to increase the MPSC frequency. Subsequently, FMRFamide was rapidly applied extracellularly by pressure ejection from a pipette for a 5 to 10 second period. Fast green, an inert dye, was included in the FMRFamide solution to allow visualization of the application of FMRFamide. Addition of 1 to 10 μM FMRFamide reliably reduced the rate of MPSCs (Figure 9A). The rate of release was reduced from a control value of 1.0 to 0.35 ± 0.11 (Figure 9B; n=9 trials from 8 synapses). Following washout of FMRFamide, the rate of release increased to a significantly greater value of 0.66 ± 0.10 (P<0.025; n=9; Figure 9B).

Fast green in culture medium was pressure ejected onto cell pairs as a control for the application of FMRFamide and was found to have no effect on the rate of release (n=2). Additionally, FMRFamide had the same inhibitory effect on the rate of secretion in synapses that were bathed in a calcium-deficient medium (n=2).

Nitr-5 is likely to be the dominant calcium buffer in the presynaptic terminal during the "calcium clamp" experiments. To confirm that the level of internal calcium was constant throughout these experiments, the fluorescent probe, fura-2, was codialyzed with nitr-5 into presynaptic neurons. The emission from fura-2 was monitored to obtain an indication of the level of free internal calcium. Neuronal pairs were exposed to ultraviolet light to photolytically raise the calcium level to approximately 400 nM to 1 μM. The addition of FMRFamide did not change the level of calcium within the presynaptic terminal as monitored with fura-2 (n=4). Thus, the peptide decreased the rate of secretion from neuron B5 under conditions of constant internal calcium.
Figure 8. Photolytically released calcium from nitr-5 promotes secretion.

(A) Presynaptic neurons were dialyzed with calcium-loaded nitr-5. Traces show the MPSCs recorded from the postsynaptic neuron. Exposure to ultraviolet light photolytically releases calcium and the background rate of MPSCs increases. The MPSC frequency remains elevated after cessation of ultraviolet illumination due to the irreversible reduction in affinity of nitr-5 for calcium. (B) Application of tubocurarine reduces synaptic transmission. (C) Neurons were dialyzed with nitr-5 which was not loaded with calcium. Ultraviolet light exposure did not increase the background rate of MPSCs demonstrating that neither ultraviolet light nor photoproducts of nitr-5 are responsible for elevating MPSC frequency in (A).
Figure 9. FMRFamide reduces the rate of MPSCs under calcium clamped conditions

(A) Presynaptic and postsynaptic neurons were whole-cell voltage clamped. The presynaptic neuron was dialyzed with a solution containing nitr-5 loaded with calcium. Ultraviolet illumination of the neurons photolytically released calcium to trigger an increase in the background frequency of MPSCs. Application of 1 μM FMRFamide reduces the frequency of MPSCs. (B) Histogram showing the normalized MPSC rate. The average rate was measured for 3 seconds for each synapse before, during, and after FMRFamide application. FMRFamide reliably reduces the rate of MPSCs which partially recovered after its removal.
A

Light On

FMRFamide

B

![Graph showing release rate (normalized)]

- Control
- FMRFamide
- Wash

Release rate (normalized)
DISCUSSION

Using an experimental system that allows direct access to the presynaptic terminal, I have examined two mechanisms by which a neuromodulator causes an inhibition of neurotransmitter release. FMRFamide reduced the amount of transmitter released through dual mechanisms. First, FMRFamide decreased the magnitude of the HVA macroscopic calcium current present in secretory membrane. FMRFamide's effects were specific in that there was no reduction in the magnitude of the LVA calcium current that is present with the HVA current in acutely-isolated cells. The subcellular mechanisms by which FMRFamide exerts its effects on the calcium channels is discussed in Section 2.

A second locus of modulation in the presynaptic neuron was revealed. Calcium was elevated to a constant level with a photolabile calcium cage to evoke secretion. When FMRFamide was applied to these calcium clamped synapses, the amount of neurotransmitter released decreased. Since the postsynaptic sensitivity to ACh is unaffected by FMRFamide, the reduced frequency of MPSCs indicates that the sensitivity of the secretory apparatus to calcium was reduced. There are numerous potential mechanisms by which FMRFamide could be affecting the secretory apparatus. One possibility is that FMRFamide is promoting the modification of vesicle-associated proteins.

A model of the presynaptic terminal has been developed to account for changes seen during synaptic depression (Zucker, 1989). This model may also be applied to the presynaptic inhibition of transmitter release caused by FMRFamide. In this model, synaptic vesicles in the nerve terminal are divided into two spatially distinct populations. One population of the vesicles lies immediately adjacent to the active zone and is available for release. The other population of vesicles is the storage pool that is not situated at active zones. In the model, the storage pool replenishes the releasable pool following presynaptic activity. FMRFamide could be exerting its inhibitory actions on the releasable pool or on the mobilization of the storage pool to the active zone.

FMRFamide may decrease the amount of transmitter released from the presynaptic neuron by reducing the size of the releasable pool through effects on vesicle-associated proteins. This type of modulation has been suggested from experiments in the squid giant axon (Llinas et al., 1985). In these experiments, the state of phosphorylation of the vesicle-associated protein, synapsin I, can alter the release of transmitter. Injection of dephosphosynapsin I had an inhibitory effect on the postsynaptic potential whereas injection of calcium/calmodulin-
dependent protein kinase II, which phosphorylates synapsin I, enhanced release. The authors propose that the injection of a kinase allows the vesicles to move to the plasma membrane to be released. Dephosphosynapsin I may prevent exocytosis by crosslinking the vesicles to the cytoskeleton such that they are unavailable for exocytosis.

Morphological studies examining the size of the releasable pool of vesicles have been performed in *Aplysia* (Bailey and Chen, 1988). These studies show that the population of vesicles immediately adjacent to the active zone is dynamic. The active zone may be a morphologically distinct region in some synapses. Vesicles fuse with the plasma membrane in this region. Following habituation training, the percent of vesicles in the active zone that are available for release in sensory neurons decreased when compared to nerve terminals that did not undergo training. The authors speculate that long-term habituation may involve a reduction in the vesicle mobilization process.

