MODULATION OF CILIARY BEAT FREQUENCY BY NEUROPEPTIDES FROM IDENTIFIED MOLLUSCAN NEURONS

A. O. DENNIS WILLOWS*, GALINA A. PAVLOVA† AND NICOLE E. PHILLIPS‡

University of Washington, Friday Harbor Laboratories, 620 University Road, Friday Harbor, WA 98250, USA

Accepted 28 February 1997

Summary

Prior work in the nudibranch Tritonia diomedea indicated that certain identifiable pedal ganglion neurons (Pd5 and 6) innervating the foot synthesize three novel peptides (TPeps) that resemble Pedal peptide (Pep) identified in the sea hare Aplysia californica. We report here that when TPeps are applied directly to isolated ciliated patches of Tritonia diomedea foot epithelium, there is an increase in ciliary beating that normally drives locomotion. Exposure to TPeps also increases the ciliary beat frequency of cells isolated from the pedal epithelium, suggesting that the observed ciliomotor effects are direct and not mediated by intervening cells. Antibodies to TPep bind to specific cells of the brain and foot and to ciliated peripheral tissues in Tritonia diomedea and in the pulmonate gastropod Lymnaea stagnalis. We suggest, therefore, that TPeps may regulate the activity of ciliated cells responsible for pedal locomotion and other functions in gastropod molluscs.

Key words: ciliated epithelia, ciliary beating rate, neuropeptides, TPeps, molluscs, Tritonia diomedea, neural control.

Introduction

Despite the ubiquity and fundamental importance of ciliated cells in animals, little is known of the underlying neural and endocrine mechanisms that regulate their activity. There is evidence that the neurotransmitter serotonin (5-HT) has a direct excitatory influence on ciliary beat frequency, both in intact epithelia and in isolated cells (Buznikov and Manukhin, 1962; Audesirk et al. 1979; Caunce et al. 1988; Syed et al. 1988; Goldberg et al. 1994). In addition, externally applied adenosine triphosphate, nitric oxide, acetylcholine, methacholine, substance P and adrenoceptor agonists stimulate ciliary transport (Dirksen and Sanderson, 1990; Aiello et al. 1991; Jain et al. 1993; Lindberg and Dolata, 1993; Mars et al. 1995; Tarasiuk et al. 1995). However, except for 5-HT, it is not known which of these effects are direct (as opposed to being mediated by intervening cells) and which, if any, mimic natural physiological mechanisms.

The bilaterally paired pedal ganglia of gastropod molluscs mediate the sensory and motor processes of the ciliated foot epithelium upon which these animals crawl (Kaiser, 1960; Caunce et al. 1988; Deliagina and Orlovsky, 1990; Lohmann et al. 1991; Murray and Willows, 1996). In Tritonia diomedea and other opisthobranch molluscs, two pairs of unusually large (>500 μm in diameter) and distinctively pigmented (white, under epi-illumination) neurons (Pd5 and 6) are identifiable in each of these ganglia (Willows et al. 1973; Willows, 1985). Previous work indicated that these neurons send axonal processes ipsilaterally in the peripheral left and right pedal nerves into the foot of the animal (Willows et al. 1973; Willows, 1985). Stimulating and recording from neurons Pd5 and 6 in semi-intact animal preparations indicate that both these and another identifiable pair of neurons (Pd21) drive an increase in ciliary beat frequency (Audesirk, 1978a,b; Cain and Foerstmann, 1996; von Dassow and Popescu, 1996). We have also found that neurons Pd5 and 6 are electrophysiologically active during behavioral responses to water currents and geomagnetic fields (Lohmann et al. 1991; Murray and Willows, 1996). Thus, there is both physiological and anatomical evidence suggesting that neurons Pd5 and 6 have a role in the orientation and locomotion of Tritonia diomedea.

Recent work has shown that Pd5 and 6 synthesize three similar 15-amino-acid peptides called TPeps-NLS, -PLS and -PAR (Lloyd et al. 1996) which differ from each other by substitution of residues N-P, L-A and S-R at three sites (positions 1, 8 and 12, respectively, from the N terminus). Of the total TPep length of 15 residues, seven are identical with the neuropeptide Pep (here called APep to distinguish it from...

