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Comparative aspects of structure and action of molluscan neuropeptides

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Abstract. A number of neuropeptides were isolated from the ganglia and muscles of molluscs, and their actions were examined. Diverse neuropeptides, in addition to several classical neurotransmitters, were suggested to be involved in the regulation of the anterior byssus retractor muscle of *Mytilus*. A wide structural variety of members of the *Mytilus* inhibitory peptide family was observed in each of the genera *Mytilus, Achatina* and *Helix*. Gly-Trp-NH_2, the C-terminal dipeptide fragment of the neuropeptide AGPWamide, showed a more potent action than the parent peptide in all of the muscles examined. Peptides related to some molluscan neuropeptides were found to be distributed interphyletically. Some neuropeptides containing a D-amino acid residue were found in *Achatina* and *Mytilus*. These aspects of molluscan neuropeptides are thought not to be exceptional.
Key words. Neuropeptide; Mollusca; ABRM; *Mytilus; Achatina; Helix*; D-amino acid residue.

Introduction

Over the last 15 years, a large number of neuropeptides have been isolated from molluscs. According to the structure and the action of the peptides, they have been classified into family groups, such as the FMRFamide family, the SCP family and the myomodulin-CARP family [25]. In collaboration with other laboratories, we have also isolated a number of neuropeptides mostly from muscles and ganglia of molluscs, and have examined their actions on muscles and neurons. Most of the isolated peptides were found to be members of families described previously. However, some comparative aspects of the structure and the action of the peptides were found to be unique. Here, we review those unique aspects.

A diversity of neuropeptides controlling a muscle

It has been well documented that acetylcholine and serotonin are involved in the regulation of the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*[33,40]. Acetylcholine has been shown to be the principal excitatory neurotransmitter in the muscle[12,47], and serotonin has been shown to be a catch-relaxing neurotransmitter[44,47]. In addition to these biogenic amines, dopamine which relaxes catch[13] and potentiates contraction[33,35] like serotonin, has been shown to be present in the ABRM[45]. Furthermore, noradrenaline[35,39,46], adrenaline[35,39,47] and octopamine[35,39] have also been shown to relax catch and potentiate contraction, though their presence in the ABRM has not been confirmed. By using an HPLC system coupled with an electrochemical detector, we recently detected considerable amounts of these three amines, in addition to serotonin and dopamine, in the ABRM (fig. 1). Thus, six classical neurotransmitters, acetylcholine, serotonin, dopamine, noradrenaline, adrenaline and octopamine, are suggested to be involved in the regulation of the ABRM (table 1). The latter three biogenic amines may be contraction-potentiating neurotransmitters, because they are less potent than serotonin and dopamine in relaxing catch. In particular, noradrenaline and adrenaline are far less potent in relaxing catch, although the strength of their contraction-potentiating actions is considerable[35,39,46,47].

In 1987, Hirata et al. isolated an octapeptide from the pedal ganglia of *Mytilus* and designated it "catch-relaxing peptide" (CARP)[17]. CARP powerfully relaxes catch tension of the ABRM (fig. $2A_1$), and inhibits contraction, though at around 10^{-9} M it slightly potentiates contraction (fig. $2A_2$)[14,17]. CARP is an analogue of myomodulin, which is found in *Aplysia*[2]. After CARP, two cogeneric hexapeptides termed *Mytilus* inhibitory peptides (MIPs) were isolated from the pedal ganglia[16]. MIPs powerfully inhibit contraction of the ABRM (fig. 2B) but do not affect catch tension[15].

Table 1. Bioactive substances in the ABRM of *Mytilus edulis*

Biogenic amines												Acetylcholine
												Serotonin
												Dopamine
												Noradrenaline
												Adrenaline
												Octopamine
Peptides	MIPs					G	S	P	M	F	V	amide
						G	A	P	M	F	V	amide
						D	S	P	L	F	V	amide
						Y	A	P	R	F	V	amide
				A	S	H	I	P	R	F	V	amide
	FaRPs							F	M	R	F	amide
		A	dL	A	G	D	H	F	F	R	F	amide
	CARP				A	M	P	M	L	R	L	amide

MIPs: *Mytilus* inhibitory peptides, FaRPs: FMRFamide-related peptides, CARP: catch-relaxing peptide, dL: D-Leu.

The presence of CARP and the two MIPs in the pedal ganglion, which is the main control center of the ABRM[34], and their strong actions on the muscle, led us to speculate that the peptides are neuromediators which regulate muscle function. On the basis of this suggestion, Fujisawa et al. attempted to isolate bioactive peptides from the ABRMs themselves, and found five MIPs including the above two, FMRFamide and a FMRFamide-related decapeptide, and CARP[9,34]. The structures of the peptides are shown in table 1. All of the MIPs found in the ABRM show similar inhibitory action on the muscle[9]. FMRFamide shows a contraction-potentiating action at low concentrations, and at higher concentrations (higher than 10^{-7} M) it evokes a contraction in the muscle. Further, at 10^{-8}–10^{-7} M it relaxes catch tension[37,38]. The FMRFamide-related decapeptide shows contraction-potentiating and contractile actions which are similar to but more potent than those of FMRFamide, but does not relax catch tension at any concentration[8]. These two peptides may be contraction-potentiating neuromediators in the ABRM[33].

