PEPTIDERGIC REGULATION OF CHROMATOPHORE FUNCTION IN THE EUROPEAN CUTTLEFISH *SEPIA OFFICINALIS*

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Summary

Color patterning in cephalopod molluscs involves activation of a peripheral chromatophore system that is under neuromuscular control. The complex behavior of individual chromatophores is mediated by a specific set of muscles, the chromatophore muscles, that receive direct innervation from the central nervous system. To date, glutamate is the only excitatory transmitter that has been proposed to act at the chromatophore neuromuscular junction of cephalopods. We present data demonstrating that the chromatophore muscles in the European cuttlefish *Sepia officinalis* are also regulated by the FMRFamide family of neuropeptides. Using an *in vitro* chromatophore bioassay, it has been determined that several FMRFamide-related peptides (FaRPs) are potent excitors of the chromatophore muscles, causing chromatophore expansion. Immunocytochemical analyses of the central nervous system using an FMRFamide antibody revealed the presence of FMRFamide-like immunoreactive cell bodies in the posterior chromatophore lobes, the region of the brain containing the chromatophore motoneurons of the fin and mantle. FMRFamide-like immunoreactivity was also seen in the periphery, in the nerves around the chromatophores and in close apposition to the muscles in the chromatophore layer of the fin. HPLC analysis of the fin dermis isolated four bioactive peaks that were FMRFamide-immunoreactive when tested on an immunoblot assay. Two of these peaks co-eluted with known FaRPs, FMRFamide and ALSGDAFLRFamide, a decapeptide isolated from squid. Taken together, these data suggest that the FaRPs are likely to be endogenous excitors of the chromatophore muscles in cephalopods.

Key words: cephalopod, cuttlefish, *Sepia officinalis*, FMRFamide, FMRFamide-related peptides, chromatophores, chromatophore muscle.

Introduction

Many organisms alter body color in response to environmental cues, but none matches the coloration changes associated with body patterning behavior in cephalopods. Cephalopods, especially cuttlefish, produce numerous body patterns noted for their intricacy and speed of formation (Holmes, 1940; Hanlon and Messenger, 1988). These patterns may be static or dynamic; one of the most intriguing and complex is the 'passing clouds' display, a series of narrow, lateral stripes across the dorsal surface of the mantle that appear to move anteriorly towards the head and down the arms (Hanlon and Messenger, 1988). Although postural and textural elements are important features of many body patterns, the predominant component is chromatic coloration. Cephalopod coloration is generated by the retraction, expansion and/or pulsation of millions of chromatophores that, unlike those of other organisms, are under direct neuromuscular control (Florey, 1966; Cloney and Florey, 1968; Florey and Kriebel, 1969).

Each chromatophore is a multicellular organ, consisting of a central pigment-containing cell attached to a set of 6–20 radially arrayed chromatophore muscles (Fig. 1; Cloney and Florey, 1968). These muscles are innervated by motoneurons whose cell bodies are thought to be centrally located in the chromatophore lobes of the cephalopod brain (Sereni and Young, 1932; Boycott, 1953, 1961; Dubas *et al.* 1986). Expansion or retraction of each pigment-containing cell occurs as a result of the contraction or relaxation of its chromatophore muscles, which are capable of producing either phasic or tonic contractions. Although the complex nature of cephalopod patterning has been reported for many years (see Holmes, 1940), little is known about the underlying neuronal mechanisms. As a first step towards understanding how cephalopod body patterns are generated, this study focuses on the regulation of individual chromatophores and, in particular, on the identification of the endogenous neurotransmitters involved in mediating neuronal signalling at the chromatophore neuromuscular junction.

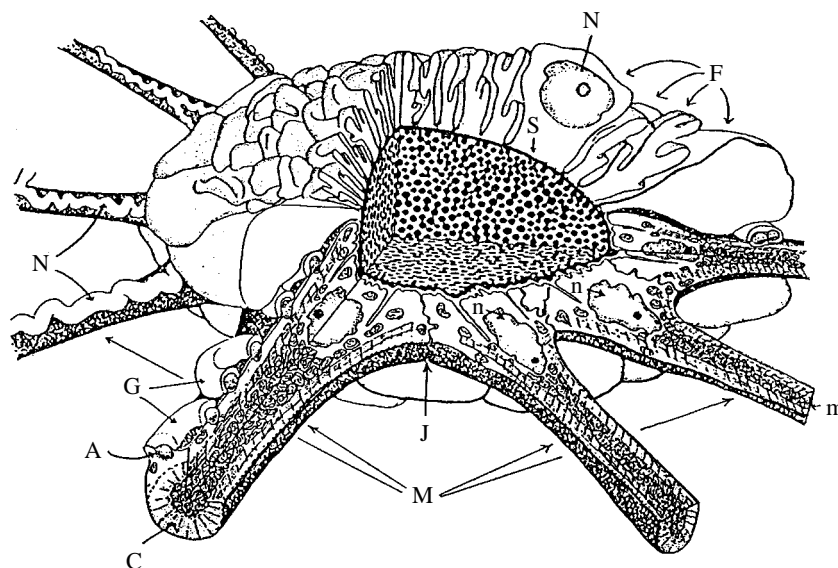Studies on squid chromatophores have identified glutamate

Fig. 1. Diagram of the ultrastructure of a retracted cephalopod chromatophore organ. A, axon; C, contractile cortex of muscle fiber; F, folds of cell membrane of chromatophore; G, glial cell; N, nerve terminals; n, nucleus of muscle cell; M, muscle fibers; m, mitochondria; J, junction between adjacent muscle fibers; S, elastic sacculus. The sheath cells that cover the chromatophore and the muscle fibers are not shown (slightly modified from Cloney and Florey, 1968).

as a putative excitatory transmitter at the chromatophore neuromuscular junction (Bone and Howarth, 1980; Florey *et al.* 1985; Messenger *et al.* 1991; Cornwell and Messenger, 1995). However, glutamate alone does not account for the complex physiological repertoire of the chromatophore muscles, suggesting the presence of other transmitters in this system. This study addresses this issue by identifying one family of endogenous neuropeptides that directly modulate chromatophore function. Using bioassay, immunocytochemical and biochemical techniques, we show that FMRFamide-related peptides (FaRPs) are present and functionally active at the chromatophore neuromuscular junction in the European cuttlefish *Sepia officinalis*. On the basis of these data, we propose that the FaRP family of neuropeptides performs an important physiological role in mediating color patterning behavior in *S. officinalis*.