Hall and Lloyd (1990) reported that one physiological function of APeP is to mediate locomotory behavior by modulating the muscular contraction waves over the surface of the foot, which drive crawling in Aplysia californica. In contrast, crawling in Tritonia diomedea is propelled by cilia beating in a thin mucus layer secreted on the surface of the foot (Audesirk, 1978a,b), and contractile waves have not been observed to contribute to this process. Thus, physiological roles for the TPEps synthesized by Pd5 and 6 have not been established.

We report here that TPEps increase cilary beat frequency on isolated patches of Tritonia diomedea pedal epithelium and also on isolated single cells. Serotonin, which promotes ciliary beating in other species, also elicits responses in Tritonia diomedea over a similar range of concentrations. Other molluscan peptides (APep, SCPb, FMRFamide) and seawater controls elicited no significant changes in ciliary beating in epithelial patches and in isolated Tritonia diomedea cells. We also report immunohistological evidence that TPEp-like peptides exist in specific brain cells, axons and target tissues in Tritonia diomedea and in the pulmonate gastropod Lymnaea stagnalis.

Materials and methods
Influence of TPEps on isolated ciliated epithelium
Tritonia diomedea Bergh (37–350 g) were trawled from Bellingham Bay or captured using SCUBA near Tacoma, WA, USA, and maintained in aquaria with continuously flowing sea water at the Friday Harbor Laboratories. All were fed on sea pens (Ptilosarcus gurneyi). Specimens of the pulmonate gastropod snail Lymnaea sp. were collected in freshwater ponds on San Juan Island, WA, USA, maintained in aquaria filled with pond water and fed on lettuce.

To measure the effects of various peptides on the cilia of the foot of Tritonia diomedea, we dissected 1.5 cm x 0.5 cm patches of foot epithelium and pinned them into Sylgard-coated Petri dishes with the ciliated surface uppermost and immersed in 5 ml of sea water (SW). Multiple patches (3–6) from one or two animals were pinned in each dish. Each dish was considered as a separate experiment. For thermal stability, the dishes were suspended in a 21 aquarium in continuously flowing SW at 13°C. Ciliary activity was monitored by direct measurement of the rate of transport of a 1 μl droplet of Sumi drawing ink for more than 5 min after solution changes. This ink tends to remain in an intact droplet, forming a visible ‘front’ whose movement can be precisely monitored against landmarks at measured intervals on the ciliated epithelium. Preparations were selected for moderately slow basal transport rate (0.01–0.06 cm s⁻¹, from a range of 0–0.3 cm s⁻¹) and, if stored at 5°C, were robust and stable for 3–5 days. Storage of preparations in SW at 5°C for 24 h prior to experiments cleared mucus secretions from patches and prevented them from interfering with transport rate measurements. Ink transport was video-taped, and measurements were made blind from these tapes at a later time.

Samples consisting of 10 μl of filtered sea water (FSW) containing serial dilutions of 50 nmoles of peptide or control samples of 10 μl of SW were pipetted directly into the 5 ml dish. This permitted mixing to the desired final concentration (by diffusion and the mixing caused by the beating of the cilia themselves) in under 30 s judging by the addition of visible dyes. We exposed patches to peptides and controls in the following sequence: SW wash (three times, 5 min each), 10 μl SW control added; 5 min equilibration, ink added, response video-recorded; 10 μl peptide added; wait 5 min, ink added, response recorded; SW wash (three times, 5 min each), then 10 μl of peptide added; 5 min of equilibration, ink added, response recorded; SW wash, etc. In experiments to determine the dose–response relationship, peptides or serotonin were added in ascending series of concentrations.