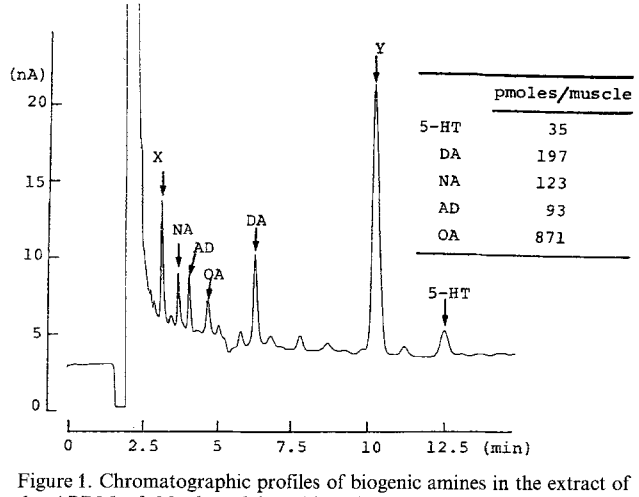

Figure 1. Chromatographic profiles of biogenic amines in the extract of the ABRM of *Mytilus edulis* subjected to HPLC separation with an electro-chemical detector. 5-HT: serotonin, DA: dopamine, NA: noradrenaline, AD: adrenaline, OA: octopamine, X and Y: unidentified substances. An aliquot (1/500) of the extract of 10 ABRMs obtained from 10 animals was used. The insert shows estimated amounts of the biogenic amines per one ABRM.

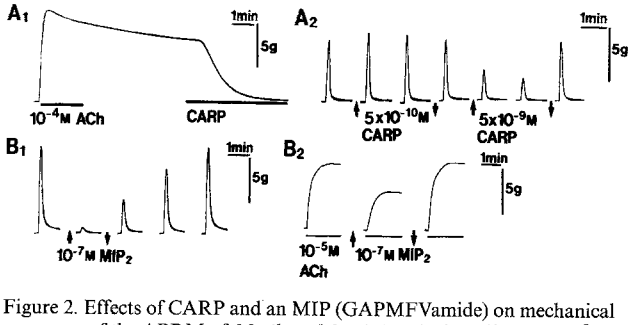

Figure 2. Effects of CARP and an MIP (GAPMFVamide) on mechanical responses of the ABRM of *Mytilus edulis*. A_1) Relaxing effect of 10^{-8} M CARP on catch tension, A_2) potentiating effect of a low dose and inhibitory effect of a high dose of CARP on phasic contraction, B_1) inhibitory effect of MIP on phasic contraction, B_2) inhibitory effect of MIP on ACh contracture. The phasic contraction was elicited by repetitive electrical stimulatory pulses.

In addition to the foregoing peptides, Fujisawa recently purified more than 15 other species of peptides from the ABRM (unpublished). They included two MIPs; one MIP-related inhibitory peptide; two SCP-related peptides, one of which showed contraction-potentiating action whereas the other showed contraction-inhibiting action; and two novel contractile peptides. Thus, it can be supposed that the ABRM of *Mytilus* is regulated by more than 20 species of peptides – playing contractile, contraction-potentiating, contraction-inhibiting or catch-relaxing roles – in addition to several classical neurotransmitters. A similar diversity of neuromediators may well be involved in the control of many other invertebrate muscles as well as vertebrate visceral muscles.

A wide variety of members of a peptide family in an animal

Table 2 shows the structures of MIPs isolated from the ABRM of *Mytilus edulis*[9] and the ganglia of the land snails *Achatina fulica*[22] and *Helix pomatia*[19,20]. All of the MIPs, except one of the *Helix* peptides, have -Pro-Xaa-Phe-Val-NH_2 as a common structure at their C-terminal portions. The exceptional one has Ile instead of Val in the common sequence. Therefore, this peptide can also be regarded as a member of the MIP family.

Almost all of the isolated MIPs are hexapeptides. However, one from *Mytilus* is an octapeptide, and three from *Achatina* are pentadecapeptides which consist of two hexapeptides linked by -Gly-Arg-Arg-. We designated the pentadecapeptides twin-type MIPs.

In addition to the MIPs shown in table 2, we recently isolated two other MIPs from *Mytilus* and six other from *Helix*. All of them were found to have -Pro-Xaa-Phe-Val(Ile)-NH_2 (unpublished). One of the peptides isolated from *Helix* was a twin-type MIP. The others were hexapeptides. Thus, we obtained four twin-type MIPs in total from *Achatina* and *Helix*. Their structures are highly homologous with each other. The common sequence of the peptides might be necessary to prevent cleavage into hexapeptides.