Materials and methods

Animals

Cuttlefish *Sepia officinalis* were purchased from the National Resource Center for Cephalopods, Marine Biomedical Institute, Galveston, TX, USA. Animals were maintained in two 474 l recirculating tanks containing artificial salt water (ASW). The tanks were housed in an environmentally controlled chamber at 20 °C with a photoperiod of 13 h:11 h L:D. Both tanks were equipped with protein skimmers and wet/dry filters containing activated carbon to hold ammonia and nitrite concentrations below detectable levels. Nitrate levels were monitored and controlled using weekly water changes. Bacterial contamination was eliminated by an in-line ultraviolet sterilizer (150 W, Erwin Sander, Osterberg, Uetze-Eltze, Germany). Individual cuttlefish used in this study were maintained for up to 10 months and fed freshly thawed shrimp and live freshwater fish. Occasionally, the diet was supplemented with chloramphenicol sodium succinate as a preventitive measure against bacterial infection.

In vitro *chromatophore bioassay*

An *in vitro* chromatophore bioassay, modified from Florey and Kriebel (1969), was used to identify putative neurotransmitters in the chromatophore system. The bioassay consisted of an isolated 1 cm^2 piece of swimming fin removed from an adult cuttlefish that had been anesthetized by immersion in 0.1 % ethanol in ASW for at least 5 min prior to surgery. The ventral dermal layer of the isolated fin patch containing chromatophores was surgically separated from the fin musculature and epidermis, and pinned onto a thin layer of Sylgard (Dow Corning) in a Plexiglas flow chamber. The volume of the chamber was adjustable and averaged about 100 μl. The flow chamber was placed on the stage of an Olympus CK-2 inverted microscope and adjusted manually to center a single chromatophore in the field of view. Of the three types of pigments found in cephalopod chromatophores (orange, yellow and dark brown), only dark-brown chromatophores were analyzed in this study.

Changes in the size of individual chromatophores were monitored quantitatively using a photocell set in the optical pathway of the microscope. The photocell output was inversely proportional to the size of the chromatophore in the field of view: chromatophore expansion caused a decrease in photocell output, whereas the output increased when the chromatophore retracted. The output of the photocell was recorded onto a chart recorder (Gould 2200s) and on an FM tape recorder (Vetter model 2) for later analysis.

Unstimulated chromatophores in the isolated patch of skin generally remained fully condensed and quiescent for the duration of the bioassay, which often lasted 6 h or longer. Bioassays were performed at room temperature and preparations were continuously perfused with ASW at a rate of approximately 120 ml h^{-1} except during the application of test substances, when perfusion was stopped. Starting or stopping the perfusion for up to 30 min had no effect on the responsiveness or the sensitivity of the bioassay. Because this study focused on the identification of putative excitatory

transmitters, test substances were analyzed for their ability to cause chromatophore expansion from an initial, fully retracted condition.

To validate chromatophore viability, each bioassay began with a 2 min pulse of 10^{-3} mol l^{-1} glutamate, a suprathreshold concentration for chromatophore expansion. The experiment was continued only if the chromatophore responded to glutamate stimulation by expanding. Prior to the application of any test substance, the ASW flow was stopped and the chamber was rapidly rinsed three times with 100 μl of the test solution to rinse out the ASW in the chamber. Immediately following the rinses, 100 μl of the test solution was added to the chamber for 10 min. After the incubation period, the ASW flow was reinitiated to allow wash-out of the test solution. The next sample was applied either at least 10 min after the wash-out had started or 2 min after the chromatophore had returned to its resting (i.e. fully retracted) state, whichever was longer. All test substances (see Table 1) were applied in increasing molar concentrations, usually ranging from 10^{-6} to 10^{-3} mol l^{-1}, except when threshold was lower than 10^{-6} mol l^{-1}, in which case lower concentrations were tested. If the test substance failed to cause chromatophore expansion, the chromatophore was again challenged with a 2 min application of 10^{-3} mol l^{-1} glutamate to ensure continued viability.

All non-peptide test substances were purchased from Sigma Chemical Company and all peptides were obtained from Bachem Corporation, with the exception of crustacean cardioacceleratory peptide (CCAP; American Chemical Co.), buccalin, small cardioactive peptide A (SCP$_a$), myomodulin (Peninsular Labs), and FLRFamide and ALSGDAFLRFamide (Research Genetics). All test substances were hydrated and diluted with ASW, or diluted in ASW from stock solutions. Stock solutions of 10^{-1} mol l^{-1} dopamine and 10^{-2} mol l^{-1} α-melanocyte stimulating hormone (α-MSH) were made with 10^{-3} mol l^{-1} acetic acid, 10^{-2} mol l^{-1} melatonin with 10 % ethanol in ASW, and 10^{-3} mol l^{-1} red pigment concentrating hormone (RPCH) in 2 % (v/v) dimethyl sulfoxide (DMSO). Applications of ASW, acetic acid, ethanol or DMSO at their respective working concentrations were without effect on the chromatophore bioassay.

Immunocytochemistry

Immunocytochemistry was performed using a polyclonal antibody raised against synthetic FMRFamide and generously donated by Dr Paul Taghert. The specificity and technical details concerning its production have been described elsewhere (Taghert and Schneider, 1990).

Immunostaining was performed on brains from 12 adult *S. officinalis* to identify neurons expressing FMRFamide-like immunoreactivity (FLI). Adults were anesthetized with 0.5 % ethanol for at least 5 min before decapitation. Following decapitation, tissues surrounding the cranial cartilage were removed. The brain with the undisturbed cranial cartilage was fixed in 4 % paraformaldehyde for at least 2 days. Fixed brains were placed in 20 % sucrose for an additional 2 days prior to sectioning. Each brain was frozen and serially sectioned on a cryostat at a section thickness of 30 μm, and sections were mounted on subbed slides.

Peripheral tissues were analyzed immunocytochemically using small pieces of fin removed from six adults anesthetized in 0.1 % ethanol and fixed as described above for a minimum of 2 days. To study the morphology of nerve distribution, 50–100 μm thick sections of the fin were obtained using a vibratome. Sections were collected in 0.1 mol l^{-1} phosphate-buffered saline (PBS). Immunostaining was performed on free-floating sections. To investigate finer structures, fixed pieces of fin were placed in 20 % sucrose for 1 day before cryostat sectioning at a thickness of 30 μm. Sections were mounted on subbed slides and immunostained as described below.

