Novel excitatory neuropeptides isolated from a prosobranch gastropod, *Thais clavigera*: The molluscan counterpart of the annelidan GGNG peptides

F. Morishita[a], H. Minakata[b], K. Takeshige[a], Y. Furukawa[c], T. Takata[a], O. Matsushima[d], S.T. Mukai[e], A.S.M. Saleuddin[e], T. Horiguchi[f]

[a]Dept. of Biol. Sci., Grad. Sch. of Sci., Hiroshima Univ., Higashi-Hiroshima, Hiroshima, Japan,
[b]Suntory Inst. for Bioorganic Res., Shimamoto, Osaka, Japan,
[c]Fac. of Integr. Art & Sci., Hiroshima Univ., Higashi-Hiroshima, Hiroshima, Japan,
[d]Fac. of Envern. Stud., Hiroshima Inst. of Technol., Hiroshima, Hiroshima, Japan,
[e]Dept. of Biology, York University, Toronto, Ontario, Canada,
[f]Natl. Inst. for Envern. Stud., Tsukuba, Ibaraki Japan.

Key words: HPLC, purification, reproduction, imposex, organotin

Short title: The molluscan GGNG peptides

Corresponding Author: Fumihiro Morishita
Department of Biological Science
Graduate School of Science
Hiroshima University
1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526 Japan
e-mail: fumi425@hiroshima-u.ac.jp
Phone/fax : +81-82-424-7439

Abstract

The GGNG peptides are excitatory neuropeptides identified from earthworms, leeches and polychaeta. Two structurally related peptides were purified and characterized from a mollusk, *Thais clavigera* (prosobranch gastropod). The peptides designated as TEP-1 (*Thais* excitatory peptide-1) (KCSGKWAIHACWGGN-NH_2) and TEP-2 (KCYGKWAMHACWGGN-NH_2) are pentadecapeptides having one disulfide bond and C-terminal GGN-NH_2 structures, which are shared by most GGNG peptides. TEP augmented the motilities of *Thais* esophagus and penial complex. TEP-like immunoreactivity is distributed in both the neurons of the central nervous system and nerve endings in the penial complex. Thus, the involvement of TEP in the contraction of the digestive and reproductive systems is suggested. Substitution of amino acids in TEP revealed that two tryptophan residues in TEP are important for maintaining bioactivity.

1. Introduction

The organotins triphenytin and tributyltin which have been used as anti-fouling agents for ships or fishing nets, are known to be released into the seawater and induce masculinization of female prosobranch gastropods [7, 10, 11, 14, 19, see 22 for review]. This worldwide phenomenon, termed imposex [2, 36], includes formation of male sexual organs such as the penial complex and vas deferens, as well as induction of spermatogenesis in the ovary [12, 13]. Since these changes result in infertility, imposex threatens the ecological balance in marine environments. Despite the clear influence of organotins on female prosobranchs, the mechanism by which imposex is induced remains controversial. Several mechanisms have been proposed, e.g., inhibition of aromatase activity [1], modification of excretion of steroid metabolites [35], and over-production of penis morphogenic factor in female [6].

Recently, it was reported that an injection of a molluscan neuropeptide, APGWamide, into a mud snail, *Ilyanassa obsoleta*, accelerated the elongation of the penial complex in females [30]. Moreover, it was demonstrated that organotins activated the retinoid X receptor (RXR) in *T. clavigera* [29]. Since an injection of a RXR ligand, 9-*cis* retinoic acid, induced imposex in the animal, activation of the RXR is a key mechanism for the induction of imposex. Considering that RXR is involved in the regulation of gene expression, it is plausible to assume that organotins modify the expression of neuropeptides/peptide hormones through the activation of the RXR. Therefore, it is likely that the malfunctioning of neuropeptide/peptide hormone systems caused by organotins is one of the mechanisms in the imposex induction [31]. Evaluation of this hypothesis however, requires a detailed knowledge of the neuropeptides/neurohormones that regulate the reproductive systems of prosobranch mollusks. To establish a foundation for understanding the functional roles of neuropeptides in the reproductive system of the prosobranch, we initiated a peptide identification project in the rock-shell *Thais clavigera*, a representative species affected by imposex.

As a strategy to identify bioactive peptides from *T. clavigera*, we adopted a screening of HPLC fractions using an immunological assay with antibodies to neuropeptides that might be involved in the reproduction of the rock-shell. In this study, we used the anti-LEP (leech

excitatory peptide) antibody. LEP is a neuropeptide originally identified in the leech, *Witmania pigra* [24], and structurally related peptides were discovered in oligochaeta (earthworm excitatory peptide; EEP) [32], and polychaeta (polychaeta excitatory peptide; PEP) [20]. These peptides have one intramolecular disulfide bond and a C-terminal GGN-NH_2 (LEP and PEP) or GGNG (EEP) structure (Table 1). Because C-terminal amidation requires a glycine residue at the C terminus, both LEP and PEP have a GGNG sequence in their precursor protein [25]. Therefore, these peptides are collectively named the GGNG peptide family.

The physiological function of LEP is not well established, however LEP is involved in the contraction of penial complex movements in *Witmania* [28]. LEP is particularly interesting because it is the only GGNG peptide that can stimulate molluscan tissues, such as the rectum of *Anodonta* and the esophagus of *Aplysia* and *Euhadra*, within the physiological concentration range [21]. Pilot studies revealed that neurons in the ganglia of *Aplysia* and a land snail, *Euhadra congenital*, are immunopositive to the anti-LEP antibody (Morishita, unpublished observations), suggesting that LEP-like peptides are endogenous molecules in mollusks. LEP may be involved in some aspects of reproduction in mollusks, as is the case in the leech [28].

In this study, we chemically identified the molluscan counterparts of the GGNG peptide for the first time. We describe the purification, structural analysis, distribution and physiological actions of novel GGNG peptides of *Thais clavigera* (*Thais* excitatory peptides: TEPs). The results suggest that TEPs are neuropeptides of *Thais clavigera*, and are involved in the contraction of the digestive and reproductive systems.

2. Materials and Methods

2.1 Animals

The rock-shells, *Thais clavigera*, were collected on the seashore around Hiraiso, Ibaraki, Japan. In this area, the relative penis length (RPL) index of females, which indicates imposex level, was less than 1%, suggesting that the influence of organotins on the animal was negligible [11]. Animals were kept in a tank filled with artificial seawater (ASW) maintained at 15 ^{o}C, and fed with mussels *Septifer virgatus*.

2.2 Purification of peptides

Rock-shells were anesthetized by immersing in 0.33M $MgCl_2$, and the shell was removed manually. The animal was pinned on a Sylgard-coated plastic dish, and the mantle, gill, ctenidium, osphradium, kidney, heart and hypobranchial glands were removed. Subsequently, the dorsal part of body wall, salivary gland, digestive gland, reproductive gland and most of the digestive tract were removed. The remaining tissues that consisted of the central nervous system (CNS), esophagus, and foot were rapidly frozen by immersion in liquid N_2. Tissues were kept frozen at -80^{o}C until extraction.

Five hundred pieces of frozen tissue obtained from male snails were pulverized in liquid N_2, and then plunged into boiling 2% acetic acid for more than 10 min. After cooling with running tap water, tissues were further homogenized with a Waring blender for 1 min at maximum speed, after which they were centrifuged for 20 min at 10,000x*g*. The supernatant was saved, and the precipitate was resuspended in 2% acetic acid, homogenized and centrifuged as before. The pooled supernatants were condensed with a rotary evaporator. This peptidic extract was divided into two parts, and each of them passed through a Sep-Pak Vac C_{18} cartridge column (20 cc, 5 g, Waters Corporation, Milford, MA) which had been equilibrated with 0.1% trifluoroacetic acid (TFA). The columns were washed with 3% methanol/0.1% TFA, and the retained materials were eluted with 70% methanol/0.1% TFA. The eluted materials were pooled and condensed to <10 ml, and subjected to HPLC separation.

For the first step separation, the extract was fractionated with an HPLC system (Shimadzu,

Kyoto, Japan) equipped with a cation-exchange column (TSK-GEL SP-5PW, 4 mm x 120mm, Tosoh, Tokyo, Japan). The column was equilibrated with 10 mM phosphate buffer (pH. 6.1) and retained materials were eluted using a linear gradient of 0-1 M NaCl over 100 min. The flow rate was 0.5 ml/min and 2-min fractions were collected. An aliquot (1/500) of each fraction was analyzed by dot blot assay using anti-LEP antibody [25]. Fractions containing immunoreactivity to anti-LEP antibody were further purified by sequential HPLC separations as shown in table 2.