Table 2. MIP (*Mytilus* inhibitory peptide) family in some molluscs

Mytilus edulis								G	S	P	M	F	V	amide
								G	A	P	M	F	V	amide
								D	S	P	L	F	V	amide
								Y	A	P	R	F	V	amide
							A	S	H	I	P	R	F	V	amide
Achatina fulica								A	A	P	K	F	V	amide
								G	A	P	K	F	V	amide
								G	A	P	V	F	V	amide
								G	A	P	Y	F	V	amide
								A	A	P	Y	F	V	amide
								G	P	P	M	F	V	amide
								G	A	P	F	F	V	amide
D	A	P	K	F	V	G	R	R	D	P	P	Y	F	V	amide
A	A	P	K	F	V	G	R	R	G	S	P	Y	F	V	amide
A	A	P	K	F	V	G	R	R	G	A	P	Y	F	V	amide
Helix pomatia								G	A	P	A	F	V	amide
								A	A	P	R	F	V	amide
								G	A	P	M	F	V	amide
								G	A	P	L	F	V	amide
								G	S	P	Y	F	V	amide
								G	A	P	Y	F	V	amide
								R	A	P	Y	F	V	amide
								S	V	P	I	F	V	amide
								G	V	P	Y	F	V	amide
								A	A	P	F	F	V	amide
								R	A	P	F	F	V	amide
								G	P	P	M	F	I	amide

Fujisawa et al. found that all of the MIPs isolated from the ABRM of *Mytilus* exhibited a similar contraction-inhibiting action on the muscle[9]. They considered that all of the MIPs may act on the same class of receptors in the ABRM. Fujisawa et al. also examined the relationship between structure and activity in the action of an MIP, Gly-Ala-Pro-Met-Phe-Val-NH_2, on phasic contraction of the ABRM[9,10]. They found that the fragment Pro-Met-Phe-Val-NH_2 was only 10–30 times less potent than the parent, while Met-Phe-Val-NH_2 was 3000–10,000 times less potent and Phe-Val-NH_2 was about 100,000 times less potent. We compared the inhibitory actions of two other MIPs, Arg-Ala-Pro-Tyr-Phe-Val-NH_2 and Gly-Ala-Pro-Lys-Phe-Val-NH_2, with those of their fragments, Pro-Tyr-Phe-Val-NH_2 and Pro-Lys-Phe-Val-NH_2, on the ABRM (fig. 3). We found that each of the fragments was about 10 times less potent than its parent. We also found that the twin-type MIPs showed considerable inhibitory actions on molluscan muscles. The actions of a twin-type MIP on phasic contraction of the ABRM of *Mytilus* and spontaneous contractions of the crop of *Achatina* are shown in figure 4.

The foregoing findings suggest that the common structure -Pro-Xaa-Phe-Val(Ile)-NH_2 is important for the inhibitory activity and that the C-terminal dipeptide, -Phe-Val(Ile)-NH_2, may be essential[9]. It can be supposed that the ancestral MIP has changed to give various structures of MIPs, but they all conserve the important structure. It should be very interesting to study the MIP-receptor systems in molluscs from the point of view of molecular evolution.

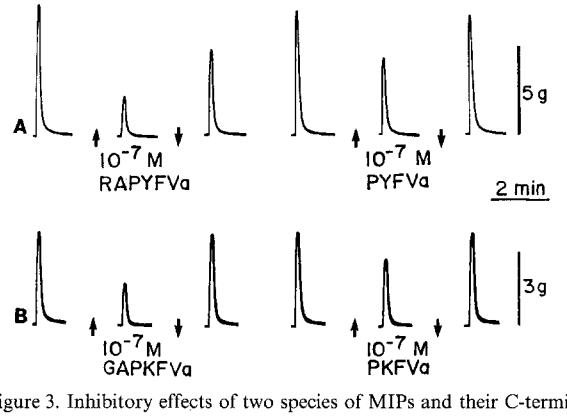

Figure 3. Inhibitory effects of two species of MIPs and their C-terminal tetrapeptide fragments on phasic contraction of the ABRM of *Mytilus edulis* in response to repetitive electrical stimulatory pulses.

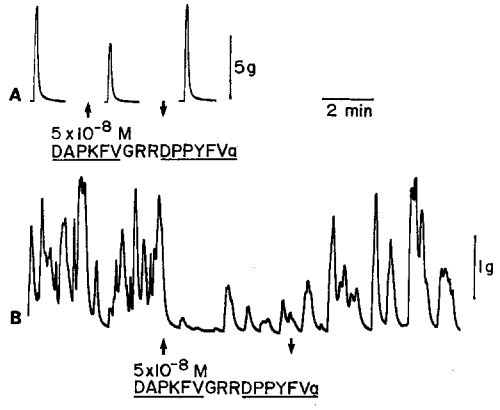

Figure 4. Inhibitory effects of a twin-type MIP on phasic contraction of the ABRM of *Mytilus edulis* in response to repetitive electrical stimulatory pulses (A), and on spontaneous contractions of the crop of *Achatina fulica* (B).