Alternatively, FMRFamide could exert effects on the releasable pool by affecting the release process itself. Proteins directly involved in the secretory event could be modified. A model involving a "fusion pore" in the exocytotic event has been proposed (reviewed in Aimers, 1990). Although the molecular composition of the presynaptic active zone is completely unknown (Kelly, 1989), this model involves the docking of vesicles at the plasma membrane at specific sites via a protein(s). Binding of calcium to these proteins triggers a conformational change, resulting in the formation of an open pore that connects the vesicle lumen and extracellular space. The contents of the vesicle can escape through the pore. The pore gradually dilates and eventually the vesicle and plasma membranes merge, fully releasing the vesicle contents. FMRFamide may reduce the number of fusion pores capable of opening in response to calcium.

FMRFamide could also have effects on the process of vesicle replenishment in the active zone, that is, the mobilization of the storage pool. This situation would be analogous to the model involving synaptic depression. This reduction in transmitter release following sustained presynaptic activity may indicate a limited store of releasable transmitter. Once depleted, this pool of vesicles requires time to be replenished from the storage pool (Zucker, 1989). FMRFamide may prevent the movement of vesicles from the storage pool to the active zone, perhaps through vesicle/cytoskeleton interactions.

The dual regulation of transmitter release may allow FMRFamide to have a greater range of inhibitory effects on neurotransmitter release. In separate studies, Haydon et al. (1991) have determined that the inhibition of the presynaptic calcium current and the secretory machinery
differ in their dose responses to FMRFamide in that the secretory machinery is inhibited at doses that do not affect the calcium current. At higher concentrations, both the calcium current and the secretory apparatus are affected by FMRFamide which may result in a greatly enhanced inhibitory effect on secretion.
REFERENCES


SECTION 2. MODULATION OF THE HVA CALCIUM CURRENT BY FMRFamide IS MEDIATED BY A PERTUSSIS TOXIN-SENSITIVE G PROTEIN AND MIMICKED BY MICROINJECTION OF THE \( \alpha \)-SUBUNIT OF G\( \alpha \)2
INTRODUCTION

Neuromodulators of synaptic transmission can act through a variety of messenger systems to modulate ionic currents and cause changes in excitability and synaptic transmission. There is increasing evidence that G proteins play an important role in mediating agonist modulation of potassium, calcium, and sodium channels (for review, see Dunlap et al., 1987; for example, Pfaffinger et al., 1985, Holz et al., 1986, Hescheler et al., 1987, Nakajima et al., 1988; Schubert et al., 1989; Toselli and Lux, 1989). β-Adrenergic stimulation of dihydropyridine-sensitive calcium channels is mediated by phosphorylation via cAMP-dependent protein kinase (Kameyama et al., 1986) and by a direct action of Gs on the channel (Yatani et al., 1987, 1988). A variety of agonists have been shown to inhibit voltage-dependent calcium channels via G1 - or Go-mediated processes (for reviews, see Rane and Dunlap, 1990 and Hescheler et al., 1990). The demonstration of two forms of Go (Scherer et al., 1987; Goldsmith et al., 1988) that arise from alternate splicing of a single gene product (Hsu et al., 1990; Strathmann et al., 1990) raises the question as to whether both forms of Go couple to the same or different effector systems. Go has been shown to couple the stimulation of phospholipase C (Kikuchi et al., 1986; Moriarty et al., 1990) as well as inhibition of calcium channel activity.

In an attempt to determine the mechanism(s) by which FMRFamide reduces a calcium current in cultured Helisoma neurons and whether the different molecular forms of Go demonstrate effector specificity, I analyzed some of the actions of guanine nucleotides, pertussis toxin (PTX) and purified G proteins on the FMRFamide-mediated reduction of the HVA calcium current. Using whole-cell voltage clamp and microinjection, I demonstrate that FMRFamide reduces the magnitude of a HVA calcium current through a PTX-sensitive G protein. Additionally, the reduction of the action potential-evoked PSC was found to be mediated by a pertussis toxin-sensitive G protein. Finally, I show that the activated α-subunit of Go2, but not of Go1, reduces the magnitude of the HVA calcium current.
MATERIALS AND METHODS

Cell Culture

Individual identified neurons were isolated using standard techniques (Haydon et al., 1985; Haydon, 1988; Haydon and Man-Son-Hing, 1988). Briefly, buccal ganglia were acutely isolated from the animal and enzymatically treated for 25 minutes in 0.2% trypsin (type III, Sigma Chemical Company, St. Louis, MO). A small cut was made in the connective sheath overlying neuron B5 with an electrolytically-sharpened tungsten microknife. The neuron was removed from the ganglion with suction from a fire-polished glass pipette. In initial experiments to study somatic calcium currents, neurons were plated into a nonadhesive environment. The isolated neuron was plated into a 50 μl culture well containing 10% unfiltered snail hemolymph in Helisoma defined medium (50% Leibowitz-15 with Helisoma salts; GIBCO) or into a bovine serum albumin-coated culture well containing brain-conditioned medium (Wong et al., 1981). In both cases, adhesion to the substratum and subsequent neurite extension were prevented and the neurons maintained a spherical geometry. Under these conditions, the somata gain the ability to release neurotransmitter in response to action potentials (Haydon, 1988). The culture medium was changed daily. After two days of culture, the cells were transferred to a Falcon #1008 dish for immobilization and the calcium currents of secretory neuronal somata were examined.

In some experiments, synapses were studied. Neurons B5 and B19 were isolated as described above. Synaptic partners were made to irreversibly contact in the culture well. A unidirectional chemical synapse forms after two days in culture.

In some experiments, the calcium currents of growth cones were examined. Neuron B5 was plated into a poly-L-lysine coated Falcon #1008 dish containing defined medium. The soma and its original adjoining axon rapidly adhered to the substratum. Within hours, a large growth cone (up to 50 μM length) formed at the axon terminal. The axon was severed with a micropipette to physically isolate the growth cone and the macroscopic calcium currents were examined. Successful recordings were obtained from 18 of greater than 250 growth cones attempted. The remainder of the growth cones were not viable.