2.3 Structural determination

The amino acid sequences of the purified peptides were determined using a gas-phase sequencer (PPSQ-10, Shimadzu. Kyoto, Japan) based on automated Edman degradation. Peptide mass was determined with the Q-Tof^{TM} (Micromass UK Ltd., Manchester, U.K.). The synthesis of peptides was performed by Fmoc chemistry using an automated solid-phase synthesizer (PSSM-8, Shimadzu) [26]. The accuracy of peptide synthesis was determined by amino acid sequencing and/or mass-spectrometry. Other peptides used in this study were synthesized following similar procedures. To confirm that the corresponding native and synthetic peptides had the same structure, the chromatographic behavior of the native and synthetic peptides was compared on two HPLC systems equipped with a reversed-phase (ODS-80Ts) and a cation-exchange column (SP-5PW) respectively.

2.4 Physiological assay

The physiological activity of the peptides was tested on the esophagus, penial complex and prostate gland of *T. clavigera*, and the large hermaphroditic duct of *Aplysia kurodai*. One end of each tissue was connected to the bottom of the chamber (2 ml) and the other to a force transducer. Testing solution (20 µl) was added to a chamber containing 2 ml of ASW (445 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM HEPES, pH 7.9-8.0), which was continuously agitated by gentle aeration. The tension generated by the contraction of the tissue was recorded on a chart recorder.

When the effects of TEPs, APGWamide and GWamide were tested on the penial complex,

responses were normalized relative to the tension generated by 10^{-4} M ACh (100%). APGWamide or GWamide was applied to the tissue for 5 min before the application of TEP-1. Statistical significance was assessed by Student's t-test. When the effects of GGNG peptides and TEP-analogs were tested on the esophagus, responses were normalized taking the tension generated by TEP-1 at 10^{-7} M as 100%. By contrast, when the effects of GGNG peptides on *Aplysia* LHD were examined, the responses were expressed as an increase in tension taking the spontaneous rhythmic contractions in ASW as 100%.

2.5 Enzyme-linked immunosorbent assay (ELISA)

The cross-reactivity of anti-LEP antibody to GGNG peptides was determined by a competitive ELISA [27]. For this assay, LEP that had been conjugated to bovine serum albumin (BSA) was immobilized on the bottom of 96-well assay plate, and then blocked with 2% BSA dissolved in phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM phosphate buffer, pH 7.2) for 2 hr at room temperature. Anti-LEP antibody (1000X dilution) was added to the assay well in the absence or presence of various concentrations of competitor peptides. After an extensive wash with PBS containing 0.1% Tween20 ($PBST_{20}$), horseradish peroxidase (HRP)-tagged anti-Rat IgG $F(ab')_2$ fragment (Rockland, Gilbertsville, PA) was added to each well (2000 x dilution). Each well was washed with $PBST_{20}$ as before. Finally, 100 µl of 0.1 M citrate buffer (pH 5.2) containing 0.4 mg/ml o-phenylenediamine and 0.01% H_2O_2 was added as the substrate for HRP-catalyzed coloration. The reaction was stopped by the addition of 50 µl of 2M H_2SO_4 and coloration was quantified with a plate reader (MPR-A4iII, Tosoh, Tokyo, Japan) by measuring absorbance at 492 nm.

2.6 Immunohistochemisty

Procedures for immunostaining of thin paraffin sections have been described previously [27]. In brief, dissected tissues were fixed with Zamboni's fixative (15% saturated picric acid, 4% paraformaldehyde in PBS) overnight at 4^{o}C, and washed with PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA). Tissues were dehydrated in an ascending series of

ethanol, cleared with xylene, and embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). Sections (4 μm) were mounted on silicon-coated slides, rehydrated, and blocked with PBS containing 2% BSA and 5% normal rabbit serum (blocking buffer; BB). Anti-LEP antibody was diluted 100X with 10% BB (BB diluted 10-fold with PBS). Sections were incubated with antibody for 60 min at 37 ^{o}C. The secondary antibody was biotinylated anti-rat IgG (1000X dilution with 10% BB; Vector Laboratory Inc., Burlingame, CA) and was incubated for 60 min at 37^{o}C. The preparation was then extensively washed using $PBST_{20}$. Signals were visualized with the Elite ABC kit (Vecter Laboratory Inc., Burlingame, CA), where 0.1 M Tris-HCl (pH 7.4) with 3,3'-diaminobenzidine (1 mg/ml), $NiCl_2$ (2 mg/ml) and 0.05% H_2O_2 was given as the substrate. Sections were lightly stained with hematoxylin-eosin, and mounted with Marinol mountant (Muto Pure Chemical, Tokyo, Japan). Preparations were observed under an Olympus microscope (Olympus Co., Tokyo, Japan) and images were recorded with a digital camera. The digitized images were compiled using Adobe Photoshop 7.0 (Adobe systems, Tokyo, Japan).

3. Results

3.1 Identification of LEP-like peptide from *Thais clavigera*.

To chemically identify the LEP-like peptide, we prepared a peptidic extract from the organs dissected from male *Thais clavigera*. The extract was applied to a cation-exchange HPLC and eluted with a linear gradient of NaCl (0-1 M /100 min). An aliquot of each fraction was subjected to a dot blot assay using anti-LEP antibody, and fractions containing immunopositive substances were further purified by RP-HPLC. After two additional HPLC fractionation steps and the dot blot assay, two immunopositive peaks were obtained (Fig.1).

The results of the amino acid sequencing of the substances subjected to automated Edman degradation are shown in table 3. Some amino acid residues were not determined by this analysis. Mass spectrometry with Q-Tof revealed that the molecular mass of peak 1 was 1613.48, while that of peak 2 was 1707.63. A comparison of the partial sequence of peak 1 to the structure of LEP (Table 1) suggested that unknown residues at 2 and 11 positions were cysteines. Assuming a disulfide bond between the two cysteine residues and the C-terminal amidation of aspargine (Asn), the calculated mass of peak 1 (1613.48) suggests that the residue at 12 is tryptophan. Thus, the structure of peak1 is predicted to be KCSGKWAIHACWGGN-NH_2. Similarly, the partial sequence of peak 2 is predicted to be KCYGKWAMHACWGGN-NH_2. These predictions were confirmed by co-elution of the native and synthetic peptides by RP-HPLC and cation-exchange HPLC (Fig. 2). For both peak 1 and peak 2, the retention time of the synthetic peptide was identical to that of the native peptide. In each case, a mixture of the two eluted as single absorbance peak. Thus, the predicted structures of peak 1 and peak 2 were confirmed. Peak 1 and peak 2 were therefore designated TEP-1 (*Thais* excitatory peptide-1) and TEP-2, respectively.

3.2 Physiological actions of the TEPs on reproductive systems.

One of the purposes of this study was to elucidate the role of neuropeptides regulating reproductive systems. Therefore, the actions of the TEPs were tested on the penial complex and prostate glands of *T. clavigera*. Most of the isolated penial complex preparations were quiescent in ASW. TEP concentrations between 10^{-7} and 10^{-6} M induced tonic contractions in this tissue (Figs. 3A and 4A).

At higher concentrations, strong repetitive contractions were superimposed on tonic contractions. These movements appeared to mimic the peristaltic movement of the penis for sperm discharge.

In the pulmonate snail, *Lymnaea stagnalis*, motility of the penial complex is regulated by two neuropeptide having opposing effects; APGWamide mediates the relaxation of the penial complex while conopressin mediates the contraction of the tissue [3]. APGWamide, which is suggested to be a putative penis-morphogenic factor in prosobranchs [30, 31], was chemically identified in a related prosobranch, *Fusinus ferrugineus* [18]. Therefore, we tested the effects of APGWamide on the penial complex of *T. clavigera*, especially on the TEP-induced contraction of the tissue. APGWamide alone had no apparent effect on the *Thais* penial complex, except for a slight decrease in basal tension (Fig. 4A). By contrast, APGWamide at 10^{-5} M reduced TEP-1-induced contractions (at 10^{-5} M) to less than half of controls (Fig. 4B). Therefore, APGWamide had an inhibitory action on *Thais* penial complex, antagonizing the excitatory action of the TEPs. GWamide, a degradation product of APGWamide, is also known to have a bioactivity [23]. However, this peptide had no apparent effect on this tissue (Fig. 4B).

TEPs induced the contraction of the prostate gland in a dose-dependent manner (Fig. 3B). The effect of TEP-1 on this gland was tonic, while acetylcholine, a classic neurotransmitter of mollusks, induced phasic contractions at 10^{-4} M. *In vivo*, these contractions may be involved in the transport of secretory products from the prostate gland. Although TEPs were purified from male tissue, they were myoactive on female tissues as well. For example, TEP-1 induced contractions of female sex-accessory glands and esophagus (data not shown). There was no significant difference in the effective concentration range of TEPs between female and male tissues.