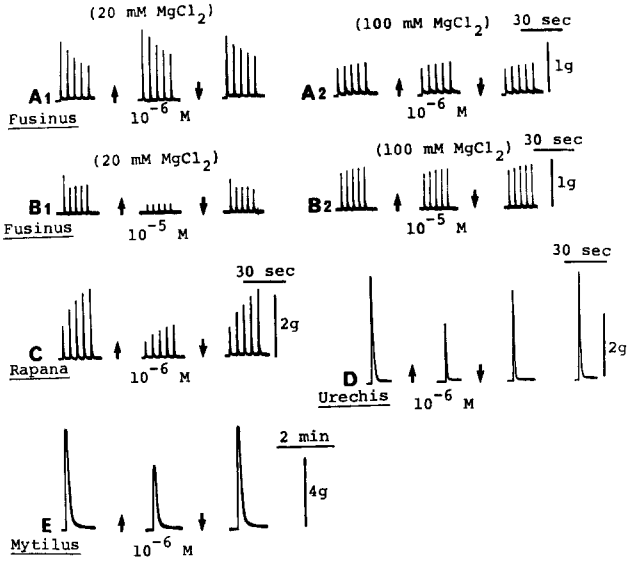

Figure 5. Effects of LSSFVRIamide on several invertebrate muscles. A) Effects of a low dose (10^{-6} M) of the peptide on twitch contractions of the radula retractor muscle of *Fusinus ferrugineus* in a low-Mg^{2+} (1) and a high-Mg^{2+} (2) ASW. B) Effects of a high dose (10^{-5} M) of the peptide on twitch contractions of the radula retractor muscle of *Fusinus ferrugineus* in a low-Mg^{2+} (1) and a high-Mg^{2+} (2) ASW. C) Effect on twitch contractions of the radula retractor muscle of *Rapana thomasiana*. D) Effect on twitch contraction of the inner circular body-wall muscle of *Urechis unicinctus*. E) Effect on phasic contraction of the ABRM of *Mytilus edulis* in response to repetitive electrical stimulatory pulses.

Interphyletically distributed members of a peptide family

Kuroki et al. isolated a bioactive peptide from a prosobranch mollusc *Fusinus ferrugineus* and determined its structure to be Leu-Ser-Ser-Phe-Val-Arg-Ile-NH_2 [28]. Ikeda, in our laboratory, in collaboration with the Balaton Limnological Institute of Tihany (Hungary) and the Suntory Institutes of Osaka and Gunma (Japan), recently isolated several peptides related to the *Fusinus* peptide from the ganglia of pulmonate molluscs, *Achatina* and *Helix*, and the ventral nerve cords of an echiuroid worm, *Urechis unicinctus*. All of the peptides have -Ser-Ser-Phe-Val-Arg-Ile-NH_2 as a common structure (table 3). Only one or two amino acid residues at the N-terminal portions are different from each other. Therefore, they are regarded as members of a family. That is, peptides of this family are distributed interphyletically. We termed these peptides S-Ia peptides. Very recently, Ikeda found another related peptide from the ganglia of *Achatina*. The structure of the peptide is Ala-Pro-Ser-Asn-Phe-Ile-Arg-Ile-NH_2. That is, the peptide can be regarded as a member of the S-Ia peptide family.

The S-Ia peptides show potent action on various molluscan muscles. In each of the muscles, all the peptides show qualitatively similar modulatory action, contraction-potentiating or contraction-inhibiting action. In most of cases, these actions are opposite to those of FMRF-amide. In *Urechis*, however, the peptides show an inhibitory action on twitch contraction of the inner circular body-wall muscle, while FMRFamide has little or no effect on this muscle.

Table 3. Peptides having SSFVRIamide structure

Fusinus ferrugineus		L	S	S	F	V	R	I amide
Helix pomatia		T	S	S	F	V	R	I amide
Achatina fulica	S	P	S	S	F	V	R	I amide
Urechis unicinctus		V	S	S	F	V	R	I amide
(Echiuroidea)		A	S	S	F	V	R	I amide
		P	S	S	F	V	R	I amide

Figure 5 shows the actions of the *Fusinus* S-Ia peptide on several invertebrate muscles. In the radula retractor muscle of *Fusinus*, the peptide potentiates twitch contraction at low concentrations (10^{-9}–10^{-6} M), but at higher concentrations (10^{-5} M or higher) it markedly inhibits the contraction. Neither of the actions are observed in high-Mg^{2+} (100 mM $MgCl_2$) artificial seawater. The peptide may act on the presynaptic sites in the muscle.

Ikeda et al. isolated two novel peptides, Phe-Arg-Val-Phe-OH and Phe-Arg-Phe-OH, from the ventral nerve codes of *Urechis* [21]. These peptides, which are termed FR peptides, showed a weak inhibitory action on twitch contraction of the inner circular body-wall muscle of the animal. In collaboration with other laboratories, Ikeda also isolated several FR-related peptides from the ganglia of *Helix*. Their structures were as follows: Phe-Arg-Thr-Phe-OH, Phe-Arg-Pro-Leu-OH, Phe-Arg-Thr-Phe-Glu-OH, Phe-Arg-Thr-Phe-Gln-OH and Phe-Arg-Thr-Phe-Gln-Lys-OH. All of these *Helix* peptides showed a weak inhibitory action on phasic contraction of the ABRM of *Mytilus*. Further, the *Urechis* peptide Phe-Arg-Val-Phe-OH and the *Helix* peptide Phe-Arg-Thr-Phe-OH were found to have a potentiating action on spontaneous contractions of the small intestine of the African clawed toad *Xenopus laevis* (fig. 6). The threshold concentration for the action was approximately 10^{-7} M in both cases.