We exposed patches to 10 μmol l⁻¹ TPEp-NLS, -PLS and -PAR, APep and 5-HT using the same protocol. To test whether ciliated cells are sensitive to peptides non-specifically, we also tested SCPb and FMRFamide, two peptides of a similar size to the TPEps and of molluscan origin that are known to occur and to have active physiological roles in Tritonia diomedea and in other species (Lloyd, 1982; Church and Lloyd, 1991). To determine the relationship between ciliary beat frequency and T-Pep and 5-HT concentration, we also exposed patches to TPEp-NLS and 5-HT (0.01–10 μmol l⁻¹) following a similar protocol.

Action of TPEps on beating rate of isolated ciliated cells
Epithelial patches have adhering nerve and muscle tissue which might be sensitive to TPEps. It is possible that these tissues might indirectly elicit responses in ciliated cells of the epithelium. To distinguish between direct and indirect effects of peptides, we prepared isolated ciliated cells following the methods of Pavlova and Bakeeva (1993). Ciliated cells were isolated by manual dissection of the most superficial layer of the foot epithelium. Small pieces were repeatedly flushed in and out of the tip of a glass eye dropper in FSW to separate individual cells. Ciliated cells were then transferred into 20 μl of FSW on a microscope slide. Cottonwool fibers under the glass coverslip prevented cells from being washed away during solution changes. Individual, suitably anchored cells were observed under video-recorded differential interference contrast optics.

We were able to prepare and to make measurements on five isolated, single cells exposed to 10 μmol l⁻¹ TPEp-NLS, -PLS and -PAR, APep, SCPb and FMRFamide and two cells exposed to 10 μmol l⁻¹ 5-HT. For all experiments, FSW controls were alternated with peptides and with intervening FSW washes, as in the experiments on epithelial patches. To change solutions, 50 μl was placed drop by drop at one side of the coverslip while fluid was simultaneously drawn off by filter paper from the other side. All measurements were made at room temperature (18–20°C).
Ciliary beat frequency was determined by recording movement with a phototransistor placed on the video monitor directly over an active cell. The resulting signal was amplified and digitally filtered to separate raster scan components. Cells were found with spontaneous rates of 0–25 Hz. As in the experiments with patches, we selected for study those with intermediate (5–16 Hz) baseline rates.

Immunolabelling of brain cells and peripheral axons in Tritonia diomedea and other animals with TPep antiserum

Antiserum was made by N-terminal conjugation of a carrier protein (bovine serum albumin), to TPep-NLS, followed by immunization of two rabbits by R&R Rabbity (Stanwood, Washington, USA). Antisera harvested at 6, 8 and 12 weeks were compared by enzyme-linked immunosorbent assay (ELISA) and found to be similarly active. Tissues were fixed for immunofluorescence studies in 4% paraformaldehyde in Sorenson’s buffer for 12 h, exposed for 24 h to primary antiserum diluted 1:500, then to secondary fluorescein-conjugated goat anti-rabbit immunoglobulin (Jackson Immunoresearch Labs, PO Box 9, West Grove, PA 19390, USA,) for 12 h, diluted 1:100. Controls in which tissues were not exposed to the antiserum and others in which the antiserum was pre-incubated with TPep-NLS (1 mmol l\(^{-1}\)) were made to test for non-specific immunolabelling. Observations were made using a Bio-Rad MRC 600 laser scanning confocal microscope (LSCM).

Results

Action of TPeps on isolated ciliated epithelium

The four patches described in Fig. 1 had basal transport rates between 0.03 and 0.06 cm s\(^{-1}\), a threshold response to TPep-NLS between 0.01 and 0.1 \(\mu\)mol l\(^{-1}\) and an increased transport rate of approximately 100% as the concentration increased from 0.01 to 10 \(\mu\)mol l\(^{-1}\). Exposure of patches to other peptides (in this case, SCPb and FMRFamide) at comparable concentrations after TPep-NLS did not elicit increases in transport rate. Although SCPb and FMRFamide appear to have reduced the transport rate slightly in Fig. 1A, this reduction was not consistently observed with different patches (the averaged responses of different patches shown in Fig. 3B indicate no significant rate change). Commonly, the rate increase of individual patches reached a plateau between 1 and 10 \(\mu\)mol l\(^{-1}\). Individual patches responded consistently both over time and to peptide exposure (Fig. 1B). SW controls did not elicit detectable rate changes. We observed that ink alone had no effect on inactive cilia, nor did serial ink applications elicit any cumulative change in transport rate. Thus, in Fig. 1B, each bar represents an average of 9–14 serial ink exposures. The standard deviation within each series is small, indicating little change from one SW exposure to the next in the series.