3.3 Effects of TEPs on *Thais* esophagus and *Aplysia* LHD

As we described earlier, LEP, but not other GGNG peptides, was myoactive on several kinds of contractile tissues in mollusks. In this experiment, the effect of TEPs was compared to that of LEP and other GGNG peptides on the *Thais* esophagus. TEP-1 augmented the movement of the isolated esophagus in a dose-dependent fashion (Fig. 3C). Threshold concentration of TEP-1 fell between 10^{-10} and 10^{-9} M (Fig. 5A). The effective concentrations of TEP-1 and -2 were much

lower for the esophagus than for the penial complex. In this experiment, the effective concentrations of TEP-2 were found to be slightly lower than that of TEP-1. Other GGNG peptides, including LEP, had notably reduced activity on this tissue (Fig. 5A). When the same batch of peptides was applied to the *Aplysia*'s large hermaphroditic duct (LHD), TEP-1, -2 and LEP were almost equipotent to each other, whereas other of GGNG-peptides had very limited effects (Fig. 5B). This result confirmed a previous study which showed LEP was myoactive on *Aplysia* tissue [21].

Unlike other GGNG peptides, TEPs have two tryptophan residues on the inside and outside of the ring structure (Trp^6 in TEP-1, and Trp^{12} in TEP-2 respectively). The tryptophan residue within the ring structure is conserved among all the GGNG peptides, whereas the one outside of the ring is exclusive to TEPs (Table 1). Therefore, we hypothesized that Trp^{12} at the C-terminal region was involved in the difference between the effects of TEP and LEP on the *Thais* esophagus. To evaluate this hypothesis, we synthesized LEP and TEP-1 analogs by substituting tryptophan with other amino acids, and tested their actions on the *Thais'* esophagus (Fig. 6). LEP with two tryptophan residues (Trp^7 and Trp^{13}; [Trp^{13}]LEP) was virtually equipotent to TEP-1. By contrast, the substitution of Trp^{12} to Leu^{12} in TEP-1 ([Leu^{12}]TEP-1) resulted in a marked reduction of activity which was comparable to that of LEP. The substitution of Trp^6 with Ala^6 ([Ala^6]TEP-1) also reduced the bioactivity of TEP-1, suggesting that both tryptophan residues are important to the bioactivity of TEP-1. There was little difference in the actions of the analog peptides at 10^{-6} M, when they were tested on the *Aplysia* LHD (data not shown).

3.4 Distribution of TEPs in the *Thais'* nervous system.

Following on from the above results, the distribution of TEPs in the CNS and peripheral nervous system was examined. TEP-specific antibodies were not currently available, and therefore the distribution of immunoreactive TEPs was examined immunohistochemically with the anti-LEP antibody that had been used previously for the HPLC screening of TEPs. The relative affinity of the LEP-antibody to EEP and other GGNG peptides was quantified by a competitive ELISA (Fig. 7A). LEP, which was added as a competitor, effectively decreased the binding of

anti-LEP antibody to the LEP-BSA complex that had been immobilized on the bottom of the assay plate. The IC_{50} of LEP was around 10^{-9} mol/well. Contrastingly, EEP and a tetradecapeptide isolated from a prosobranch, *Fusinus* [15], failed to decrease the binding. Inhibition of the immunoreaction by TEPs was intermediate, and required about 100-fold higher concentrations than LEP did. There was little difference between the inhibition by TEP-1 and TEP-2. These results indicated that the LEP-antibody could recognize TEPs, although the affinity to TEPs was relatively low.

When paraffin sections of *Thais* CNS were immunostained with the anti-LEP antibody, positive signals were found in neurons in several ganglia, including the subesophageal ganglion and pedal ganglion. TEP-immunoreactive neurons had relatively small cell bodies (around 20 μm), and formed clusters consisting of a few neurons (Fig. 7C). Positive signals were detected in the neuropil region as well. In the peripheral regions, muscular tissue of the penial complex contained immunopositive nerve endings (Fig. 7E), which was found to branched out into these tissues. All the signals appeared to be specific, because pre-incubation of the antibody with excess LEP diminished the positive signals (Figs 7B and D).

Discussion

The GGNG peptides are excitatory neuropeptides of annelids. Structurally related peptides have been identified from earthworms (EEP) [32], leeches (LEP) [24] and sandworms (PEP) [20] (Table 1). In this study, we identified two novel bioactive peptides (TEP-1, TEP-2) from the prosobranch *Thais clavigera*, through a combination of HPLC fractionation and screening with anti-LEP antibody. The peptides have one intramolecular disulfide bond and C-terminal –GGN-NH_2 structure, and share structural features with LEP and PEP. Of the 15 amino acids in TEP-1 and -2, eleven are identical to those in LEP. Based on their structural similarity, it was concluded that TEPs are the molluscan counterparts of the GGNG-peptides. Mollusks and annelids share some structurally related peptides, such as allatotropin-like tetradecapeptides [9, 37] and inhibitory penta-FVamide peptides [8, 38], and TEPs are another example demonstrating the kinship of neuropeptides between the two animal phyla.

Immunostaining with anti-LEP antibodies demonstrated the nerve-processes like structures branching into the muscular tissues of the penial complex. Although there was no direct evidence, it can be assumed these nerve processes are a releasing site of TEPs, increasing the motility of the penial complex. In the CNS, some smaller neurons in subesophageal and pedal ganglia were found to contain TEPs. Since the affinity of anti-LEP antibody to TEPs is relatively low, an underestimation of the TEP-containing neuron density may have occurred. However, these results suggest that TEPs are neuropeptides in both the central and peripheral nervous systems of *Thais clavigera*. Further studies are needed to trace the connection between TEP-containing neurons in the CNS, and TEP-containing nerve processes in peripheral tissues for the elucidation of the control centre in the CNS for male sexual behavior.

The physiological actions of TEPs on *Thais* tissues are mostly excitatory. The peptides increased the motilities of the esophagus, penial complex and prostate gland. Effective concentrations of TEPs were between 10^{-9} and 10^{-7} M in the esophagus, which were within the physiological range. Contrastingly, the effective concentrations of TEPs were much higher in the penial complex and prostate gland. This may be partly because these tissues, especially the penial complex, are covered with a thick integument that limits penetration of TEPs from the bathing

saline solution. Another possible reason is that the tissues have a reduced sensitivity to TEPs in none-breeding season. It is likely that TEPs are more effective in the mating season (early summer in Japan). Therefore, we assume that, despite the high concentration ranges required, TEPs are the neuropeptides that are involved in the positive regulation of male sexual activities of *T. clavigera*, such as transportation of sperm for copulation. This hypothesis should be verified with additional studies, including the detailed localization of TEPs among male sexual organs and detection of released TEP among the tissues.

The peptidic regulation of male sexual behavior has been thoroughly studied in the freshwater snail, *Lymnaea stagnalis* [3]. In this animal, the preputium and penis are everted just before copulation, and inserted into the female gonopore to induce sperm transfer [3, 4]. Among the several neuropeptides distributed in the tissues, conopressin and APGWamide play key roles in the eversion of these tissues. For example, APGWamide relaxes the preputium so that the hydrostatic pressure generated by the re-distribution of hemolymph can evert the tissue, whereas conopressin functions to counteract the effects of APGWamide [39].

In *Thais*, the penial complex is not retractable, but compactly coiled to the right of the tentacle in the resting state. It is extended toward the gonopore of the female partner during copulation. Thus, the structure of the penial complex of *Thais* is somewhat different from that of *Lymnaea*. Nevertheless, multiple peptides displaying antagonizing properties appear to control the motility of the penial complexes in *Thais*, because the TEPs had the excitatory effect on the tissue, which in turn was decreased by APGWamide. In fact, APGWamide was chemically identified from a related species, *Fusinus* [18]. Furthermore, DeLange *et al* reported that APGWamide-containing neurons were located in the lateral side of cerebral ganglia of *Littorina*, where there is a putative control center of reproduction [5]. Thus, it is likely that APGWamide is an endogenous peptide in many prosobranch gastropods, including *T. clavigera*. These results suggest that APGWamide and TEPs are important peripheral regulators of male sexual activity in this animal.