In addition to the S-Ia and FR peptides, some other peptides related to molluscan neuropeptides, such as small cardioactive peptides (SCPs), were shown to be

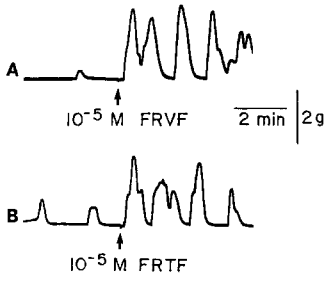

Figure 6. Effects of FRVF (A) and FRTF (B) on spontaneous contractions of the small intestine of the toad *Xenopus laevis*.

present in the ventral nerve cord of *Urechis* (unpublished). The neuropeptides of the Echiuroidea, as well as the Annelida, seem to be closely related to those of the Mollusca.

A fragment of a neuropeptide shows more potent action than the parent peptide

The neuropeptide APGWamide (Ala-Pro-Gly-Trp-NH_2), which is closely related to the tetrapeptide fragment of the crustacean hormone RPCH (table 4), was first found in the ganglia of the prosobranch mollusc *Fusinus*[27]. APGWamide was also found in the fresh water snail *Lymnaea stagnalis*, by the group working at the Free University of The Netherlands[1], and in the land snails *Achatina*[29] and *Helix* (unpublished). The peptide, as well as RPCH, shows interesting modulatory actions in many molluscan muscles and neurons[27,29,30]. To examine the structure-activity relationships of APGWamide, we synthesized several analogues (Phe-Ala-Pro-Gly-Trp-NH_2, Pro-Gly-Trp-NH_2, Gly-Trp-NH_2 and Trp-NH_2) and tested them in various molluscan muscles. Their actions were compared with those of APGWamide and RPCH. In every one of the muscles examined, all the compounds except Trp-NH_2 showed a qualitatively similar action. The potency order was Gly-Trp-NH_2 $\geqq$ Ala-Pro-Gly-Trp-NH_2 > Phe-Ala-Pro-Gly-Trp-NH_2 > RPCH > Pro-Gly-Trp-NH_2 (table 4). That is, in all the muscles, the dipeptide fragment Gly-Trp-NH_2 is equipotent to, or more potent than, the parent peptide APGWamide[30]. Trp-NH_2, even at 10^{-4} M, showed little or no effect on any of the muscles.

In the ABRM of *Mytilus*, APGWamide and the related peptides inhibited tension development and relaxation of phasic contraction in response to repetitive electrical stimulation. Gly-Trp-NH_2 showed the most potent effects (fig. 7A). However, Gly-Trp-NH_2, as well as the other related peptides, did not inhibit contraction in response to acetylcholine but slightly potentiated it (fig. 7B). The peptides also did not inhibit FMRFamide contracture of the muscle. Furthermore, relaxation of catch tension of the ABRM in response to serotonin was not affected by Gly-Trp-NH_2 and related peptides, though relaxation in response to repetitive electrical stimulation was markedly inhibited by the peptides (fig. 8). The relaxing responses to dopamine, octopamine and CARP were not affected by the peptides either. These results suggest that Gly-Trp-NH_2 and the related peptides inhibit release of excitatory and relaxing neurotransmitters by acting on presynaptic sites in the ABRM[30].

Table 4. Equipotent molar ratios (EPMRs) of APGWamide and related compounds estimated on several molluscan muscles

Compounds									EPMRs
pQ	L	N	F	S	P	G	W	amide (RPCH)	30–100
			F	A	P	G	W	amide	3–10
				A	P	G	W	amide	1
					P	G	W	amide	100–10000
						G	W	amide	0.5–1
							W	amide	No effect

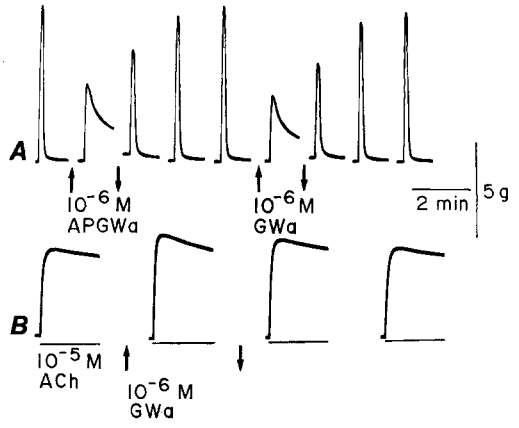

Figure 7. Effects of APGWamide and its C-terminal dipeptide fragment, GWamide, on phasic contraction of the ABRM of *Mytilus edulis* in response to repetitive electrical stimulatory pulses (A), and effect of APGWamide on ACh contracture of the ABRM (B).