Standard antibody staining protocols using the biotin/avidin system from Vector Laboratories were used for all FMRFamide immunostaining (Clausen *et al.* 1991). After sectioning, tissues were briefly rinsed in PBS with 3 % Triton-X and incubated in normal goat serum (1:100 PBS with 3 % Triton-X) for 1–3 h to block non-specific binding by the primary antibody. The blocking solution was replaced with the primary antibody at a concentration of 1:2000 (PBS with 3 % Triton-X) and 1:1000 for sections with counterstaining. Sections were incubated in the diluted primary antibody overnight and washed three times in PBS with 3 % Triton-X prior to the application of the biotinylated anti-rabbit secondary antibody (ABC Kit, Vector Labs) at a concentration of 1:200 (PBS with 3 % Triton-X) for 1–2 h. After three washes in PBS without Triton-X, a solution of biotin/avidin complex was added for 1–2 h. After an additional three washes in PBS, the reaction product was visualized using diaminobenzadine and standard visualization protocols. Immunostained sections were dehydrated and mounted in Permount. All immunostaining procedures were carried out at room temperature (23 °C) and with gentle agitation using a rotary shaker.

Extraction and biochemical analyses of FaRPs

The ventral dermis of the right and left fin were dissected from a single adult *S. officinalis* and heat-treated at 80 °C for 10 min in 4 vols of 0.1 % acetic acid in a glass tissue homogenizer. Following heat treatment, the tissue grinder with its contents was immediately chilled on ice and the fin was homogenized. The homogenate was transferred to microfuge tubes and centrifuged at high speed (14 000 g) in a microcentrifuge. The resultant supernatant was loaded onto a pre-activated C-18 Sep-pak cartridge (Waters), which was then sequentially washed with water, 10 % acetonitrile (MeCN) and 60 % MeCN. All chromatophore-stimulating activity was found in the 60 % MeCN fractions, which were lyophilized, resuspended and loaded onto a second Sep-pak cartridge. This second Sep-pak cartridge was sequentially treated with water, 15 % MeCN and 40 % MeCN. The 40 % fractions were lyophilized and frozen for HPLC analyses.

The 40 % fractions from the second Sep-pak step were rehydrated in 20 % MeCN containing 0.1 % heptafluorobutyric acid (HFBA) and subjected to a three-step HPLC purification protocol. The first HPLC step used a 2.1 mm (i.d.), 220 mm

column (C-18 Aquapore OD300, Brownlee) installed on an Isco HPLC (model 2350). The solvents used were water and MeCN with 0.1 % HFBA as the counterion and the flow rate was $500\,\mu l\,min^{-1}$. This first HPLC step used a three-segment program, an initial isocratic segment (20 % MeCN for 5 min) followed by shallow (an increase in MeN of 0.5 % min^{-1} for 30 min) and steep (1.5 % min^{-1} increase for 10 min) gradient segments. Fractions were collected at 1 min intervals and samples bioassayed on the *in vitro* chromatophore assay to identify fractions that contained chromatophore-expanding bioactivity. Bioactive peaks were separated and individually rechromatographed a second time using the same hardware and conditions as in step 1, with the exception that 0.1 % trifluoroacetic acid (TFA) was used as the counterion. A similar three-segment chromatography program was utilized, with an initial isocratic step (10 % MeCN for 5 min) followed by shallow (1 % min^{-1} increase for 50 min) and steep (8 % min^{-1} increase for 5 min) MeCN gradient segments. Fractions from the second HPLC step were collected and bioassayed as described for the first HPLC step. Bioactive peaks were individually rechromatographed on a syringe pump HPLC (Applied Biosystems model 130A), using a 1.0 mm i.d., 250 mm column (C-18, Aquapore 300, Brownlee Corp.), a flow rate of $100\,\mu l\,min^{-1}$, and the same solvents and counterion as in the second HPLC step. The three-segment run on the syringe pump consisted of an initial isocratic segment (18 % MeCN for 5 min) followed by two MeCN gradient segments (A, 0.33 % min^{-1} increase for 60 min; B, 2.8 % min^{-1} increase for 15 min). Fractions from this run were collected at 1 min intervals, divided into samples, lyophilized, frozen and stored for subsequent bioassay and immunoblot analyses.

FaRP immunoblot analyses

A quantitative immunoblot assay was developed to analyze FaRP-containing fractions (Salzet *et al.* 1993). Lyophilized samples from the final HPLC step were rehydrated in $5\,\mu l$ of double-distilled water. $1\,\mu l$ of each fraction was blotted onto a $0.22\,\mu m$ nitrocellulose membrane (Micron Separation Inc.) and baked for 30 min at 100 °C (Li *et al.* 1988). The membrane was washed in $0.1\,mol\,l^{-1}$ Tris-buffered saline containing 0.05 % Tween 20 (v/v; pH 7.4). Prior to incubation with the primary antibody, the membrane was treated with 3 % bovine serum albumin (BSA) in $0.1\,mol\,l^{-1}$ Tris buffer (pH 7.4) for 1 h. Following removal of the BSA-containing solution, the primary FMRFamide antibody was added at a concentration of 1:30 000 and the membranes were left to incubate overnight. Visualization of the primary antibody was accomplished using a standard avidin/biotin protocol (Peroxidase ABC kit, Vector Labs) using $0.1\,mol\,l^{-1}$ Tris containing 0.05 % Tween 20 and True Blue (Kirkegaard and Perry Labs) as the substrate. All steps were carried out at room temperature using a rotary shaker for gentle agitation. Positive and negative controls were run in tandem with all replicates using synthetic FMRFamide and SCP$_a$, respectively. Immunoblots were visually quantified using arbitrary units of 0–4, where 0 was no visible staining, 1 was the faintest visible staining and 4 was the darkest spot

for any individual trial. Staining of positive control stripes with 0.1, 1.0 and 10 ng of FMRFamide was used to normalize inter-trial differences by arbitrarily scoring them as 1, 2 and 3, respectively. SCP$_a$ produced no visible spots on this assay in amounts up to 10 ng.

Results

Effect of various substances on chromatophore expansion

As a first screen to identify the neuronal factors that may be involved in the regulation of chromatophore activity, various neuronally derived substances were applied to the *in vitro* chromatophore bioassay. The assay consisted of applying test substances while monitoring the size of an individual dark-brown chromatophore. Because the focus of this study was to identify factors that induce chromatophore expansion at the chromatophore neuromuscular junction, each chromatophore studied was always initially in a retracted form, i.e. the chromatophore muscles were fully relaxed and the diameter of the pigment-containing chromatophore cell was at a minimum.