Voltage Clamp

The calcium currents of neurosecretory, day two B5 somata were examined with whole-cell patch clamp techniques (Hamill et al., 1981). The cells were transferred to Falcon #1008
dishes to immobilize the cells for electrophysiological recording. Calcium currents were pharmacologically and ionically isolated for study with a standard external saline consisting of (in mM): 0 NaCl, 4.1 CaCl2, 1.5 MgCl2, 1.7 KCl, 30 tetraethylammonium (TEA) Br, 10 4-aminopyrididine, 10 HEPES, and 30 sucrose (pH 7.3 with TEA OH).

Patch pipettes had resistances of 2 MΩ when filled with an internal saline containing (in mM): 35 CsCl, 5 MgCl2, 5 EGTA, 5 HEPES, 1 GTP (tris salt) (pH 7.3 with CsOH). The internal solution usually contained 5 mM ATP (Na+ salt); similar results were obtained with or without ATP. Neurons were voltage clamped using a Dagan 8900 patch clamp amplifier and signals were filtered with a corner frequency of 1 kHz. In all figures, leakage and capacitative currents have been digitally subtracted using appropriately scaled hyperpolarizing pulses. Series resistance compensation was employed.

When the effect of FMRFamide (Bachem Inc.) on the calcium current was studied, a standard stimulus paradigm was used. Neurons were repetitively depolarized at 30 second intervals for nine episodes from a holding potential of -60 mV to a command potential that evoked the maximum current in an individual cell. The current magnitude was monitored for three depolarizations in standard external saline to ensure the stability of the current. If the peak calcium current decreased by >5% in this time period, analysis of the cell was terminated. If the current remained stable, the average magnitude of the control HVA current evoked by the three depolarizations was assigned a value of 1.0. The mean peak calcium current from three depolarizations in 1 μM FMRFamide, and the mean peak current from three depolarizations following washout of the peptide, were calculated and normalized to the control value of 1.0. Differences were tested using 1- and 2-tailed student's t-tests. The values expressed are the mean ± s.e.m. unless otherwise stated. Unless stated otherwise, 1 μM FMRFamide was used throughout the experiments.

During electrophysiological recordings from synapses, the culture wells were filled with DM or 1 μM FMRFamide in DM. Postsynaptic neurons were voltage clamped using Dagan Corporation 8900 patch amplifiers. Postsynaptic pipettes contained (in mM): 50 KCl, 5 MgCl2, 5 EGTA, 5 K+HEPES, pH 7.3 and typically had resistances of 4 MΩ.

Action potentials were evoked in the presynaptic neuron to stimulate secretion using microelectrodes with resistances of 10 to 40 MΩ when filled with 1.5 M KCl. The microelectrodes were connected to a Getting Model 5A amplifier. To quantify the effect of FMRFamide on action potential-evoked release, the experiment was divided into three periods: before the addition of FMRFamide, in the presence of FMRFamide, and following washout of
FMRFamide. In each synapse in the period before addition of FMRFamide, the amplitude of the three evoked postsynaptic currents (PSCs) were averaged and normalized to a value of 1.0. The amplitude of the three PSCs in the presence of FMRFamide, and the three PSCs following washout of FMRFamide were averaged and compared to the control value of 1.0.

Chemicals

To test for the involvement of arachidonic acid metabolites in the mediation of FMRFamide's action on the calcium current, stock solutions of arachidonic acid (Sigma) and nordihydroguaiaretic acid (NDGA; Sigma) were made in DMSO at concentrations of 10 mM and 1 mM respectively. Stock solutions were diluted in standard external saline to yield final concentrations of 50 μM arachidonic acid and 5 μM NDGA.

When the involvement of G proteins in FMRFamide's action on the calcium current was tested, guanine nucleotide derivatives replaced GTP in the following concentrations: 100 μM GTPγS (guanosine-5'-O-(3-thiotriphosphate), Boehringer Mannheim Biochemicals); 100 μM Gpp(NH)p (5'-guanylyl-imidodiphosphate, Sigma); 500 μM, 2 mM, and 5 mM GDPβS (guanosine 5'-O-(2-thiodiphosphate), Boehringer Mannheim Biochemicals).

Pertussis toxin A protomer (List Biological Laboratories, Inc.) was made up to a final concentration of 0.1 μg/μl in normal internal solution that also contained 20 mM dithiothreitol, 20 mM NAD+, and 5 mM ATP. The toxin was pressure injected into neuronal somata with beveled microelectrodes.

All G proteins and α-subunits were kindly provided by Dr. Juan Codina. The purified activated α-subunits were diluted to 50 nM in a buffer containing (in mM): 10 TrisHCl, 1 EDTA, 1 dithiothreitol, 7.5 MgCl2, 20 KCl and stored at -70°C. Immediately prior to use, the α-subunits were diluted with buffer to 5 nM or 10 nM.

G proteins and pertussis toxin were pressure injected using a Picospritzer II (General Valve Corporation). The final concentration of α-subunits in the neuron was estimated by performing parallel microinjections of fluorescein (Molecular Probes). The fluorescence of the cell following the microinjection was compared to a standard curve constructed from the fluorescence of known concentrations of fluorescein. Using this method, I estimate the dilution of the α-subunits to be 100-fold following microinjection.
RESULTS

Arachidonic acid

In *Aplysia* sensory neurons, FMRFamide increases the probability that a class of potassium channels, the "S" channels, are in an open state (Belardetti et al., 1987). The effects of FMRFamide on sensory neurons have been found to be mediated by lipoxygenase metabolites of arachidonic acid (Piomelli et al., 1987). In addition, arachidonic acid has been implicated in G protein activation of cardiac potassium channels (Kurachi et al., 1989; Kim et al., 1989). Therefore, to test for the involvement of a similar pathway in mediating FMRFamide's effect on the HVA current in *Helisoma*, 50 μM arachidonic acid was bath applied to the neurons (Figure 1A). In 11 cells tested, arachidonic acid did not change the magnitude of the calcium current. Furthermore, the inhibitors of arachidonic acid metabolism, indomethacin (5 μM; Figure 1B; n=6) and nordihydroguaiaretic acid (5 μM; Figure 1C; n=7), did not prevent the FMRFamide-evoked reduction of the HVA calcium current. FMRFamide alone caused a 17% reduction in the calcium current in the neuron shown in Figure 1. In the presence of indomethacin and NDGA, FMRFamide caused a 16% and a 17% reduction of the calcium current, respectively. To ensure that arachidonic acid and NDGA were fully dissolved, additional experiments were performed using DMSO as the solvent. In three cells tested, arachidonic acid did not reduce the magnitude of the current. NDGA did not prevent FMRFamide from exerting its effect (n=3). Furthermore, arachidonic acid does have modulatory effects on the secretory apparatus (J. Richmond, unpublished observations). Thus, under conditions of whole-cell recording, arachidonic acid does not contribute to the FMRFamide-induced reduction of the HVA calcium current.