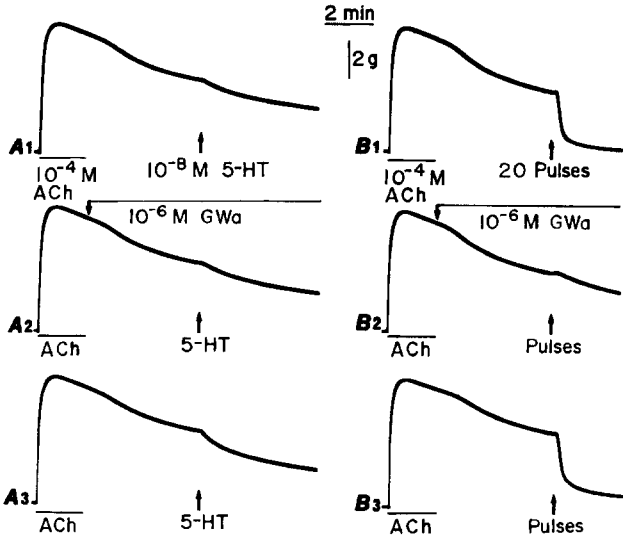

Figure 8. Effects of GWamide on relaxation of catch tension of the ABRM of *Mytilus edulis* in response to serotonin (A) and repetitive electrical stimulatory pulses (B).

In the radula retractor muscle of the prosobranch mollusc *Rapana thomasiana*, Gly-Trp-NH_2 and the related peptides showed marked potentiating action on twitch contraction, and in the pharyngeal retractor muscle of the pulmonate mollusc *Euhadra congenita*, they showed marked inhibitory action on tetanic contraction. It has also been suggested that these effects could be brought about by presynaptic action of the peptides[30]. In the radula retractor muscle of *Fusinus*, the peptides showed a potentiating action on twitch contraction which was suggested as being brought about by postsynaptic action of the peptides[30,36]. In the central nervous system of *Achatina*, APGWamide was shown to hyperpolarize the membranes of various neuron types[29]. Thus, APGWamide and the related peptides seem to exhibit their effects in various molluscan tissues by acting on presynaptic sites or postsynaptic sites. In all of the tissues sensitive to the peptides, the dipeptide fragment Gly-Trp-NH_2 is probably equipotent to, or more potent than, the parent peptide APGWamide.

It has been known that some groups of organisms have a class of endopeptidase termed dipeptidylaminopeptidases, which remove N-terminal Xaa-Ala and Xaa-Pro dipeptides by hydrolyzing peptidyl bonds following Ala and Pro[26]. Such a peptidase might also be present in molluscs. APGWamide might be processed into Gly-Trp-NH_2 by the peptidase, and Gly-Trp-NH_2 might be released as a neuromediator, though there is no evidence available for this speculation.

It has been shown that substance P(5-11), which lacks the N-terminal tetrapeptide of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), exhibits the contractile activity on guinea pig ileum to a much higher degree than does the parent peptide[24]. The presence of substance P(5-11)[18], and an active uptake system for the fragment[41], have been shown in some mammals. The N-terminal tetrapeptide of substance P consists of two Xaa-Pro structures. Therefore, substance P(5-11) might be produced by the action of a dipeptidylaminopeptidase.

In relation to these structures and actions of APGWamide and substance P, it is interesting that most of the foregoing MIPs have Xaa-Ala or Xaa-Pro structure at their N-terminal portions. It is necessary to compare the activities of the C-terminal tetrapeptide fragments of the MIPs with those of the parent peptides. For some tetrapeptide fragments we have already shown that their inhibitory activities on the ABRM of *Mytilus* are less potent than those of the parent peptides.

Peptides containing a D-amino acid residue

It has been shown that dermorphins and deltorphins, the bioactive peptides isolated from the skin of some frogs, have D-Ala or D-Met at position 2[31,32,43]. Recently, it has also been shown that some molluscan neuropeptides have a D-amino acid residue at position 2. These neuropeptides are achatin-I, fulicin and *Mytilus*-FFRFamide. Their structures are shown in table 5.

Table 5. Peptides containing a D-amino acid residue

Achatina	G	dF	A	D									(Achatin-I)
fulica	F	dN	E	F	V	amide							(Fulicin)
Mytilus edulis	A	dL	A	G	D	H	F	F	R	F	amide		(*Mytilus*-FFRFamide)

dF: D-Phe, dN: D-Asn, dL: D-Leu.

Achatin-I was first found in the ganglia of *Achatina*[23]. Following this finding, the peptide was also purified from the atria of the animal by Fujimoto et al.[7]. Achatin-I induces a voltage-dependent Na^+-current in the identified giant neuron, periodically oscillating neuron (PON)[23], which is known to be a potent heart excitor in this animal[11]. Although achatin-I is present in the atrium, the peptide does not affect its activity. In contrast, the peptide strongly enhances the activity of the ventricle. Furthermore, achatin-I potentiates contractions of the radula retractor muscle and the penis retractor muscle of *Achatina*. Thus, it is considered that achatin-I is involved in the regulation of the heart and other muscles, such as the radula and penis retractor muscles, by acting on both the peripheral and central sites as excitatory neuromodulators[7]. Substitution of L-Phe for D-Phe^2 in achatin-I eliminates the activities of the peptide, indicating that D-Phe^2 is essential for the activities.