The substances tested included classical transmitters, neuropeptides that affect chromatophores in other organisms and invertebrate myoactive neuropeptides (Table 1). Glutamate was the only classical transmitter effective in triggering chromatophore expansion (Table 1). The response to glutamate always consisted of rapid and complete chromatophore expansion occurring within seconds after application (Fig. 2A). The delay between bath application and the response was probably due to the time necessary for glutamate to gain access to the chromatophore muscles. Glutamate wash-out with ASW resulted in the retraction of the chromatophore within a few minutes to its initial pre-test size. The threshold concentration for glutamate-mediated expansion was between 10^{-4} and $10^{-3}\,mol\,l^{-1}$. This relatively high threshold was probably the result of glutamate uptake by other cells in the preparation. All effective doses of glutamate produced full expansion of the chromatophore which was completely and rapidly reversible. Little or no desensitization was observed, even when suprathreshold concentrations were applied for up to 10 min (Fig. 2A).

Besides glutamate, many of the neuropeptides tested also caused chromatophore expansion (Table 1). Myomodulin, α-MSH, buccalin, SCP$_a$ and five FaRPs, including FMRFamide, all elicited chromatophore expansion on the *in vitro* bioassay. As a group, the threshold concentrations ranged between 10^{-4} and $10^{-9}\,mol\,l^{-1}$, with the FaRPs having the lowest thresholds (Table 1). The delay between peptide application and the chromatophore response was usually about 1 min or longer (e.g. Fig. 2Bi), a period substantially greater than that measured for glutamate. Application of some peptides at threshold concentrations resulted in a delayed response taking up to 2 min; the delay was substantially reduced at higher concentrations. As with glutamate, all effective peptides produced maximal chromatophore expansion at all suprathreshold concentrations. However, unlike glutamate,

Table 1. *The effects of various neuronal factors on the* in vitro Sepia officinalis *chromatophore bioassay*

Neuronal factors tested on the *in vitro* chromatophore bioassay	Response	Lowest effective concentration $(mol\,l^{-1})$
Classical neurotransmitters		
GABA	–	–
Dopamine	–	–
Acetylcholine	–	–
Serotonin	–	–
Octopamine	–	–
Glutamate	+	10^{-3}
Neuropeptides that affect chromatophore function in other organisms		
Red pigment concentrating hormone (RPCH)	–	–
Melatonin	–	–
α-Melanocyte stimulating hormone (α-MSH)	+	10^{-4}
Invertebrate myoactive neuropeptides		
Crustacean cardioacceleratory peptide (CCAP; Cheung *et al.* 1992)	–	–
Proctolin (Hui *et al.* 1985)	–	–
Buccalin (Cropper *et al.* 1988)	+ (irreversible)	10^{-4}
Small cardioacceleratory peptide A (SCP$_a$) (Lloyd *et al.* 1987)	+	10^{-4}
Myomodulin (Cropper *et al.* 1987)	+	10^{-4}
FMRFamide (Price and Greenberg, 1977)	+	10^{-7}
FLRFamide (Lesser and Greenberg, 1993)	+	10^{-9}
pGDPFLRFamide (Price *et al.* 1987)	+	10^{-6}
TNRNFLRFamide (Peptide F1) (Trimmer *et al.* 1987)	+	10^{-8}
ALSGDAFLRFamide (Price *et al.* 1994)	+	10^{-8}

many of the peptide responses required much more time for the chromatophore muscles to return fully to their pre-test relaxation state. For example, chromatophores expanded by TNRNFLRFamide application took up to 4 min to return to their initial resting state (Fig. 2Bi). All peptide-induced effects were reversible, with the exception of the effects of buccalin which remained even after 2 h of perfusion with ASW. Peptide-mediated desensitization was occasionally observed, especially after repeated applications (Fig. 2Bii).

Although neurotransmitters such as serotonin and acetylcholine (Fig. 2C) did not cause chromatophore expansion, there is some circumstantial evidence suggesting that they inhibit excitation of the chromatophore muscles (Florey and Kriebel, 1969). A longer wash-out period or higher concentration of glutamate was needed to cause chromatophore expansion immediately after serotonin or acetylcholine application. Occasionally, chromatophores in their normal resting state retracted even further in response to serotonin application.

FMRFamide-like immunoreactivity (FLI) in chromatophore motoneurons

The bioassay results suggested that several neuropeptides may be involved in controlling chromatophore activity. Because the most potent response was seen with FMRFamide and its relatives, the FaRPs, we focused our attention on the FaRPs to pursue the possibility that they are endogenous activators of the chromatophore muscles in *S. officinalis*. This
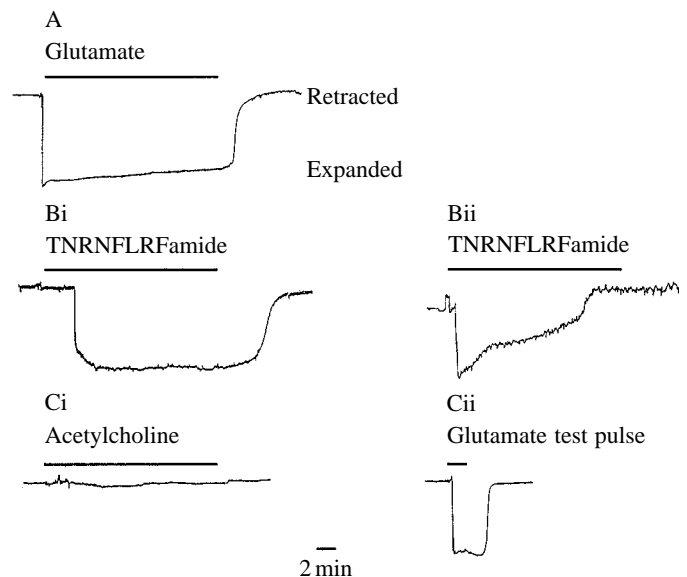