TEP-1 and -2 contain two tryptophan residues within their structures. The tryptophan residue within the ring structure of TEPs is conserved among all the GGNG-peptides, whereas the

other in the C-terminal region is exclusive for TEPs. In this study, we found that both were important for TEP bioactivity, because substitution of either tryptophan residue with other amino acids significantly reduced their bioactivity. It has been reported that tryptophan residues determines the bioactivity of many endogenous peptides. For example, the D-configuration of tryptophan is essential for the cardio-excitatory action of a molluscan neuropeptide, NdWFamide on the heartbeat of *Aplysia* and pulmonate snails [26, 27]. In a porcine myeloid peptide (PMAP-23), the C-terminal tryptophan, but not the N-terminal one, is required for antibacterial activity [16]. In this peptide, the C-terminal tryptophan is required for the establishment of the C-terminal alpha-helical structure, which is crucial for the interaction of the peptide with the lipid bilayer of target organisms [33]. Therefore, it is possible that tryptophan residues contribute to the formation of a unique configuration in these peptides, which is recognized by putative TEP receptors in *Thais'* tissues. This presumably explains why other GGNG-peptides including LEP had a low impact on *Thais'* esophagus. Since those peptides do not have a tryptophan residue in the C-terminal region, the configurations of those peptides are different from those of the TEPs. The observation that a LEP analog with two tryptophan residues was equipotent to TEP-1 reinforces this hypothesis. However, this hypothesis does not explain why both LEP and TEPs are effective on *Aplysia* tissues. Perhaps a shorter region of the peptide, such as the C-terminal -GGN-NH_2 structure, is essential for receptor binding in *Aplysia* tissues. Identification of the putative GGNG-peptide of *Aplysia* is necessary to verify this hypothesis. Studies into the tertiary structure of TEP are now being undertaken.

Besides the aforementioned peptides, some other bioactive peptides, such as allatotropin-like tetradecapeptide, FRFamide and myomodulin-related peptides have been chemically identified in the prosobranch *Fusinus* [15, 17]. Immunohistochemical distributions of egg-laying hormones of *Aplysia* or *Lymnaea* have been reported in the CNS of prosobranchs such as *Busycon, Concholepas* and *Tegula* [34]. However, their roles in the regulation of the reproductive activities are yet unknown. Further identification of structures and functions of neuropeptides are required for elucidation of the peptidic regulation in the reproduction of prosobranch gastropods.

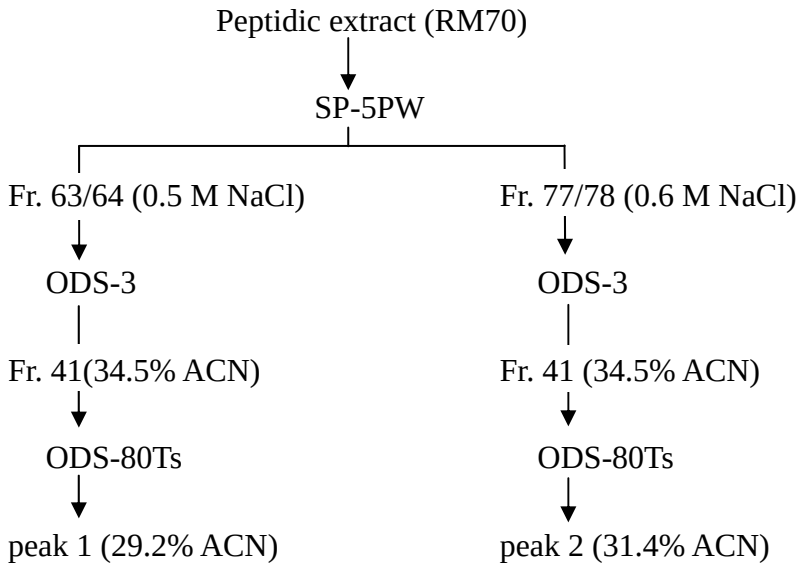
Acknowledgements

This work was supported by a grant-in-aid from the Japan Society for the Promotion of Science to F. M. and from the Ministry of the Environment, Japan, to T. H.

Table 1. Peptides used in this study

Animals	Structures	Abbreviations
GGNG-peptides		
Mollusc		
prosobranch	KCSGKWAIHACWGGN-NH_2	TEP-1
	KCYGKWAMHACWGGN-NH_2	TEP-2
Annelid		
Oligochaeta	GKCAGQWAIHACAGGNG	EEP
Hirudunea	AKCEGEWAIHACLGGN-NH_2	LEP
Polychaeta	KCTGPWAIHACGGGN-NH_2	PEP
Analog peptides		
	KCSGKWAIHACLGGN-NH_2	[Leu^{12}]TEP-1
	KCSGKAAIHACWGGN-NH_2	[Ala^{6}]TEP-1
	AKCEGEWAIHACWGGN-NH_2	[Trp^{13}]LEP

Table 2. A flowchart of HPLC-fractionation steps of *Thais* tissues



SP-5PW: TSK GEL SP-5PW (4 x 120 mm, Tosho, Tokyo, Japan)
0-1 M NaCl/100 min
ODS-3: Inertsil ODS-3 (4.6 x 250 mm, GL Science Inc., Tokyo, Japan)
0-60% ACN/120 min
ODS-80T$_S$: TSK GEL SP-5PW (4.6 x 150 mm, Tosho, Tokyo, Japan)
28-38% ACN/80 min)
ACN: acetonitrile

Table 3. Amino-acid sequencing of immunopositive materials.

Cycle	peak 1		peak 2	
	Amino acid	Yield	Amino acid	Yield
1	Lys	25.8	Lys	11.2
2	---	---	---	---
3	Ser	5.1	Tyr	15.0
4	Gly	18.5	Gly	10.8
5	Lys	8.3	Lys	7.2
6	Trp	3.4	---	---
7	Ala	8.8	Ala	8.7
8	Ile	5.4	Met	6.1
9	His	6.4	His	6.4
10	Ala	6.4	Ala	7.7
11	---	---	---	---
12	---	---	---	---
13	Gly	4.6	Gly	6.3
14	Gly	5.2	Gly	7.2
15	Asn	3.8	Asn	5.2

Yields are expressed in pmol.

References

[1] Betten C, Oehlmann J, Stroben E, TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Holgol Meeresunters 1996; 50: 229-317

[2] Bryan GW, Gibbs PE, Hummerstone LG, Burt GR, The decline of the gastropod *Nucella lapillus* around south-west England: evidence for the effect of tributyltin from antifouling paints. J Mar Biol Ass UK 1986; 66: 611-40

[3] Chase, R., Reproduction In: Behavior and its neural control in gastropod molluscs. New York: Oxford University Press; 2002, p.170-213

[4] De Lange RPJ, Joosse J, Van Minnen J, Multi-messenger innervation of the male sexual system of *Lymnaea stagnalis*. J Comp Neurol 1998; 390: 564-77

[5] De Lange RPJ, Van Minnen J, Localization of the neuropeptide APGWamide in gastropod molluscs by in situ hybridization and immunocytochemistry. Gen Comp Endocrinol 1998; 109: 166-74

[6] Feral C, LeGall S, The influence of a pollutant factor (tributyltin) on the neuroendocrine mechanism responsible for the occurrence of a penis in the female of Ocenebraerinacea. In: Lever J, Boer H, editors. Molluscan neuro-endocrinology, Amsterdam, North Holland Publishing Co; 1983, p.173-75

[7] Folsvik N, Berge J.A, Brevik E.M, Walday M, Quantification of organotin compounds and determination of imposex in populations of dogwhelks *(nucella lapillus)* from Norway. Chemosphere 38:681-691 1999

[8] Furukawa Y, Nakamaru Y, Sasaki K, Fujisawa Y, Minakata H, Ohta S, et al. PRQFVamide, a novel pentapeptide identified from the CNS and gut of *Aplysia.* J Neurophysiol 2003; 89: 3114-27

[9] Harada A, Yoshida M, Minakata H, Nomoto K, Muneoka Y, Kobayashi M, Structure and function of the molluscan myoactive tetradecapeptides. Zool Sci 1993; 10: 257-65

[10] Horiguchi T, Shiraishi H, Shimizu M, Morita M, Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. Environ Pollu 1997; 95: 85-91

[11] Horiguchi T, Cho H-S, Shiraishi H, Shibata Y, Morita M, Shimizu M, et al., Themporal trend and present status on imposex and organotin contamination in Gastropods. Bull Costral Oceangraphy 2000, 37 :89-95

[12] Horiguchi T, Takiguchi N, Cho HS, Kojima M, Kaya M, Shiraishi H, et al. Ovo-testis and disturbed reproductive cycle in the giant abalone, *Haliotis madaka*: possible linkage with organotin contamination in a site of population decline. Marine Environ Res 2000; 50: 223-9

[13] Horiguchi T, Kojima M, Kaya M, Matuo T, Shiraishi H, Morita M, et al. Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, *Haliotis gigantea*. Marine Environ Res 2002; 54: 679-84

[14] T. Horiguchi, Z. Li, S. Uno, M. Shimizu, H. Shiraishi, M. Morita, et al. Contamination of organotin compounds and imposex in molluscs from Vancouver, Canada. Marine Environ Res, 57:75-88 2003

[15]Kanda T, Kuroki Y, Kubota I, Muneoka Y, Kobayashi M, (1990) Neuropeptides isolated from the ganglia of a prosobranch mollusc, *Fussinus ferrugineus*. In: Yanaihara N editor. Peptide Chemistry 1989, Osaka: Protein Research Foundation; 1990, p. 39-44

[16] Kang JH, Shin SY, Jang SY, Kim KL, Hahm KS, Effects of tryptophan residues of porcine myeloid antibacterial peptide PMAP-23 on antibiotic activity. Biochem Biophys Res Commun 1999; 264:281-6

[17] Kobayashi M, Muneoka Y, Structure and action of molluscan neuropeptides. Zool Sci 1990; 7: 801-14

[18] Kuroki Y, Kanda T, Kubota I, Fujisawa Y, Ikeda T, Miura A, et al. Molluscan neuropeptide related to the crustacean hormone, RPCH. Biochem Biophys Res Commun 1990; 167: 273-9

[19] Marshall DJ, Rajkumar A. Imposex in the indigenous *Nassarius kraussianus* (Mollusca: Neogastropoda) from South African harbours. Mar Pollut Bull 2003, 46: 1150-1155.