Fulicin, as well as achatin-I, was isolated from the ganglia of *Achatina* by Ohta et al.[42]. The peptide was found to show potent excitatory action on the penis retractor muscle, the radula retractor muscle, the ventricle and some identified neurons in the buccal ganglion of *Achatina*. In the penis retractor muscle, the threshold concentration for the excitatory action of the peptide is as low as 10^{-11} M[42]. Substitution of L-Asn for D-Asn^2 in fulicin markedly reduces its potency.

Mytilus-FFRFamide is a FMRFamide-related decapeptide containing D-Leu at position 2. The peptide was purified from the ABRM of *Mytilus* by Fujisawa et al.[9]. Kuroki et al. found a FMRFamide-related decapeptide (Ala-Leu-Thr-Asn-Asp-His-Phe-Leu-Arg-Phe-NH_2) in the ganglia of *Fusinus*[28]. This peptide is highly homologous with *Mytilus*-FFRFamide, but all of the amino acid residues in its sequence are of the L-form.

Mytilus-FFRFamide shows a strong contraction-potentiating action on the ABRM (fig. 9). The threshold is between 10^{-12}–10^{-10} M. At higher concentrations (3×10^{-8} M or higher), the peptide evokes a contracture of the muscle. *Mytilus*-FFRFamide is thought to be a contraction-potentiating neuromediator in the ABRM of *Mytilus*.

It is interesting that substitution of L-Leu for D-Leu^2 in *Mytilus*-FFRFamide does not change the activity of the peptide. Furthermore, the C-terminal tetrapeptide fragment (Phe-Phe-Arg-Phe-NH_2) is almost equipotent with, or slightly less potent than *Mytilus*-FFRFamide, indicating that the C-terminal tetrapeptide fragment structure is

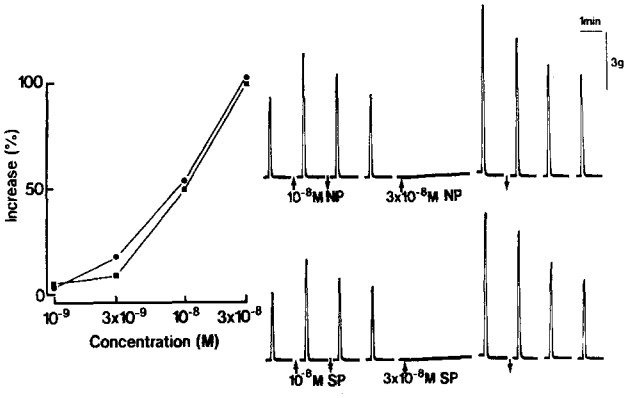

Figure 9. Comparison between the effects of the native (NP) and synthetic (SP) *Mytilus*-FFRFamide on phasic contraction of the ABRM of *Mytilus edulis* in response to repetitive electrical stimulatory pulses. ● and ■: percent increase in peak tension of the phasic contraction evoked in the native and synthetic peptides, respectively. Note: both of the peptides elicited a contracture of the muscle at 3×10^{-8} M or higher.

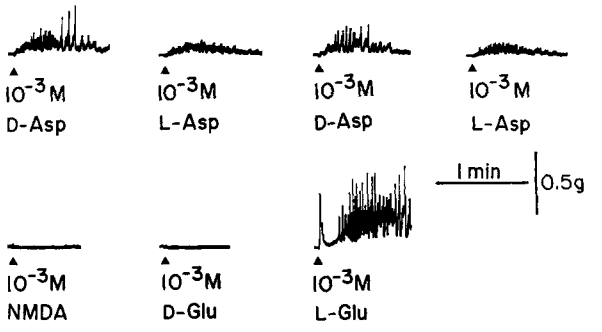

Figure 10. Contractile effects of the excitatory amino acids on the radula retractor muscle of *Fusinus ferrugineus*.

very important in the activity of the peptide on the ABRM, but the N-terminal hexapeptide portion is not essential for the activity[9]. The reason for the presence of D-Leu[2] in the sequence of *Mytilus*-FFRFamide may be to make the peptide resistant to aminopeptidases.