Fig. 2. The effect of glutamate, acetylcholine and several FMRFamide-related peptides (FaRPs) on the *in vitro* chromatophore bioassay. (A) $10^{-3}\,mol\,l^{-1}$ glutamate; (Bi) $10^{-6}\,mol\,l^{-1}$ TNRNFLRFamide; (Bii) $10^{-5}\,mol\,l^{-1}$ TNRNFLRFamide, after repeated stimulations with TNRNFLRFamide; (C) $10^{-3}\,mol\,l^{-1}$ acetylcholine (Ci) followed by $10^{-3}\,mol\,l^{-1}$ glutamate (Cii). Note that the isolated chromatophore routinely responded more rapidly to glutamate application than to any of the neuropeptides tested. The bar above each trace indicates the period of transmitter application. All applications were for 10 min except for Cii (2 min).
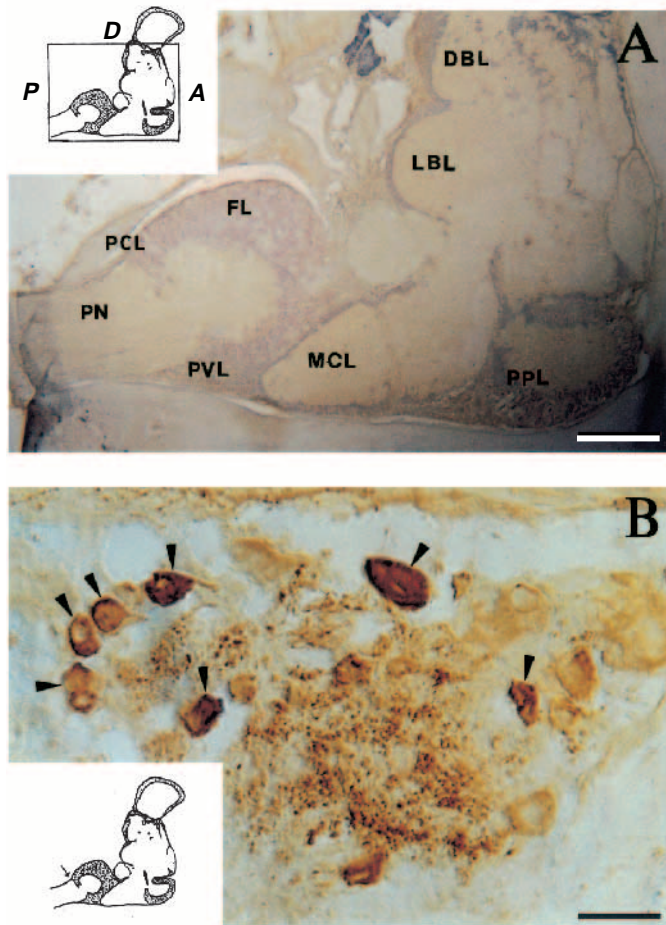
Fig. 3. FMRFamide-related peptide (FaRP) staining in the brain of *Sepia officinalis*. (A) Low-magnification lateral sagittal section of the brain from an adult male cuttlefish. The section has been counterstained with Nissl stain. (B) FaRP-positive cells in the chromatophore lobe from a non-counterstained preparation visualized with Nomarski optics. Arrowheads point to FaRP-positive cell bodies. Insets: schematic diagrams of a lateral sagittal section of the *Sepia officinalis* brain. The relative location of each photograph is indicated by a box (A) or arrow (B). A, anterior; D, dorsal; DBL, dorsal basal lobe; FL, fin lobe; LBL, lateral basal lobe; MCL, magnocellular lobe; P, posterior; PCL, posterior chromatophore lobe, PN, pallial nerve; PPL, posterior pedal lobe; PVL, palliovisceral lobe. Scale bars, 1.2 mm (A) and 50 μm (B).

issue was explored using a polyclonal FMRFamide antibody to determine whether FLI was present in the cell bodies and terminals of the motoneurons innervating the chromatophore muscles.

Neurons expressing FLI were localized to the posterior chromatophore lobes (PCL; Fig. 3A), the location of the somata of motoneurons innervating the chromatophore muscles in the fin and mantle (Boycott, 1953, 1961; Dubas *et al.* 1986). The PCL is part of the posterior subesophageal mass, a region that also includes the visceral and fin lobes (Boycott, 1961; Young, 1976). The PCL lies directly dorsolateral and adjacent to the pallial nerve, which is thought to include axons

of PCL chromatophore motoneurons (Boycott, 1961; Young, 1976; Fig. 3A). We estimate that the PCL in adult *S. officinalis* contains approximately 30 000 cells, a number that is in good agreement with other published figures (Hanlon and Messenger, 1988). The PCL contains cell bodies of several size classes, ranging from 20 μm to over 60 μm.

FLI in the PCL was restricted to a small subset of somata found in the most posteriolateral region, immediately adjacent to the pallial nerve (Fig. 3B). All somata containing FLI were approximately 20 μm in diameter, and about 20 % of PCL cells in this size class contained FLI. None of the larger PCL cells exhibited FLI. FMRFamide-immunoreactive cells with a soma diameter of 20 μm were also observed in the most posterior region of the palliovisceral lobe immediately ventral to the pallial nerve. Together with most of the FMRFamide-immunoreactive cells in the PCL, they form a cuff of FMRFamide-immunoreactive somata around the pallial nerve. FMRFamide-immunoreactive somata in the PCL not associated with this cuff were evenly dispersed among the other smaller PCL cells in this region.

Strong FLI also was observed in other regions of the *S. officinalis* brain, including the optic lobes, the dorsal basal and inferior frontal lobes of the supraesophageal mass and the posterior pedal, palliovisceral and magnocellular lobes of the subesophageal mass (data not shown). One brain region of particular interest was the anterior chromatophore lobe (ACL), the presumptive site of chromatophore neurons innervating the chromatophores on the head and arms. Unfortunately, variable staining across trials precluded any definitive conclusion about the presence or absence of FMRFamide-immunoreactive neurons in the ACL.

In addition to the FLI in the PCL and other regions of the central nervous system (CNS), FLI was also observed in the periphery. A dense meshwork of FMRFamide-immunoreactive fibers was found throughout the dermal chromatophore layer (Fig. 4A). Many FMRFamide-immunoreactive fibers appeared to traverse across or along the length of individual chromatophore muscles (Fig. 5A,B). FLI in peripheral nerves was punctate in appearance, particularly when the nerve was in close apposition to the chromatophore muscle (Fig. 5B). FLI in both the periphery and the CNS was completely absent when the primary antibody was omitted or preincubated in 10^{-4} mol l^{-1} FMRFamide or FaRPs (Fig. 4B). Pre-incubation of the primary antibody with SCP$_a$ had no apparent effect on the distribution or intensity of the staining.