The ink transport rate also increased in response to serotonin over the same range of concentrations (Fig. 2A). The threshold for the response was between 0.01 and 0.1 \(\mu\)mol l\(^{-1}\), the transport rate increased by up to 500% over the range 0.01–10 \(\mu\)mol l\(^{-1}\), and the dose–response relationships were similarly variable, with recovery to basal rate after a 5 min SW wash.

We also looked for differences between sensitivities to the three TPep variants identified in Pd5 and 6 (Fig. 2B). Individual patches exposed sequentially to 10 \(\mu\)mol l\(^{-1}\) TPep-PLS, -PAR and -NLS responded similarly, although perhaps with a greater increase in response to TPep-NLS. In this experiment, we also compared the effects of exposure to TPeps and APep. We observed no consistent response of the Tritonia diomedea tissues to APep. In three other experiments (not shown), we observed the same dose–response relationship to the three TPeps, with thresholds between 0.01 and 0.1 \(\mu\)mol l\(^{-1}\), and an increased transport rate of approximately 100% as the concentration increased from 0.01 \(\mu\)mol l\(^{-1}\) to saturation at approximately 10 \(\mu\)mol l\(^{-1}\).

Fig. 1. Responses of ciliated patches of pedal epithelium to TPep exposure. (A) The rate of ink transport of four patches from one animal in response to seawater (SW) and peptide exposures at the concentrations indicated, with intervening 5 min washes. Patches have basal rates varying from 0.03 to 0.06 cm s\(^{-1}\) and threshold responses at 0.01–0.1 \(\mu\)mol l\(^{-1}\), with the response rates approximately doubling as peptide concentration increases from 0.01 to 10 \(\mu\)mol l\(^{-1}\). Sea water added to the bath in the absence of TPeps and the addition of the molluscan peptides SCPb and FMRFamide (both at 10 \(\mu\)mol l\(^{-1}\)) elicit no comparable change in ciliary transport. (B) Mean responses + s.d. (9–14 measurements each, made at 1 min intervals) of three patches exposed to SW, then TPep-NLS (10 \(\mu\)mol l\(^{-1}\)), and then SW again after a 15 min wash. Repeated measurements (time taken for 1 cm of transport) are different for each patch, with little variation within each series for any patch or treatment.
Fig. 2. Responses of individual ciliated patches to serotonin (5-HT), TPeps (PLS, PAR and NLS) and APep. (A) Transport rate increases as 5-HT concentration increases from 0.01 to 10 μmol l\(^{-1}\) (threshold 0.01–0.1 μmol l\(^{-1}\)) and returns to the baseline level in SW. (B) Exposure to peptides TPep-PLS, -PAR and -NLS and to APep (10 μmol l\(^{-1}\)) alternating with SW controls. The transport rate increases (two- to threefold) by a comparable amount in all three TPeps (five patches), but no comparable change is observed in the presence of APep or in the SW controls.

Since the responses of individual patches were variable, we also determined averaged aggregate responses of several patches taken from different animals (Fig. 3). Baseline transport rate measurements were made during SW control exposures before and after peptide exposure (10 μmol l\(^{-1}\)) and the two were averaged. Changes were calculated for each patch separately, then averaged for all responses. We observed a large difference (>50% increase) between the responses to all TPeps and that of the SW controls. Three other molluscan peptides (APep, SCPb and FMRFamide) produced no significant change in transport rate at this concentration.

The average dose–response relationships for TPep-NLS and serotonin were found to be similar, with a response threshold of approximately 0.01–0.1 μmol l\(^{-1}\) and saturation of the response at 1–10 μmol l\(^{-1}\) (Fig. 3A).