Neuropeptides which have D-amino acid residues in their sequences have hitherto been isolated only from some molluscs, a pulmonate *Achatina* and a bivalve *Mytilus*. However, the bioactive peptides, dermorphins and deltorphins, which are found in frog skin, might play some roles as neuropeptides in the nervous system of these animals. A further search may well reveal neuropeptides containing D-amino acid residues in other animal groups. It has been shown in many invertebrates that considerable amounts of free D-amino acids are present in the tissues. The presence of D-aspartate in molluscs has been reported by several investigators[3-6]. We examined the effects of D-aspartate and some related amino acids on various molluscan muscles and obtained interesting results. The radula retractor muscle of *Fusinus* was found to be capable of responding to D- and L-aspartate and L-glutamate with contractions. The effect of D-aspartate was more potent than L-aspartate, though it was less potent than L-glutamate (fig. 10). The radula retractor muscle of *Hemifusus ternatanus*, which belongs to the same family as *Fusinus*, also responded to these amino acids, and furthermore it was capable of responding to N-methyl-D-aspartate with a weak contraction. The penis retractor muscle of *Achatina* showed considerable sensitivity to L-aspartate, but was only slightly sensitive to D-aspartate and L-glutamate. The ABRM and the pedal retractor muscle of *Mytilus* were insensitive to the amino acids. In general, muscles of plankton-feeding molluscs seem to be insensitive to excitatory amino acids. All of the results obtained in the experiments on excitatory amino acids are summarized in table 6. The results show that the muscles differ in their response to the amino acids. Some muscles show considerable sensitivity to D-aspartate but others do not. These facts lead us to suspect that, at least in certain groups of molluscs, D-aspartate or an unknown acidic substance related to D-aspartate is involved in the neural mechanisms of the animals as an excitatory neurotransmitter. We should pay more attention to D-forms of free amino acids, and to peptides containing D-amino acid residue.

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Table 6. Contractile effects of the excitatory amino acids on the muscles of several molluscs

Molluscs		Amino acids (10^{-3} M) D-Asp	L-Asp	NMDA	D-Glu	L-Glu
Mytilus edulis	ABRM	−	−	−	−	−
	Pedal retractor	−	−	−	−	−
Fusinus ferrugineus	Radula retractor	++	+	−	−	+++
	Proboscis retractor	−	−	−	−	++
Hemifusus ternatanus	Radula retractor	++	++	±	−	+++
Rapana thomasiana	Radula retractor	−	−	−	−	+++
	Radula protractor	−	−	−	−	+++
Achatina fulica	Penis retractor	±	++	−	−	±
Euhadra congenita	Pharyngeal retractor	−	−	−	−	++

+++: strong effect, ++: moderate effect, +: weak effect, ±: slight effect, −: no effect.

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Peptidergic co-transmission in *Aplysia*: Functional implications for rhythmic behaviors

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Abstract. Despite their ubiquitous presence in the central and peripheral nervous systems, the behavioral functions of peptide co-transmitters remain to be elucidated. The marine mollusc *Aplysia*, whose simple nervous system facilitates the study of the neural basis of behavior, was used to investigate the role of peptidergic co-transmission in feeding behavior. Several novel modulatory neuropeptides were purified, and localized to identified cholinergic motorneurons. Physiological and biochemical studies demonstrated that these peptides are released when the motorneurons fire at frequencies that occur during normal behavior, and that the peptides modify the relationship between muscle contraction amplitude and relaxation rate so as to maintain optimal motor output when the intensity and frequency of feeding behavior change.
Key words. Aplysia; co-transmission; modulation; motorneuron; muscle; peptide.

Our understanding of chemical synaptic transmission has been significantly influenced by the widespread finding that a single neuron may contain and release more than one transmitter substance. In many cases a classical neurotransmitter, such as acetylcholine or GABA, is co-localized with a modulatory neuropeptide[1, 13, 17]. A number of suggestions have been made to explain the functional role of the co-release of classical and peptide transmitters, but despite the impressive progress that has been made in understanding the cellular mechanisms of action of both classical and peptide transmitters, relatively little is known about the behavioral consequences of co-transmission[3, 4]. The challenging task of understanding its behavioral role has not been made any easier by recent reports indicating that many neurons contain more than one peptide co-transmitter (see, for example, Jones et al.[15] and Vincent et al.[32]). Some of the best evidence relating co-transmission to behavior has been obtained in invertebrates[17], but even in these relatively simple systems it has not yet been possible to reach unequivocal conclusions regarding the physiological contribution that even single peptide co-transmitters make to behavior.

We have chosen to study the physiological role of peptidergic co-transmission in a simple neuro-behavioral model system: the feeding behavior of the marine mollusc *Aplysia californica*. Even though the consummatory phases of feeding (biting and swallowing) are highly stereotyped, the size and frequency of these responses are strongly affected by the internal state of the animal, for example, whether the animal is satiated or aroused[28]. Parameters of feeding behavior are also influenced by the nature of the food the animal is ingesting, for example, its size and hardness[16]. In this paper we present a hypothesis, with supporting evidence, that suggests that peptidergic co-transmission plays a major role in maintaining the efficiency of the feeding behavior in the face of such changing behavioral demands.

Let us consider a simple change in feeding behavior that occurs at the beginning of a meal. Figure 1 (A and B) illustrates how the speed and strength of biting change as the animal becomes exposed to food. In the beginning, individual bites are weak and occur at a slow rate. Subsequent bites become stronger and faster until both the strength and speed of biting reach a plateau[28, 34]. At first glance, it may appear that this adjustment of the rate and magnitude of biting is a straightforward task that may be accomplished simply by increasing the biting cycle frequency and firing rates of feeding motorneurons. However, it is by no means obvious how it is achieved when we realize that if animals are to maintain their ability to ingest food, some 15 pairs of muscles must maintain proper phase and intensity relationships in the face of severe biomechanical constraints.