Biochemical analyses of FaRPs in the *Sepia officinalis* fin

The results from the bioassay and immunocytochemical experiments suggested the presence of an FaRP-like factor(s) in the nerves innervating the chromatophore muscles. This hypothesis was explored experimentally using biochemical techniques to isolate and partially characterize FaRPs from the dermis. The chromatophore layer of the fin from an adult *S. officinalis* was dissected and extracted for FaRPs using the protocol described in the Materials and methods section. The fin extract was passed through several preliminary purification
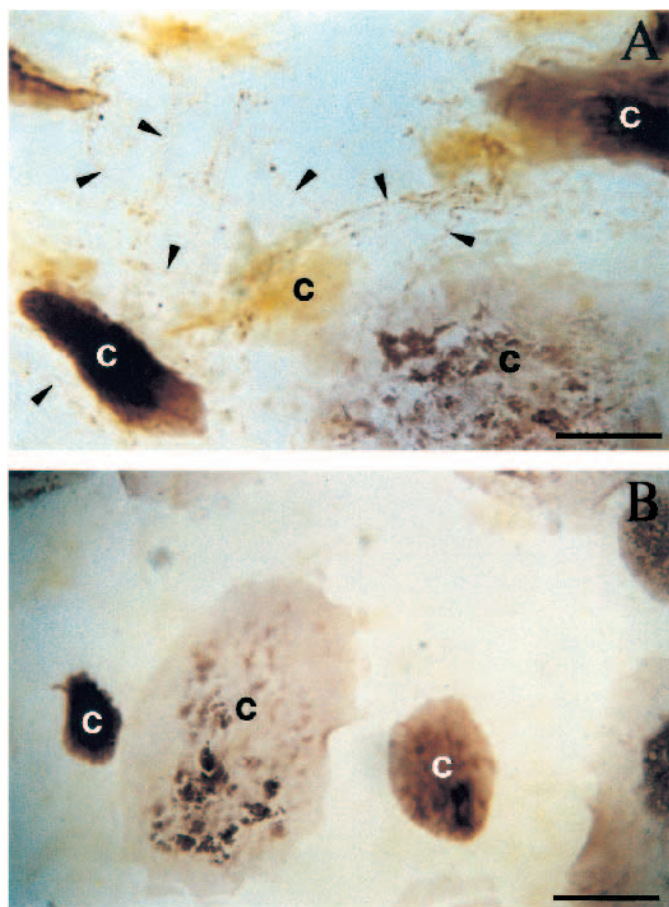
Fig. 4. FMRFamide-related peptide (FaRP) immunostaining in peripheral nerves innervating the chromatophore layer of the dermis of *Sepia officinalis*. Sections were cut horizontally and incubated with (A) or without (B) primary antibody. Arrowheads point to FaRP-immunopositive nerves. c, chromatophores. Scale bars, 50 μm.
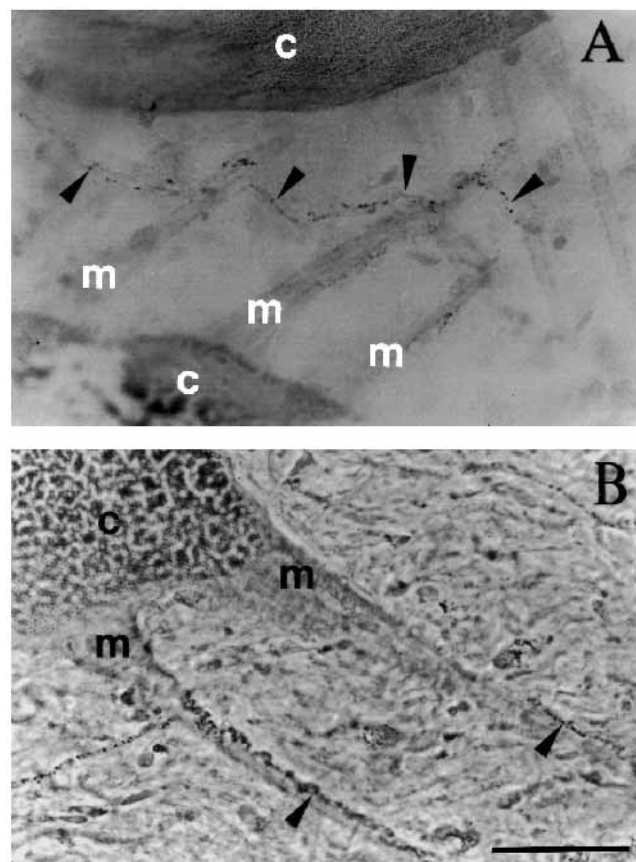

Fig. 5. FMRFamide-related peptide (FaRP) immunostaining in nerves associated with the chromatophore muscles of *Sepia officinalis*. (A) FaRP-stained nerve innervating several muscles associated with a single chromatophore. (B) Immunopositive nerves running adjacent to the chromatophore muscles. Arrowheads point to FaRP-immunopositive nerves. m, muscle; c, chromatophores. Scale bar, 50 μm.

steps using a low-pressure C-18 cartridge (Sep-pak) followed by a three-step HPLC chromatography procedure. Bioactivity, defined as the ability to produce chromatophore expansion, was followed at each step in the purification process using the *in vitro* chromatophore bioassay.

The first HPLC step revealed five distinct peaks of chromatophore-expanding bioactivity (peaks I–V; Fig. 6A). Each peak was individually rechromatographed on two subsequent HPLC steps (Figs 6B–F, 7A–E). Two of the original peaks, II and IV, split into two peaks each; peak IV split into peaks IVa and IVb at the second HPLC step (Fig. 6E), and peak II split into peaks IIa and IIb at the third HPLC step (Fig. 7B). The elution times of all peaks on the final HPLC step were compared with those of synthetic FMRFamide, several other FaRPs and SCP$_a$ (Fig. 7). Peak I co-eluted with FMRFamide and peak IVb with a FaRP decapeptide isolated from squid (ALSGDAFLRFamide; Price *et al.* 1994). Bioactivity associated with peaks IVa and V was not detected after the third HPLC step.

Because of the possibility that the bioactivity associated with these peaks was due to a component other than FaRPs, each fraction from the third HPLC step was assayed for the presence of FaRPs using a highly sensitive FaRP dot immunoblot assay. Six peaks (I, IIa, IIb, IVa, IVb and V) were immunopositive on both immunoassay replicates, implying that these peaks contained FaRP-like motifs (Fig. 7A–E). The FaRP-like nature of peak III remains equivocal since it was immunopositive in only one of the two of the immunoassays.