Guanine nucleotides

In many cell types, it has been shown that pertussis toxin-sensitive G proteins couple inhibitory transmitters to calcium currents (for example, Holz et al., 1986; Lewis et al., 1986; Dolphin and Scott, 1987; Hescheler et al., 1987; Wanke et al., 1987; Ewald et al., 1988; Harris-Warrick et al., 1988; Ewald et al., 1989; Toselli and Lux, 1989). In neurons that were dialyzed with GTP, FMRFamide caused a reversible reduction in the magnitude of the calcium current (Figure 2A). In 27 neurons, FMRFamide reduced the current magnitude by an average value of 13% (Figure 2B). Neurons dialyzed with a solution that did not contain GTP retained their responsiveness to FMRFamide throughout the recording period. The involvement of a G
Figure 1. Arachidonic acid metabolites do not mediate the actions of FMRFamide on the HVA calcium current

(A) A secretory somata was voltage clamped at a holding potential of -60 mV and repetitively depolarized to +20 mV. Superimposed current traces show that application of 50 μM arachidonic acid does not reduce the calcium current magnitude. Neither of the inhibitors of arachidonic acid metabolism, indomethacin (Indo; B; 5 μM) nor nordihydroguaiaretic acid (NDGA; C; 5 μM) prevent FMRFamide from exerting its inhibitory effects on the calcium current.
Figure 2.  FMRFamide reversibly reduces the magnitude of a HVA calcium current in neurosecretory somata

(A) A secretory soma was dialyzed with 1 mM GTP and repetitively depolarized to +10 mV from a holding potential of -60 mV. Superimposed current traces show that addition of 1 μM FMRFamide reversibly reduces the magnitude of the inward current. The current magnitude increases following washout of FMRFamide. (B) Histogram showing that 1 μM FMRFamide reversibly reduces the magnitude of the calcium current (n=27). The magnitude of the current is represented by the bars and is expressed as mean ± s.e.m.

FMRFamide promotes an irreversible reduction in the calcium current magnitude when cells were dialyzed with 100 μM GTPγS (C; n=8) and 100 μM Gpp(NH)p (D; n=6). (E) After 30 minutes of dialysis with 500 μM GDPβS, FMRFamide's action on the calcium current is blocked (n=4).
A

- FMRFamide
- \( nA \) 40 ms

**B**

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protein in mediating the reduction of the HVA current by FMRFamide was tested by substituting the guanine nucleotide derivatives Gpp(NH)p, GTPγS, or GDPβS for GTP in the pipette solution.

Nonhydrolyzable GTP analogues irreversibly activate G proteins. In the absence of agonist, these analogues can slowly activate the endogenous G proteins. I used the agonist, FMRFamide, to promote GDP/GTPγS exchange to accelerate G protein activation and then determined whether the action of FMRFamide was irreversible under this experimental condition. With 100 μM GTPγS in the pipette solution, FMRFamide (1 μM) decreased the calcium current magnitude to 0.79 ± 0.03 of the control value (Figure 2C; n=8). In contrast to the results from cells containing GTP, this reduction was irreversible as the calcium current remained at the value of 0.78 ± 0.05 after washout of the peptide (Figure 2C). This value is significantly different from the washout value of 0.96 ± 0.02 in GTP-containing cells (P-value<0.002; 1-tailed t-test). Similar results are shown in Figure 2D with 100 μM Gpp(NH)p dialyzing the cells. FMRFamide caused a decrease in the calcium current magnitude to 0.81 ± 0.03 which did not recover following washout (0.76 ± 0.03; n=6). Following washout of FMRFamide, the calcium current magnitude in GTP-containing cells was significantly different from the Gpp(NH)p-containing cells (P value<0.0005; 1-tailed t-test).

Additional evidence for the involvement of a G protein in mediating the action of FMRFamide is that a second application of the peptide to GTPγS-containing cells did not cause a further reduction in the HVA current (n=5). This indicated that the first application of FMRFamide promoted maximal, irreversible G protein activation.

The results from the previous experiments involving GTP analogues indicate that a G protein is involved in mediating FMRFamide's action on the calcium current. To further test this hypothesis, the GDP analogue, GDPβS, which competes with GTP for the guanine nucleotide binding site and prevents G protein activation, was included in the patch pipette solution.

The effect of FMRFamide was blocked in a time- and dose-dependent fashion with GDPβS dialyzing the cell. FMRFamide reduced the calcium current during the initial 15 minutes of dialysis with 500 μM GDPβS in the pipette solution. Later, the extent of the reduction caused by FMRFamide became attenuated until the effect was completely blocked. As shown by the pooled data in Figure 2E, the HVA current remained unchanged at a value of 1.0 ± 0.10 during the application of FMRFamide after 30 minutes of dialysis with GDPβS (n=4). The effect of FMRFamide was blocked immediately when higher concentrations of GDPβS were included in
the pipette solution (2 to 5 mM). The first application of FMRFamide did not reduce the magnitude of the HVA current (1.05 ± 0.06; n=4). Taken together, these data are consistent with the involvement of a G protein in mediating FMRFamide's reduction of the HVA current.

**Pertussis toxin**

To begin to identify the FMRFamide-activated G protein, the PTX A protomer was added to the interior of the cell by pressure injection through a beveled microelectrode. Cells were allowed to incubate for 4 to 18 hours before voltage clamping. As shown in Figure 3A, FMRFamide had little effect on the magnitude of the calcium current in a PTX-treated cell. FMRFamide decreased the magnitude of the calcium current in PTX-injected cells to 0.97 ± 0.02 of the control current (n=7) and in heat-inactivated PTX-injected cells to 0.71 ± 0.02 (n=11; Figure 3B). The effects of cholera toxin were not tested. Thus, a PTX-sensitive G protein is a component of the FMRFamide signal transduction pathway in reducing the magnitude of the somatic calcium current.