[20] Matsushima O, Takahama H, Ono Y, Nagahama T, Morishita F, Furukawa Y, et al. A novel GGNG-related neuropeptide from the polychaete *Perinereis vancaurica*. Peptides 2002; 23: 1379-90

[21] Matsushima O, Morishita F, Furukawa Y, Minakata H, Muneoka Y, Annelid bioactive peptides: A comparative view. Trends in Comp Biochem Biophys 2000; 7: 131-41

[22] Matthiessen P, Gibbs PE. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ Toxicol Chem 1998, 17: 37-43.

[23] Minakata H, Kuroki Y, Ikeda T, Fujisawa Y, Nomoto K, Kubota I, Muneoka Y, Effects of the neuropeptide APGW-amide and related compounds on molluscan muscle – GWamide shows potent modulatory effects. Comp Biochem Physiol C 1991; 100: 565-71

[24] Minakata H, Fujita T, Kawano T, Nagahama T, Oumi T, Ukena K, et al. The leech excitatory peptide, a member of the GGNG peptide family : Isolation and comparison with the earthworm GGNG peptides. FEBS Lett. 1997; 410: 437-42

[25] Minakata H, Ikeda T, Nagahama T, Oumi T, Ukena K, Matsushima O, et al. Comparison of precursor structures of the GGNG peptide derived from the earthworm *Eisenia foetida* and the leech *Hirudo nipponia*. Biosci Biotechnol Biochem 1999; 63: 443-5

[26] Morishita F, Minakata H, Sasaki K, Tada K, Furukawa Y, Matsushima O, et al. Distribution and function of an *Aplysia* cardioexcitatory peptide, NdWFamide, in pulmonate snails. Peptides 2003; 24: 1533-44

[27] Morishita F, Nakanishi Y, Sasaki K, Kanemaru K, Furukawa Y, Matsushima O, Distribution of the *Aplysia* cardioexcitatory peptide, NdWFamide, in the central and peripheral nervous systems of *Aplysia*. Cell Tissue Res 2003; 312: 95-111

[28] Nagahama T, Ukena K, Oumi T, Morishita F, Furukawa Y, Matsushima O, et al. Localization of leech excitatory peptide, a member of the GGNG peptides, in the central nervous system of a leech (*Whitmania pigra*) by immunohistochemistry and *in situ* hybridization. Cell Tissue Res 1999; 297: 155-62

[29] Nishikawa J, Mamiya S, Nishikawa T, Shiraishi F, Horiguchi T, Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Envirn Sci Technol 2004; 38: 6271-6

[30] Oberdorster E, McClellan-Green P, The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. Peptides 2000; 21: 1323-30.

[31] Oberdorster E, McClellan-Green P, (2002) Mechanisms of imposex induction in the mud snail, *Ilyanassa obsolete*: TBT as a neurotoxin and aromatase inhibitor. Mar Environ Res 54:715-8

[32] Oumi T, Ukena K, Matsushima O, Ikeda T, Fujita T, Minakata H, et al (1995) The GGNG peptides: Novel myoactive peptides isolated from the gut and the whole body of the earthworms. Biochem Biophys Res Commun1995; 216:1072-8

[33] Park K, Oh D, Shin SY, Hahm KS, Kim Y, Structural studies of porcine myeloid antibacterial peptide PMAP-23 and its analogues in DPC micelles by NMR spectroscopy. Biochem Biophys Res Commun 2002; 290: 204-212

[34] Ram JL, Gallardo CS, Ram ML, Croll RP, Reproduction-associated immunoreactive peptides in the nervous systems of prosobranch gastropods. Biol. Bull 1998; 195: 308-18

[35] Ronis MJJ, Mason AZ, The metabolism of testosterone by the periwinkle (*Littorina Littorea*) in vitro and in vivo: Effects of Trybutyl tin. Marine Environ Res 1996; 42: 161-6

[36] Smith BS, Sexuality in the American mud snail, *Nassarius obsoletus Say*. Proc Malacol Soc Lond 1986; 39: 377-8

[37] Ukena K, Oumi T, Matsushima O, Ikeda T, Fujita T. Minakata H, et al. A novel gut teteradecapeptide isolated from the earthworm *Eisenia foetida*. Peptides 1995; 16: 995-9

[38] Ukena K, Oumi T, Matsushima O, Takahashi T, Muneoka Y, Fujita T, et al. Inhibitory pentapeptides isolated from the gut of the earthworm, *Eisenia foetida*. Comp Biochem Physiol A 1996; 114:245-9

[39] Van Golen FA, Li KW, De Lange RPJ, Van Kesteren RE, Van Der Schors RC, et al. Co-localized neuropeptides conopressin and Ala-Pro-Gly-Trp-NH_2 have antagonistic effects on the vas deferens of *Lymanea*. Neurosci 1995; 69: 1275-87

Figure legends

Fig. 1. Final purification of LEP-like substances. Two substances immunoreactive to anti-LEP antibody were purified from *Thais clavigera* (arrows). They were tentatively designated peak 1 (A) and peak 2 (B), respectively. See Table 2 for the chromatography conditions.

Fig. 2. Co-elution of the purified and synthetic peptides. Retention times of peak 1 (A) and peak 2 (B) were compared to those of synthetic peptides on either a reversed phase (ODS-80Ts) or cation-exchange (SP-5PW) column. S: synthetic peptide; N: purified peptide; M: mixture of S and N. Conditions: A) *upper*: 19% acetonitrile (isocratic) with 0.1% TFA. *Lower*: 0.45-0.6 M NaCl in 10 mM PB (pH 6.3) over 35 min. B) *Upper*: 20.5% acetonitrile (isocratic) with 0.1% TFA. *Lower*: 0.4-0.7 M NaCl in 10 mM PB (pH 6.3) over 100 min. Flow rate was 0.5 ml/min in all cases.

Fig. 3. The actions of TEPs on *T. clavigera* tissues. The physiological actions of TEP-1 and TEP-2 were examined on the penial complex (A), prostate gland (B), and esophagus (C) of male *T. clavigera*. The effect of ACh at 10^{-4} M was tested on the prostate gland for a comparison. Applications of peptides were indicated as arrows.

Fig. 4. The effects of TEPs and APGWamide on penial complex. A) The effects of TEP-1 (open circle), TEP-2 (closed circle), APGWamide (open triangle) and GWamide (closed triangle) were tested on the *Thais* penial complex. Each point and vertical bar represents the mean of 5-6 measurements and S. E., respectively. B) The antagonistic actions of APGWamide and GWamide on TEP-1-induced contractions of penial complex. All the peptides were tested at 10^{-5} M (N=6).

Fig. 5. The actions of GGNG-peptides on Thais esophagus and *Aplysia* large hermaphroditic duct. A) The effects of TEP-1 (open circle) and TEP-2 (closed circle) were compared to those of other GGNG peptides identified from leech (LEP, closed triangle), earthworm (EEP, open triangle) and sandworm (PEP, closed square) on the *Thais* esophagus. Each point and vertical bar represents the mean of five measurements and S. E., respectively. B) The effects of GGNG-peptides at 10^{-6} M were tested on *Aplysia* large hermaphroditic duct. Note the difference in the action of LEP on *Aplysia* and *Thais*. Each point and vertical bar represents the mean of four measurements and S. E., respectively.

Fig. 6. Effects of TEP-analogs on *Thais* esophagus. The effects of analogs of LEP and TEP-1 having a substitution of Trp to Ala or Leu were examined on the *Thais* esophagus. Note that TEP-1 with single Trp showed a marked reduction in bioactivity. TEP-1 (open circle), LEP (closed triangle), [Leu^{12}]TEP-1 (open square), [Ala^{6}]TEP-1 (closed square) , [Trp^{13}]LEP (open triangle). Each point and vertical bar represents the mean of four measurements and S. E., respectively. Refer to Table 1 for structures of peptides.