The HPLC and immunoblot data taken together indicate that *S. officinalis* contains more than one FaRP. Our results are most conclusive for peaks I and IVb, which may be identical or closely related to FMRFamide and the squid FaRP decapeptide ALSGDAFLRFamide, respectively. This conclusion is further supported by preliminary molecular data (Loi and Tublitz, 1995) demonstrating the presence of three FaRPs in *S. officinalis*: FMRFamide, a 10-residue FaRP in *S. officinalis* with the same primary structure as the squid decapeptide (Price *et al.* 1994), and FLRFamide. The remaining five peaks may also be FaRPs on the basis of immunoblot assays. However, confirmation of the nature of any of these peaks will not be definitive until their primary structure has been determined.
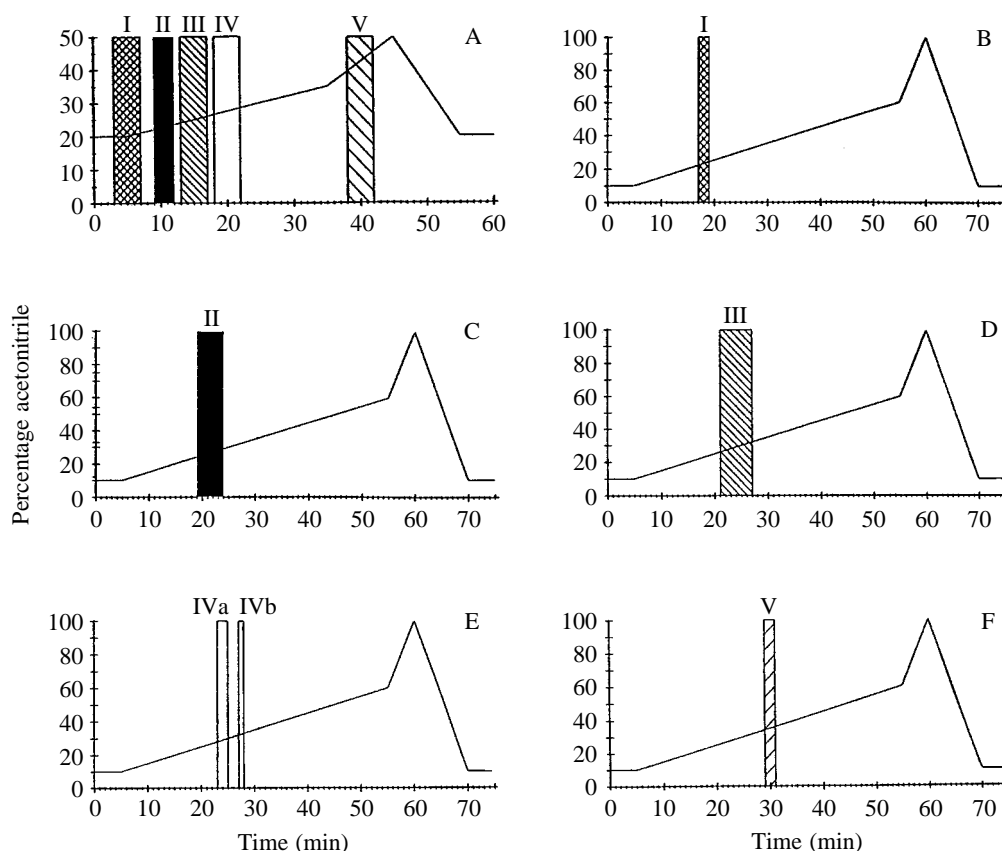
Fig. 6. Biochemical analyses of chromatophore-expanding bioactivity from the dermis of an adult *Sepia officinalis*. (A) Results from the first HPLC step showing the presence of five bioactive peaks (I–V). (B–F) Results from the second HPLC step showing the elution profiles of each of the five peaks. The bar(s) in each histogram represent the elution time of the bioactivity associated with each peak and do not represent quantification of bioactivity. The superimposed line graph illustrates the gradient profile for that particular HPLC run.

Discussion

FaRPs are putative excitatory transmitters at the chromatophore neuromuscular junction

The primary objective of this study was to determine the identity of some of the endogenous factors that act as excitatory neurotransmitters at the chromatophore neuromuscular junction. The data in this paper strongly implicate FaRPs as likely candidates since they fulfill three of the four standard criteria used to identify neurotransmitters (Jan and Jan, 1982): (1) all FaRPs tested caused chromatophore expansion when applied to the *in vitro* chromatophore bioassay (Table 1); (2) FLI was observed in the cell bodies (Fig. 3) and peripheral processes (Figs 4, 5) of putative chromatophore motoneurons; and (3) biochemical and immunological analyses of the chromatophore layer of the dermis yielded at least two factors that co-elute with known FaRPs and are FaRP-immunopositive (Figs 6, 7). Thus, we have demonstrated that FaRPs are present in *S. officinalis*, are localized to putative chromatophore motoneurons and have an excitatory effect on the chromatophore muscles. Although this evidence clearly indicates a role for FaRPs in controlling chromatophore expansion, we hesitate to label these substances unequivocally as neurotransmitters until the fourth criterion is satisfied, i.e. that these factors are released from the chromatophore motoneurons in a Ca^{2+}-dependent fashion, and will therefore refer to them as putative neurotransmitters.

The finding that *S. officinalis* contains several FaRPs is in agreement with previous immunocytochemical, biochemical and molecular studies in *S. officinalis* and in other cephalopods. FLI in the cephalopod brain has been reported in the dorsal basal and olfactory lobes of cuttlefish *Sepia officinalis* (Le Gall *et al.* 1988) and in the optic lobes of octopus *Octopus vulgaris* (Martin and Voigt, 1987). FLI has also been observed peripherally in the neuropil of the neurosecretory system of the octopus vena cava (Martin and Voigt, 1987). Several FaRPs, including FMRFamide, FLRFamide and two pentapeptides, have been biochemically isolated from the optic lobes of octopus (Martin and Voigt, 1987). That FaRPs perform a physiological role in cephalopods is further supported by a report of the presence of FMRFamide receptors in the optic lobes of the squid *Loligo pealei* (Chin *et al.* 1994). Moreover, preliminary molecular results in squid indicate the presence of at least one and possibly two FaRP-coding genes (Price *et al.* 1994). Similar studies in *S. officinalis* using the polymerase chain reaction have also isolated an FaRP-coding cDNA fragment from a brain cDNA library (Loi and Tublitz, 1995). Thus, there is ample evidence to support the hypothesis that the cephalopod CNS, like that of other molluscs, contains one or more FaRPs.