**PTX prevented FMRFamide from modulating synaptic transmission**

Since the effects of FMRFamide on the HVA calcium current and on the secretory machinery (Haydon et al., 1990) appear to be mediated by a PTX-sensitive G protein, I tested whether a PTX-sensitive G protein is also involved in the modulation of action potential-evoked synaptic transmission. Presynaptic somata were injected with the A protomer of PTX 4 hours prior to recording. As shown by the PSC traces in Figures 4A and 4C, FMRFamide had little inhibitory effect in PTX-treated synapses but did reduce the PSC magnitude in control synapses. In PTX-treated synapses, FMRFamide reduced the PSC magnitude from 1.0 to 0.88 ± 0.09 (n=5; Figure 4B). In control preparations that had been preinjected with heat-inactivated toxin, the PSC magnitude was reduced to 0.35 ± 0.09 (n=5; P<0.002; 2-tailed t test; Figure 4D). PTX-sensitive G proteins appear to be involved in mediating the effects of FMRFamide on both a voltage-sensitive calcium current and on action potential-evoked release of transmitter.

**Growth cones**

Transmitter release characteristically occurs from neuronal processes rather than from somata. Haydon and Zoran (1989) have demonstrated that FMRFamide causes presynaptic inhibition of transmitter release at synapses formed by growth cones of B5. Growth cones
Figure 3. FMRFamide's action on the calcium current is mediated by a PTX-sensitive G protein

Superimposed current traces were obtained from neurons which had been injected 4 to 18 hours earlier with either PTX (A) or heat-inactivated PTX (B). (A) FMRFamide did not cause a reduction in the magnitude of the calcium current in a soma injected with PTX. (B) FMRFamide decreases the calcium current of a heat-inactivated PTX-treated soma. (C) Histogram showing the percent reduction in the calcium current magnitude by 1 μM FMRFamide in PTX- (n=7) and heat-inactivated PTX-treated (n=11) neurons.
A  PTX

Control, FMRFamide

B  Heat-inactivated PTX

FMRFamide  Control

C

% reduction with FMRFamide

Heat-inactivated PTX
PTX
Figure 4. PTX-treatment prevents FMRFamide from exerting its inhibitory effects on action potential-evoked PSCs

The A protomer of PTX (A) or heat-inactivated PTX (C) was preinjected into the presynaptic neuron. Single traces of action potential-evoked PSCs were obtained before and after FMRFamide addition. (B) PTX pretreatment prevents FMRFamide from reducing the magnitude of the PSC. (D) Heat-inactivated PTX does not prevent FMRFamide from decreasing the PSC magnitude.
**A**

PTX-treated

Control

FMRFamide

25 ms

60 pA

**B**

PTX-treated

□ Control

■ FMRFamide

**C**

Heat-inactivated

Control

FMRFamide

25 ms

200 pA

**D**

Heat-inactivated PTX

□ Control

■ FMRFamide
contain a HVA calcium current that is similar electrophysiologically and pharmacologically to the HVA current in somata (Haydon and Man-Son-Hing, 1988). To determine whether FMRFamide has a similar action on calcium currents in growth cones as in secretory somata, a limited number of experiments were performed on growth cones (see Materials and Methods). Growth cones were physically isolated from the remainder of the cell and were then voltage clamped in the whole cell mode (see Haydon and Man-Son-Hing, 1988). Figure 5A shows that the growth cone HVA calcium current was reversibly reduced by FMRFamide when GTP was included in the pipette solution. The magnitude of the current was reduced to 0.75 ± 0.04 of the control current (n=3). As in somata, the reversal potential of the calcium current was not changed by FMRFamide and addition of Cd²⁺ decreased the extent of the action of FMRFamide but the percent reduction remained unchanged. Thus, FMRFamide reduces the HVA calcium current of growth cones as well as secretory somata.

To test if this reduction is mediated by G proteins, guanine nucleotide derivatives were included in the pipette solution. When 100 μM GTPγS or 100 μM Gpp(NH)p (Figure 5B) were dialyzed into growth cones, FMRFamide caused an irreversible reduction in the calcium current magnitude. With GTPγS in the pipette solution, FMRFamide decreased the calcium current to 0.76 ± 0.08 of the control current magnitude and the current remained reduced (0.77 ± 0.08) following washout of the peptide (n=3). With Gpp(NH)p, the current was decreased to 0.57 ± 0.12 of the control current upon application of FMRFamide and further declined to 0.48 ± 0.20 after washout (n=3). GDPγS (5 mM) prevented FMRFamide from reducing the calcium current (Figure 5C; n=3). The calcium current changed minimally to 1.02 ± 0.05 of the control current magnitude in the presence of FMRFamide. Due to the fragility of growth cones under recording conditions, the higher concentration of 5 mM GDPγS was used to see an immediate effect.

To determine whether a pertussis toxin-sensitive G protein mediates the action of FMRFamide on growth cone calcium currents, the A protomer was injected into the cell body and allowed to diffuse to the growth cone for 12 hours prior to severing the axon. Pretreatment with pertussis toxin prevented FMRFamide from reducing the calcium current (Figure 5D; n=3). Application of FMRFamide caused an insignificant change in the calcium current magnitude to 1.04 ± 0.02 of the control value. In growth cones pretreated with heat-inactivated pertussis toxin, FMRFamide reduced the magnitude of the current to 0.64 ± 0.12 of the control current (n=3). Thus, in growth cones that release neurotransmitter, FMRFamide
Figure 5. The effect of FMRFamide on the HVA calcium current in growth cones is mediated by a PTX-sensitive growth cone.