Fig. 7. Distribution of TEP-like immunoreactivity in *Thais* tissues. A) Anti-LEP antibody used for immunohistochemistry was characterized with a competitive ELISA. LEP (closed triangle), TEP-1 (open circle), TEP-2 (closed circle), PEP (closed square) and EEP (open triangle) were used

as competitors. B-C) Immunostaining with the anti-LEP antibody in paraffin sections of the subesophageal ganglia (B, C) and the penial complex (D,E). Arrows and arrowheads indicate immunopositive signals. Preincubation of the antibody with excess LEP diminished the positive signals (B,D). Scale bars represent 50 µm.

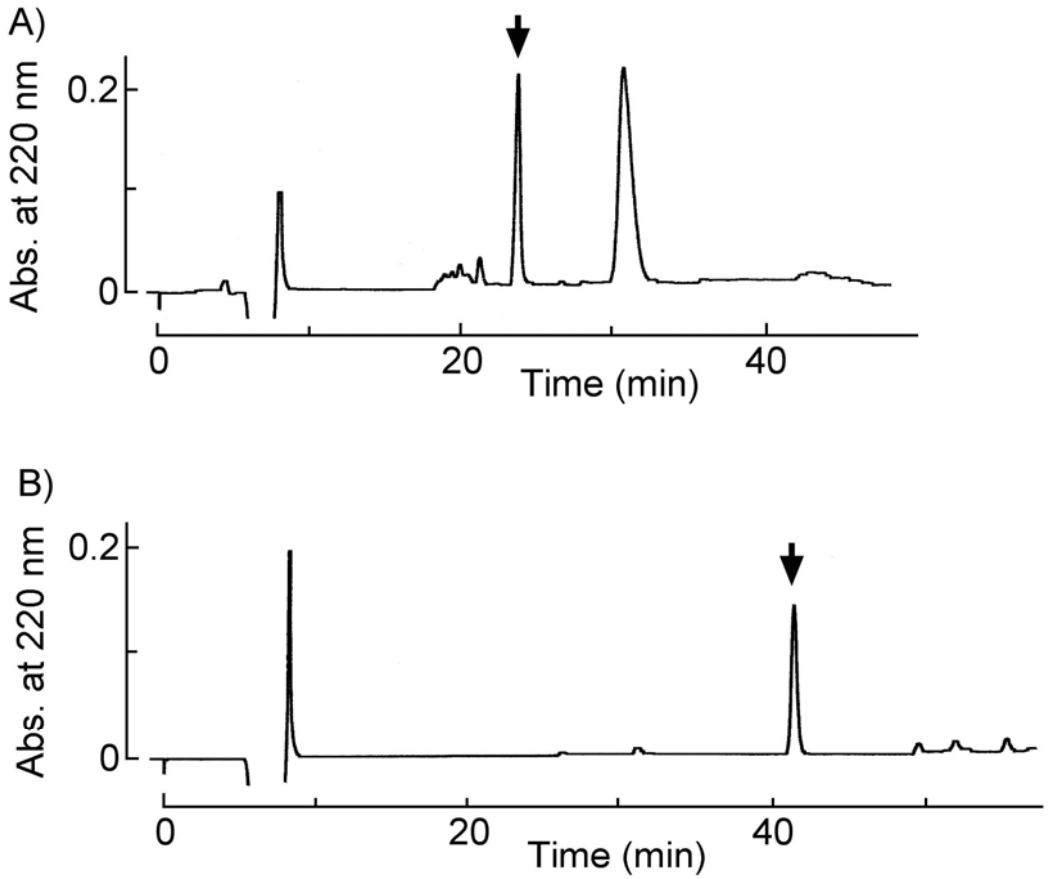

A)
Abs. at 220 nm
0.2
0
0
20
40
Time (min)
B)
Abs. at 220 nm
0.2
0
0
20
40
Time (min)

Fig. 1

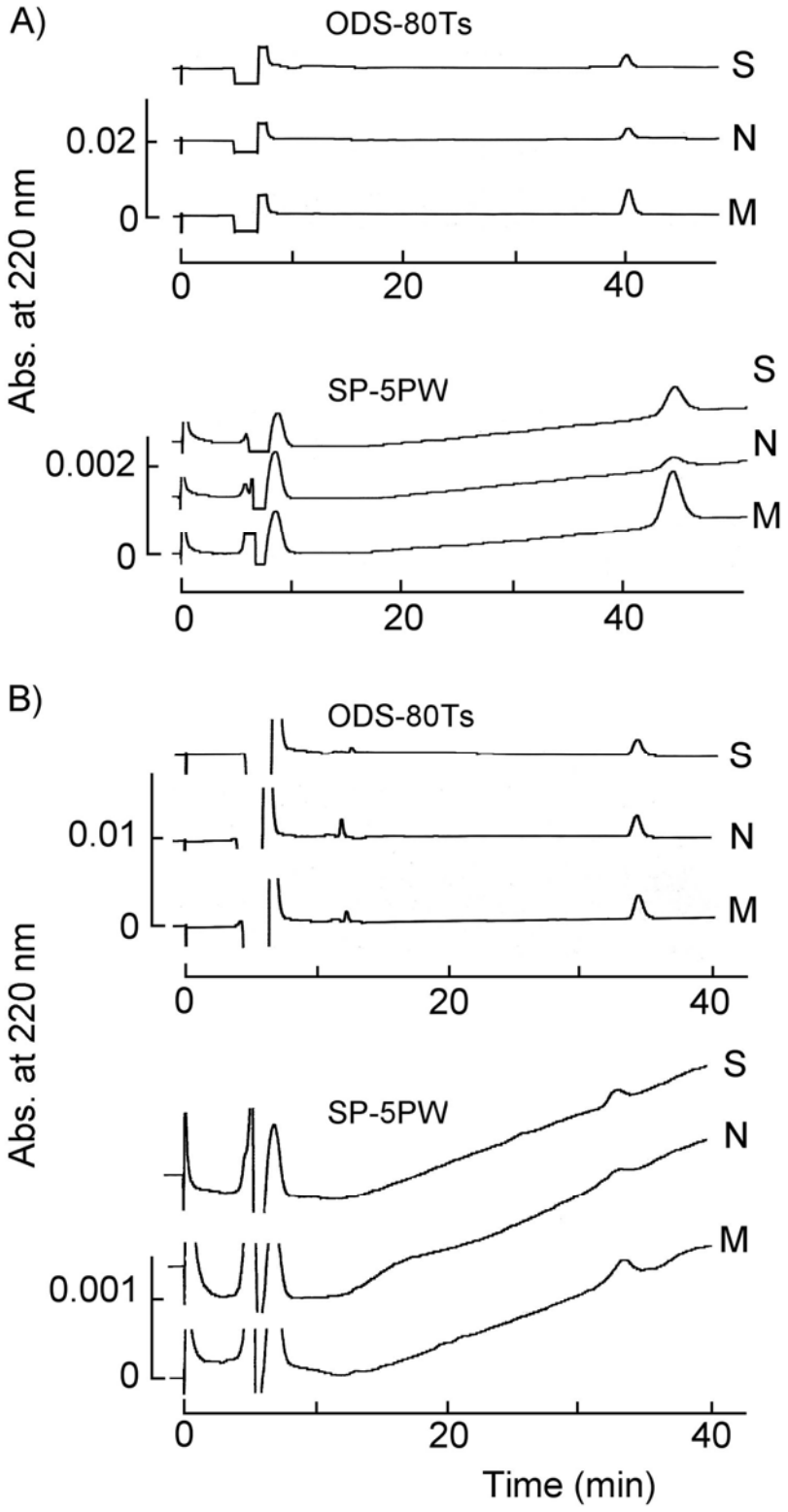

A)
ODS-80Ts
S
N
M
0.02
0
Abs. at 220 nm
0
20
40
SP-5PW
S
N
M
0.002
0
0
20
40
B)
ODS-80Ts
S
N
M
0.01
0
Abs. at 220 nm
0
20
40
SP-5PW
S
N
M
0.001
0
0
20
40
Time (min)