Chromatophore function is regulated by multiple transmitters
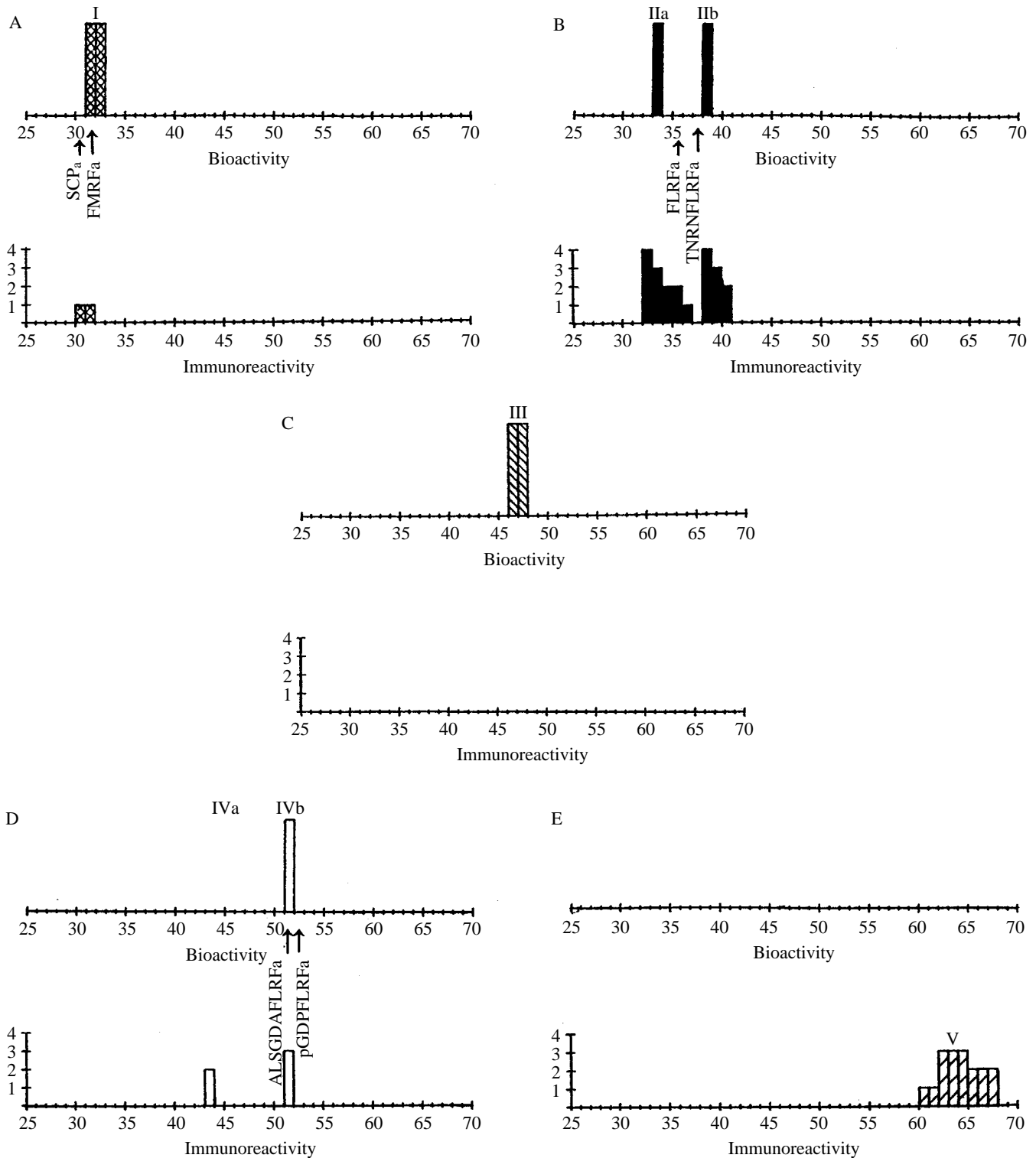
The complicated behavior of an individual cephalopod

Fig. 7. Bioassay and dot immunoblot results following the final (third) HPLC step. (A–E) Each peak (I–V) was separately chromatographed using conditions detailed in the Materials and Methods section. The resultant fractions were assayed for both bioactivity and FMRFamide-related peptide (FaRP) immunoreactivity. The bioactivity histograms refer only to elution time and do not represent quantification of bioactivity. Bar heights for the immunoassays represent staining intensity on the FaRP immunoblot assay on an arbitrary scale of 0–4, where 0 is no staining and 4 is the darkest staining. Arrows indicate the elution times of various synthetic peptides. Bioactivity for peaks IVa and V was undetectable in the third HPLC step.

chromatophore suggests an equally complex neuronal regulation. One possibility is that this complexity is mediated at the chromatophore neuromuscular junction by more than one neurotransmitter, and this hypothesis is supported by the results of the present study. Prior to this report, the only excitatory neurotransmitter thought to be active at the cephalopod neuromuscular junction and for which there was any empirical evidence was glutamate. The evidence for the involvement of glutamate at the chromatophore neuromuscular junction is based on pharmacological studies in which application of glutamate to squid chromatophores caused chromatophore expansion (Florey *et al.* 1985; Messenger *et al.* 1991; Cornwell and Messenger, 1995). The same authors also showed that chromatophore expansion induced by nerve stimulation was partially blocked by glutamate receptor antagonists. The bioassay data on glutamate presented here lend additional support to the contention that glutamate acts as an excitatory neurotransmitter at the chromatophore neuromuscular junction.

The results of this study indicate that, in addition to glutamate, several peptides also act as putative excitatory neurotransmitters at this synapse. The HPLC and immunoblot data presented here identify at least two and possibly more FaRPs involved in modulating chromatophore activity in cuttlefish. Our HPLC data indicate that *S. officinalis* skin contains at least one non-FaRP factor that also causes chromatophore expansion. This unidentified factor may be one of the peptides in Table 1 that cause chromatophore expansion and also regulate visceral and skeletal muscles in a variety of other molluscs. It is therefore highly probable that the contraction of chromatophore muscles and the resultant expansion of the central pigment-containing cell is mediated by more than one chemical transmitter, although whether these transmitters perform redundant or different functions is as yet unknown.

The focus of this paper was to identify some of the neuronal factors, in particular neuropeptides, that may be involved in chromatophore expansion. However, there is some evidence that there are other factors that may be 'inhibitory' in this system, i.e. cause chromatophore retraction. Application of acetylcholine (ACh) or serotonin (5-HT) to the isolated cuttlefish skin bioassay reduced or delayed the excitatory effect of glutamate (data not shown). In squid, 5-HT antagonizes tonic or transmitter-induced contractions of chromatophore muscles (Florey and Kriebel, 1969). The presence of 5-HT at the chromatophore neuromuscular junction is also supported by immunocytochemical identification of 5-HT-positive cells in the chromatophore lobes of *Octopus vulgaris* (Uemura *et al.* 1987) and in the nerve fibers in the skin of squid *Alloteuthis subulata* (Cornwell and Messenger, 1995). Although suggestive of an 'inhibitory' role for these substances, these results should be not overinterpreted since there are other, paradoxical effects of these substances. Florey and Kriebel (1969) report that ACh induces contraction of chromatophore muscles in the squid *Loligo opalescens*. They also showed that 5-HT facilitates chromatophore muscle contraction and

relaxation activated by nerve stimulation. Since another study (Florey, 1969) suggested that there are no inhibitory synapses on the chromatophore muscles, 5-HT and ACh may not function as neurotransmitters but as neuromodulators, modulating the excitatory effects of glutamate and FaRPs. These reports, taken together, suggest that the chromatophore system in cephalopods is regulated by multiple chemical messengers.

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