Isolated growth cones were voltage clamped at -60 mV and depolarized to +10 or +20 mV and the resulting current traces are superimposed. (A) In a GTP-containing growth cone, FMRFamide reversibly reduces the magnitude of the HVA calcium current. (B) FMRFamide irreversibly decreases the magnitude of calcium currents in growth cones dialyzed with Gpp(NH)p. (C) GDPβS prevents FMRFamide from reducing the magnitude of the current. (D) Pretreatment with PTX abolishes the action of FMRFamide on the calcium current.
A. 1 mM GTP

- FMRFamide
- Control, Wash

B. 100 μM Gpp(NH)p

- FMRFamide, Wash
- Control

C. 5 mM GDP$_{_	ext{S}}$

- Control, FMRFamide

D. PTX

- Control, FMRFamide

[Graphs showing current responses with labels for each condition]
acts through a pertussis toxin-sensitive G protein to decrease the HVA calcium current in a manner similar to that more rigorously tested in secretory somata.

**Introduction of exogenous G proteins**

To determine which G proteins are able to couple FMRFamide to the inhibition of the calcium current, purified heterotrimeric G proteins were introduced into cells that had been previously treated with PTX. After ensuring that the endogenous G proteins were inactivated by the toxin, the purified G protein was microinjected into the cell and the effect of FMRFamide on the calcium current was tested 3 to 25 minutes following injection. Addition of 5 or 10 nM $G_o1$ (n=6), $G_o2$ (n=6) and $G_i2$ (n=4) did not reconstitute the effect of FMRFamide.

Previous studies have shown that G protein $\alpha$-subunits can directly activate potassium channels (Yatani et al., 1987, 1988) and reconstitute agonist inhibition of calcium channels (Ewald et al., 1989). Therefore, I injected purified, preactivated G protein $\alpha$-subunits into secretory somata to determine their effects on whole-cell calcium currents.

Injection of the GTPyS-activated $\alpha$-subunit of $G_o2$ ($\alpha_o2^*$; 10 nM electrode concentration) into somata reduced the magnitude of the calcium current (Figure 6A). This effect was detected as soon as 30 seconds after injection, which was the time of the first voltage step following microinjection. Subsequent injections into the same cell further reduced the calcium current until it was abolished. In comparison to control injections of buffer, $\alpha_o2^*$ significantly reduced the current of untreated cells and cells that had been previously injected with PTX. Injection of $\alpha_o2^*$ did not significantly reduce the LVA calcium current compared to buffer injection ($P>0.1$).

The mean reduction in HVA calcium current per injection ($\pm$ s.e.m.) was calculated for each cell. Buffer injection reduced the current by $4.67\% \pm 1.17$ (n=5) while $\alpha_o2^*$ injection decreased the current by $11.91\% \pm 2.82$ (Figure 6A; n=9; P value < 0.05, 2 tailed student's t-test). While $\alpha_o2^*$ injection significantly reduced the calcium current, neither $\alpha_o1^*$ (10 nM) nor $\alpha_i3^*$ (10 nM) had effects significantly different from control injections (Figure 6A).
Figure 6. Microinjection of the α-subunit of G_o2, but not buffer, reduces the magnitude of HVA.

The magnitude of the HVA calcium current was measured every 30 seconds. (A) Injection of α_o2* (arrow) reliably decreases the magnitude of the current immediately following injection. (B) Injection of buffer (arrow) did not decrease the magnitude of the calcium current. Current traces from before and after three injections are shown in the insets. Application of FMRFamide decreases the current magnitude both before and after the series of injections.
Alpha\(_{\alpha2}\)\* was diluted to concentrations of 1 and 0.1 nM and injected into cells to determine the threshold concentration required to reduce the calcium current. Injection of 1 nM, but not 0.1 nM, \(\alpha_{\alpha2}\)\* significantly reduced the current (Figure 7B). Based on fluorescein dilution experiments, I estimate that a volume equal to 0.01 of the total cell volume was injected. Thus, I estimate that the threshold concentration of \(\alpha_{\alpha2}\)\* for reducing the calcium current is about 10 pM.

The effect of FMRFamide on the calcium current was measured both before and after injection of \(\alpha_{\alpha2}\)\* to determine whether the \(\alpha_{\alpha2}\)\*-mediated inhibition of the calcium channels affected the actions of FMRFamide. FMRFamide caused a 25.78 % ± 3.30 (n=9) current reduction before injection and 24.11% ± 3.53 (n=9) following injection (Figure 7C). Thus, when \(\alpha_{\alpha2}\)\* had reduced the current magnitude, FMRFamide still had the same relative effect on the current indicating that the same subpopulation of calcium channels is susceptible to FMRFamide and \(\alpha_{\alpha2}\)\* inhibition. (In these experiments FMRFamide reduced the calcium current to an extent greater than that shown in Figure 2B. The source of the variability is unknown.)

To further determine if FMRFamide and \(\alpha_{\alpha2}\)\* are acting on the same population of calcium channels, difference currents were obtained for FMRFamide and \(\alpha_{\alpha2}\)\* mediated current inhibition by digitally subtracting control currents from the current following experimental perturbation. Normalized difference traces following addition of FMRFamide and \(\alpha_{\alpha2}\)\* fully superimposed. Taken together, these data suggest that FMRFamide and \(\alpha_{\alpha2}\)\* injections target the same population of calcium channels.
Figure 7. The effect of $\alpha_{O2}^*$ injection is significantly greater than buffer, $\alpha_{O1}^*$, and $\alpha_{i3}^*$

(A) Histogram of the % reduction of the calcium current magnitude following injection of buffer (n=5), $\alpha_{O1}^*$ (n=8), $\alpha_{O2}^*$ (n=9), and $\alpha_{i3}^*$ (n=6). (B) Histogram of the % reduction in the calcium current magnitude caused by injection of buffer (n=5), 0.1 nM $\alpha_{O2}^*$ (n=5), 1 nM $\alpha_{O2}^*$ (n=5), and 10 nM $\alpha_{O2}^*$ (n=9). Injection of 1 nM and 10 nM significantly reduced the current magnitude while 0.1 nM had no significant inhibitory effect (P>0.10). (C) The extent of FMRFamide's effect is similar before (n=9) and after (n=9) injection of $\alpha_{O2}^*$ (as shown in Figure 6A).
Calcium Current Reduction with FMRFamide (%)

Reduction in current due to injection (%)

Before Injection

After Injection

Buffer 0.01 M 0.002 M 0.005 M

Reduction in current due to injection (%)

Buffer 0.1 M 1 mM 10 mM
DISCUSSION

In the present study, the involvement of a G protein in the reduction of the calcium current was demonstrated in experiments using guanine nucleotide analogues. In the presence of nonhydrolyzable GTP analogues, GTPγS or Gpp(NH)p, FMRFamide’s action on the calcium current was irreversible. GDPγS blocked the effects of FMRFamide. Relatively high concentrations of GDPγS (500 μM to 5 mM) were required to completely block FMRFamide’s effect. This may be due to a low affinity of the G protein having for GDPγS, thus requiring high concentrations to effectively compete with GTP for binding. Additionally, all of the G proteins have to be inactivated by GDPγS for FMRFamide’s effect to be blocked. Volterra and Siegelbaum (1988) have shown that in Aplysia sensory neurons, the two different G proteins involved in mediating the effects of serotonin and FMRFamide have different sensitivities to prolonged intracellular injection of GDPγS. The G protein involved in the FMRFamide response is two orders of magnitude less sensitive to the blocking effect of GDPγS.