Fig. 2

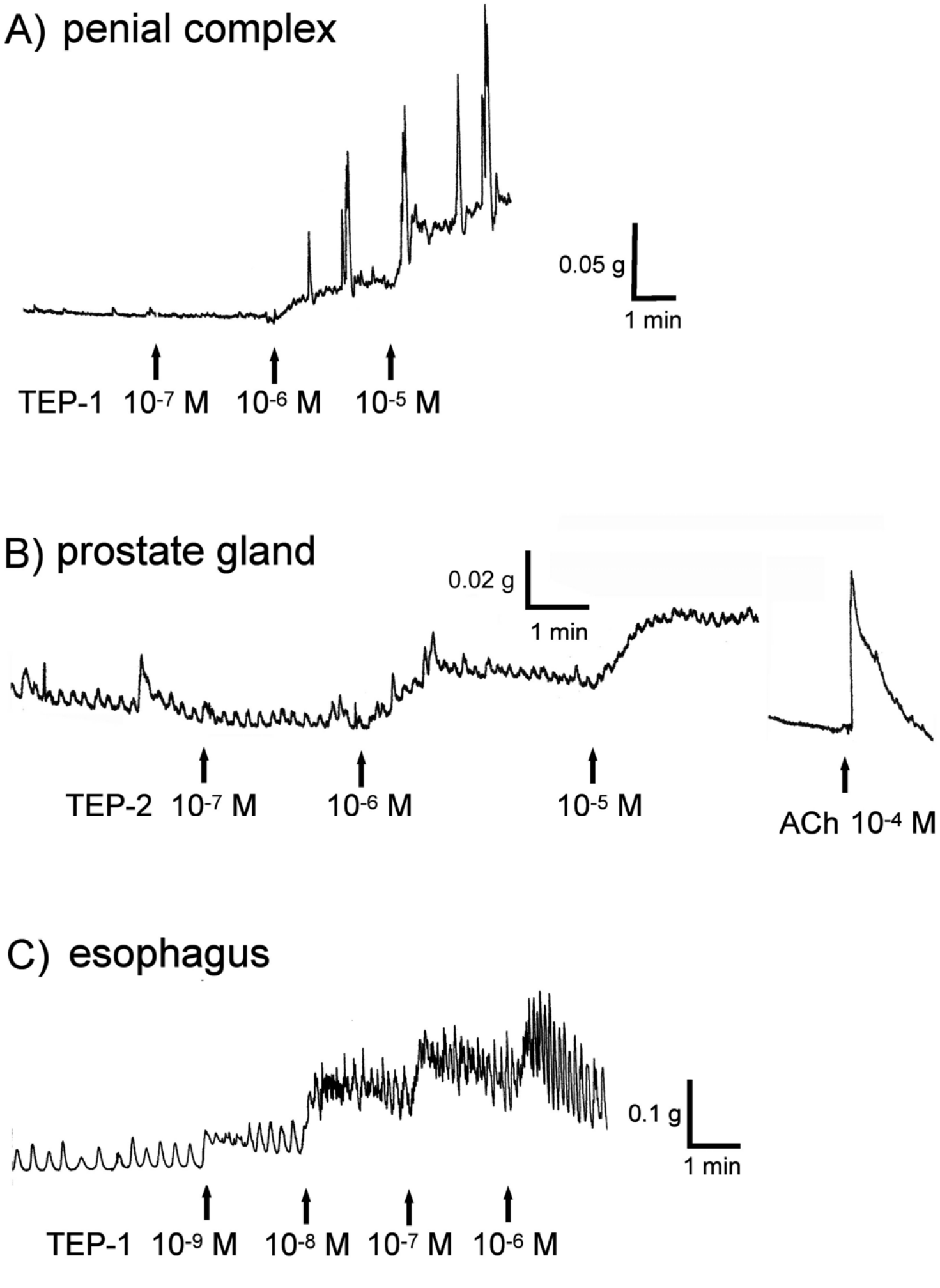

A) penial complex
0.05 g
1 min
TEP-1 10-7 M 10-6 M 10-5 M
B) prostate gland
0.02 g
1 min
TEP-2 10-7 M 10-6 M 10-5 M
ACh 10-4 M
C) esophagus
0.1 g
1 min
TEP-1 10-9 M 10-8 M 10-7 M 10-6 M

Fig. 3

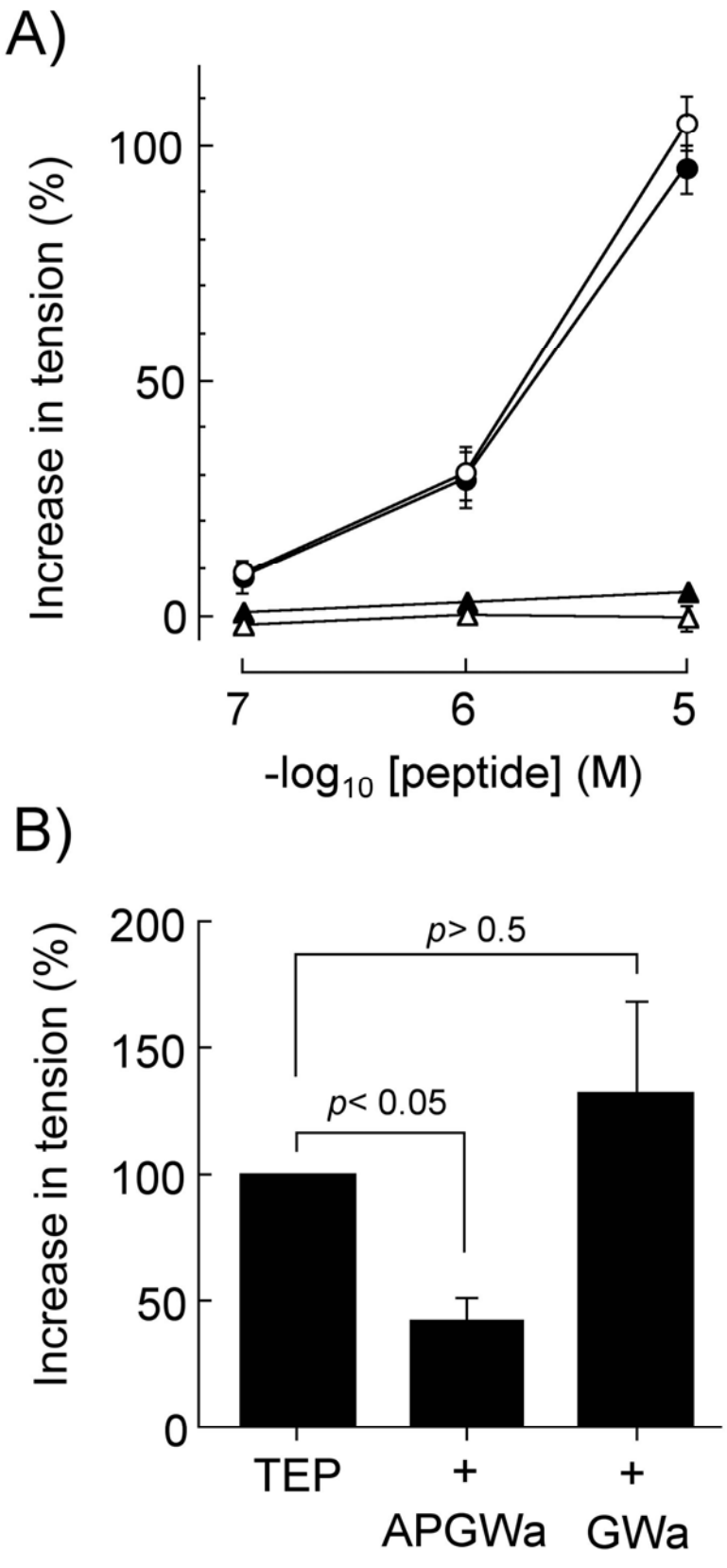

A)
Increase in tension (%)
100
50
0
7
6
5
-log10 [peptide] (M)
B)
Increase in tension (%)
200
150
100
50
0
p> 0.5
p< 0.05
TEP
+
APGWa
+
GWa