In addition to preventing FMRFamide from exerting its inhibitory effects on calcium currents, pertussis toxin reduced FMRFamide’s effects on action potential-evoked secretion. This may be due entirely to the lack of modulation of the calcium current. Alternatively, a similar class of G proteins may be involved in the modulation of another site in the presynaptic terminal such as the secretory machinery. G proteins are involved in the process of exocytosis from nonneural cells (Gomperts, 1990). Perhaps this is also the case in neurons.

Although a key role for a PTX-sensitive G protein in the FMRFamide signal transduction pathway has been demonstrated in my experiments, it remains to be established which endogenous G protein is involved. Calcium currents in another snail, Helix, are decreased by dopamine and this effect is mimicked by exogenously supplied mammalian αO (Harris-Warrick et al., 1988). Helix nervous tissue contains a G protein that is sensitive to PTX and is recognized by a bovine anti-αO. Injection of anti-αO prevents dopamine from reducing the calcium current. Muscarinic inhibition of the HVA calcium current in PTX-treated hippocampal neurons is reconstituted by dialysis with the α subunit of Go or with the holoprotein Go (Toselli et al., 1989). Calcium currents are not inhibited exclusively by Go. Hescheler et al. (1987) demonstrated that both Go and Gi can reconstitute the inhibitory effect of the opioid peptide DADLE on calcium currents in neuroblastoma x glioma hybrid cells. Go is, however, 10 times more potent than Gi in mediating such reconstitution. Similarly in hippocampal cells, Gi can reconstitute muscarinic calcium current inhibition, although Go...
performs this reconstitution more effectively (Toselli et al., 1989). Ewald et al. (1989) have shown that \( \alpha_{O}, \alpha_{I1}, \) and \( \alpha_{I2} \) individually can partially reconstitute bradykinin's inhibitory effect on calcium currents in rat dorsal root ganglia but the combination of \( \alpha_{O} \) plus \( \alpha_{I2} \) was required to fully reconstitute the effect. Interestingly, in these same studies, \( \alpha_{O} \) was more effective than \( \alpha_{I1} \) and \( \alpha_{I2} \) was almost ineffective in reconstituting neuropeptide Y-mediated inhibition. Furthermore, recent studies by Linder et al. (1990) using recombinant proteins expressed in E. coli indicate that recombinant \( \alpha_{O}, \alpha_{I1}, \) and \( \alpha_{I3}, \) but not \( \alpha_{I2} \) reconstitute neuropeptide Y-mediated inhibition of calcium currents in rat dorsal root ganglia while all four recombinant proteins reconstitute bradykinin-mediated inhibition.

Studies by Haydon and Man-Son-Hing (1988) and Haydon and Zoran (1989) have shown that growth cones of B5 can release neurotransmitter and that FMRFamide causes presynaptic inhibition of transmitter release from growth cones. As a second approach to studying the mechanism of presynaptic inhibition, voltage clamp studies on neuronal processes were performed. The effects of FMRFamide and G protein analogues are not restricted to the soma. The HVA current in growth cones was reduced in magnitude by FMRFamide and this process was also mediated by a PTX-sensitive G protein. Thus, the model system of secretory somata accurately reflects the properties of secretory membrane located on neuronal processes.

Using Helisoma neurons, I have further examined the role of \( G_{O} \) in regulating calcium currents. Injection of \( \alpha_{O2} \) can reduce the magnitude of the calcium current and is specific for the HVA current. Injected \( \alpha_{O1} \) or \( \alpha_{I3} \) did not effectively decrease the calcium current. Previous published work in which \( G_{O} \) was able to effectively reconstitute hormonal inhibition was likely to have used a mixture of \( G_{O1} \) and \( G_{O2} \). Thus, it is yet unknown whether calcium currents are differentially sensitive to \( G_{O1} \) and \( G_{O2} \) in a variety of tissues and organisms.

Studies by Moriarty et al. (1990) suggest another possibility. These authors found that \( \alpha_{O} \) but not \( \alpha_{I1}, \alpha_{I2} \) or \( \alpha_{I3} \), stimulates the PTX-sensitive phospholipase C-phosphatidylinositol pathway in Xenopus oocytes. Furthermore, \( G_{O} \) has been shown to stimulate five distinct classes of potassium channels in rat hippocampal cells under conditions not thought to activate phospholipase C (VanDongen et al., 1988). Thus, it is possible that one isoform of \( \alpha_{O} \) couples to inhibition of calcium currents while the other couples to stimulation of phospholipase C or potassium channels.

Recent molecular cloning experiments have identified two forms of \( \alpha_{O} \) in HIT (hamster insulin-secreting tumor) cells (Hsu et al., 1990) and mouse brain (Strathmann et al., 1990) cDNA libraries. The deduced amino acid sequence demonstrates that the expressed proteins
would differ in only 25 (mouse) or 26 (hamster) amino acids. These amino acid substitutions are located in the carboxyl third of the protein between amino acids 249 and 354. The amino acid substitutions largely occur within domain III of the α-subunit which is proposed to be the region involved in G protein α-subunit interactions with receptors and effectors (Masters et al., 1988). Assuming that these two cDNAs correspond to the proteins αo1 and αo2, the locations of these amino acid substitutions can guide future experiments designed at identifying critical sites for Gα-effector interaction. At the present time, I do not know which of the two forms of αo identified by cloning corresponds to αo1 and αo2 isolated from bovine brain. Additionally, there may be other forms of Gαo that can be resolved from the known forms of G0 (Kobayashi et al., 1989).