Fig. 4

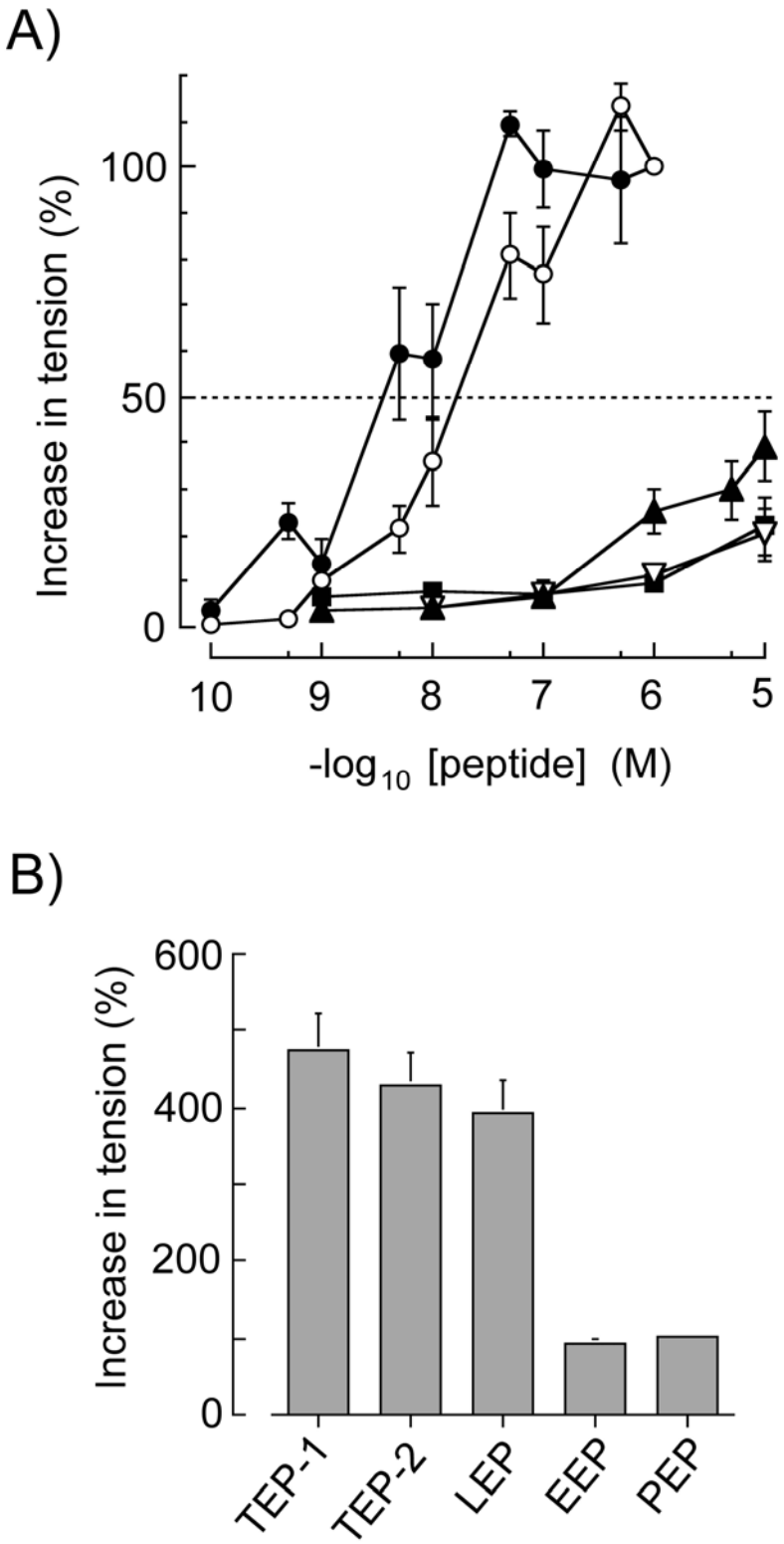

A)
Increase in tension (%)
100
50
0
10
9
8
7
6
5
-log10 [peptide] (M)
B)
Increase in tension (%)
600
400
200
0
TEP-1
TEP-2
LEP
EEP
PEP

Fig. 5

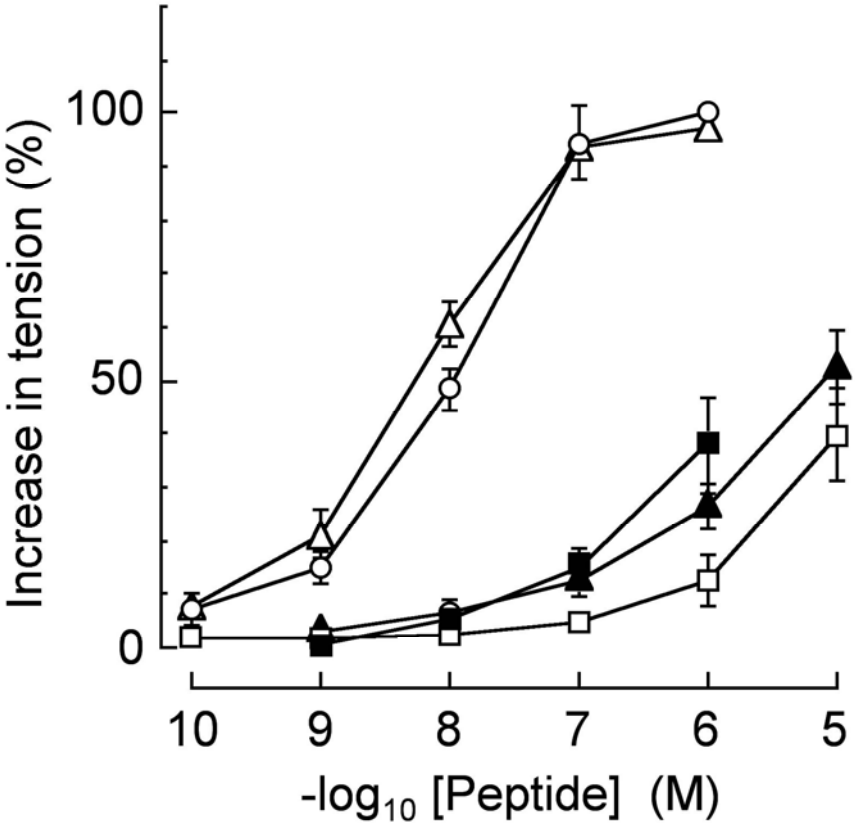


Fig. 6

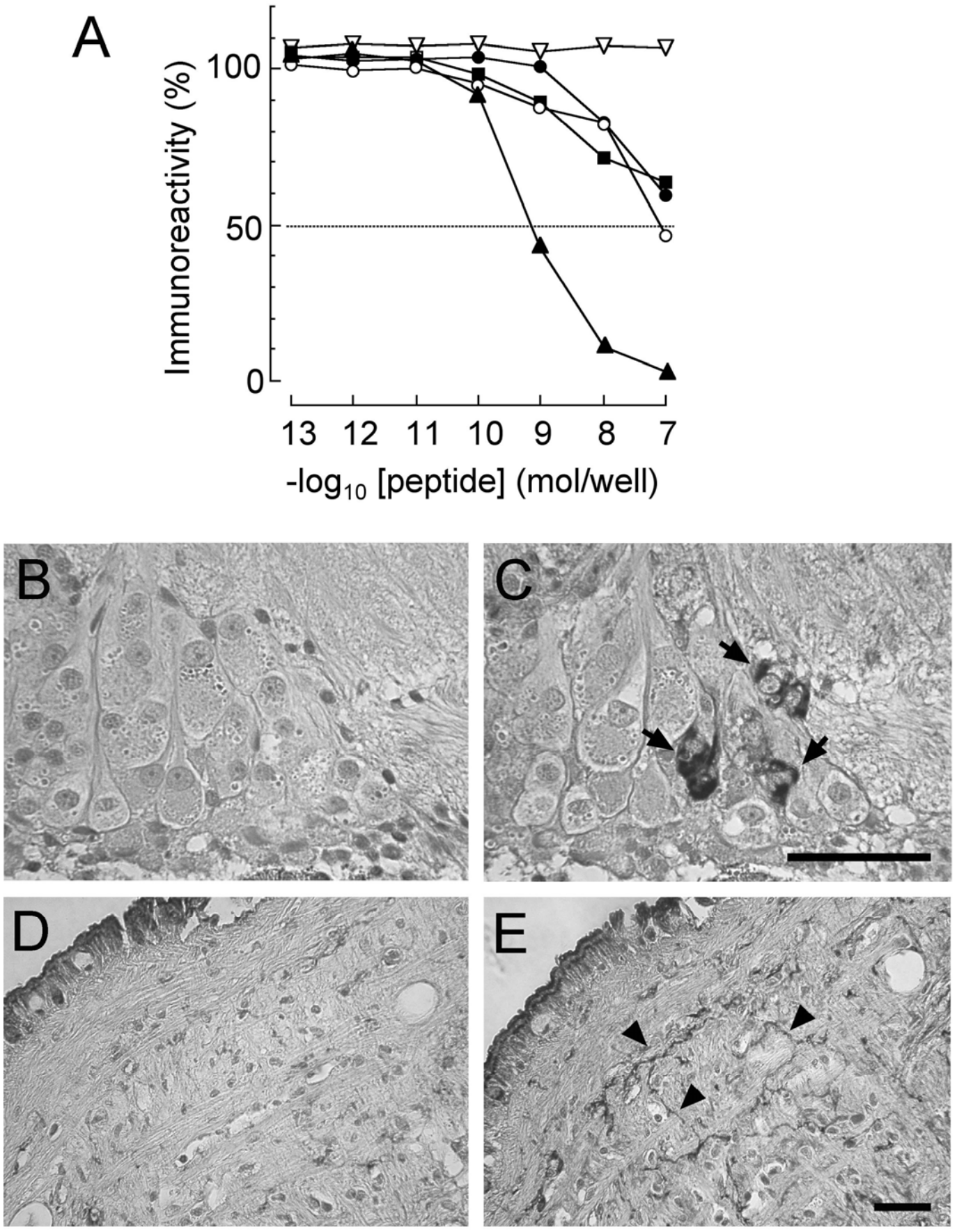

A
Immunoreactivity (%)
100
50
0
13
12
11
10
9
8
7
-log10 [peptide] (mol/well)
B
C
D
E

Fig. 7