My data are consistent with the hypothesis that αo2 acts on the same calcium current as the peptide FMRFamide. Both agents reduce the HVA current. Injection of αo2* reduces the calcium current and causes a reduction in the absolute magnitude of FMRFamide's effect, but leaves the percentage reduction in the current by FMRFamide unchanged. Finally, based on difference current analysis, αo2* and FMRFamide both reduce the HVA current that has the same inactivation kinetics as the control macroscopic current. Thus, these data are consistent with αo2* acting on the same target as FMRFamide, although unequivocal proof awaits single channel analysis. It is unknown if the injected α-subunits are acting directly on the calcium channels or through second messengers. Single channel analysis will distinguish between the possibilities.

It is unclear why my attempts to reconstitute the inhibitory action of FMRFamide in PTX-treated cells with injection of heterotrimeric G proteins failed. Differences between β subunits of mammals and snails may have prevented effective receptor G protein interactions (Homburger et al., 1987). In Helisoma, I found it necessary to inject PTX into the neuron since bath application of the holotoxin did not uncouple FMRFamide's action on the calcium current. Due to the concern that I may have a high concentration of PTX within the cell that may be rapidly ADP-ribosylating the injected G protein, I diluted PTX for injection until a threshold concentration was achieved. At this concentration, heterotrimeric G protein injection still failed to reconstitute FMRFamide's action on the calcium current. In other studies in which G protein reconstitution was successful, patch pipette dialysis on small cells was used which leads to rapid washout of soluble intracellular proteins. Since neuron B5 is large (60μm diameter), dialysis of proteins is very slow. Perhaps PTX is effectively washed out of the
cytoplasm of the smaller cells so that added G proteins do not become ADP-ribosylated but sufficient PTX remains in B5 to ADP-ribosylate the injected G protein.

In _Aplysia_ neurons, FMRFamide decreases the amount of neurotransmitter released by coordinately decreasing a calcium current and enhancing a potassium current (Belardetti et al., 1987; Brezina et al., 1987). It has been proposed that a G protein activates a phospholipase that generates arachidonic acid. In _Aplysia_, certain presynaptic inhibitory actions of FMRFamide are mediated by lipoxygenase metabolites of arachidonic acid (Piomelli et al., 1987). For example, the "S" potassium current is enhanced by application of arachidonic acid. Furthermore, arachidonic acid also has been implicated in G protein-mediated activation of mammalian cardiac potassium channels (Kurachi et al., 1989; Kim et al., 1989). In my studies, application of arachidonic acid had no effect on the HVA calcium current. Therefore, in neuron B5, FMRFamide-mediated inhibition of a calcium current does not appear to involve phospholipase generation of arachidonic acid.

In summary, in the present study, I have demonstrated that FMRFamide's inhibition of the HVA calcium current is mediated by a PTX-sensitive G protein and that the mammalian α subunit of Go2 can mimic the actions of FMRFamide on neuronal calcium currents.
REFERENCES


GENERAL SUMMARY

FMRFamide is a neuropeptide that is endogenous to the *Helisoma* nervous system (Murphy et al., 1985; Bulloch et al., 1988). The actions of FMRFamide on synaptic transmission were studied using a system of somatic synapses. FMRFamide was found to cause a reduction in the magnitude of the postsynaptic current in response to presynaptic action potentials. The locus of FMRFamide's effects was examined. Long duration (4 seconds) voltage steps were delivered to the presynaptic neuron to evoke desynchronized release of neurotransmitter that was detected as MPSCs. Application of FMRFamide reduced the frequency of these MPSCs probably by a presynaptic locus of action. Postsynaptic responsiveness was also tested by extracellularly applying ACh in the presence and absence of FMRFamide (Zoran et al., 1989). The sensitivity of postsynaptic neurons to ACh remained unchanged in the presence of FMRFamide. Together, these two lines of evidence indicate that the presynaptic terminal is the locus of FMRFamide's inhibitory action.

The system of "giant" synaptic terminals allowed me to examine the subcellular mechanisms of FMRFamide in the presynaptic terminal. Since neurotransmitter release is triggered by the influx of calcium through voltage-dependent channels, I first examined the effect of FMRFamide on the presynaptic calcium current. Voltage clamp studies revealed that the magnitude of the macroscopic HVA calcium current in the presynaptic membrane was reduced in the presence of FMRFamide. This inhibitory effect on calcium channels should result in a reduced influx of calcium into the presynaptic terminal during an action potential and a decrease the amount of transmitter released. Along with the calcium current, there is at least one additional site of action in the presynaptic terminal, the secretory apparatus. This was determined in experiments using a photolabile calcium cage. Presynaptic neurons were held at a constant, elevated level of calcium to trigger secretion of neurotransmitter. Application of FMRFamide to calcium clamped synapses reduced the amount of transmitter released without changing the intracellular calcium levels. Thus, the sensitivity of the secretory machinery to calcium was reduced by FMRFamide. This may be a critical site of modulation in the processes of learning and memory.

I further examined the subcellular mechanisms of FMRFamide on the presynaptic calcium current. FMRFamide has been shown to act through lipoxygenase metabolites of arachidonic acid in *Aphysia* sensory neurons (Piomelli et al., 1987). In *Helisoma* neurons however, neither arachidonic acid nor its metabolites mediate the action of FMRFamide on the calcium current.
A pertussis toxin-sensitive G protein was found to be involved in the inhibition of the calcium current from studies using guanine nucleotide analogues and pertussis toxin. Nonhydrolyzable analogues of GTP caused FMRFamide to irreversibly reduce the calcium current magnitude and the analogue of GDP, GDPβS, prevented FMRFamide from exerting its effect. Microinjection of pertussis toxin also prevented FMRFamide from having its effects on the current. Purified α-subunits from Go1, Go2, and Gi3 were microinjected into presynaptic somata to determine which were able to inhibit the calcium channels. Only the α-subunit of Go2 reduced the magnitude of the calcium current and inhibited the same population of calcium channels that were susceptible to the effects of FMRFamide.
GENERAL REFERENCES


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