**Fig. 3.** Mean changes in transport rate of ciliated patches of foot epithelium exposed to peptides and serotonin (5-HT). (A) Mean values (+s.d.) of the percentage increase in the rate of ink transport by isolated patches exposed to increasing concentrations of TPep-NLS (N=5) and 5-HT (N=13). The threshold of the response for both peptides is 0.01 μmol l\(^{-1}\), and the peptides show similar responses up to concentrations of 10 μmol l\(^{-1}\) where the transport rate is increased by more than 60%. (B) Changes in the rate of transport elicited by TPep-PLS, -PAR and -NLS (10 μmol l\(^{-1}\)). All three peptides cause increases of more than 50%, (N=21); peptides APep, SCPb and FMRFamide elicit no comparable increase (N=5).

**Action of TPeps on beating rate of isolated ciliated cells**

The direct effects of TPep-NLS, 5-HT, SCPb and FMRFamide on the beating rate of an isolated ciliated cell were measured by optical recording as illustrated in Fig. 4. We observed that, as with patches, TPeps and 5-HT promote increased activity (>100% in the examples shown in Fig. 4B,C). We exposed five individual cells to 10 μmol l\(^{-1}\) TPep-NLS and observed 50–150% increases in ciliary beat frequency over SW controls in each case. In other experiments (not shown), similar excitatory responses were observed to TPep-PLS and -PAR. As with patches, although SCPb and FMRFamide produced small frequency changes (e.g. Figs 3B, 4D), no substantial nor consistent changes in ciliary beat frequency were observed. Changing solutions sometimes elicited a transient change in beating rate, due presumably to direct mechanical disturbance; however, this did not persist for more than a few seconds.

**Immunolabelling brain cells and peripheral axons with TPep antiserum**

To begin to ascertain the extent of the phylogenetic distribution of TPep-like peptides, we prepared an antiserum to TPep-NLS in rabbits, which was then tested against diverse cells and tissues from Tritonia diomedea and Lymnaea stagnalis.

Using indirect immunofluorescence, we observed cell-specific binding of TPep antiserum to neurons in the brain and to cells in the oviduct and foot in both species. In Tritonia diomedea brain, we observed specific immunoreaction in Pd5 and 6 and in other brain cells, including Pd7 (Fig. 5A) and several other neurons of unknown function that form a loose cluster on the ventral surface of the pedal ganglia (Fig. 5B). Additionally, a smaller number of cerebral, pleural and buccal ganglion cells of unknown function stained consistently. Tissue was also examined to look for axons of immunoreactive brain cells, centrally and in peripheral nerves and in other tissues. Labelled axons were observed emerging from labelled cell bodies, within nerve trunks (Fig. 5A,B) and in the foot epithelium of Tritonia diomedea (Fig. 5C). These latter fibers ended in a meshwork near the basal surfaces of the ciliated epithelium. We also observed immunoreactive nerve fibres in the oviduct of Tritonia diomedea (not shown) similar to those shown in Fig. 5F for Lymnaea stagnalis.
To determine whether TPep-like molecules occur in other gastropods, we incubated the central nervous system, foot and oviducts of the pond snail *Lymnaea stagnalis* with antiserum against TPep-NLS. We found strong immunoreactivity especially in the pedal (Fig. 5E) and buccal ganglia and in their axonal fibres in three pairs of pedal nerves known to innervate the rostral medial and caudal regions of the foot. Fewer (2–6) neurons were immunolabelled in each of the cerebral, pleural, parietal and visceral ganglia. We also observed peripheral axonal fibers ending in the pedal epithelium (Fig. 5D) and in the oviduct (Fig. 5F) of *Lymnaea stagnalis* adjacent, in both cases, to ciliated epithelium.

In all experiments, two standard controls for specificity of immunolabelling were used. None of the cells or structures mentioned was labelled if the primary antiserum was omitted from the technique or if the primary antiserum was exposed overnight to 100 μmol l⁻¹ TPep-NLS, prior to incubation with the tissue.

**Discussion**

There is physiological and anatomical evidence that certain pedal neurons have a role in the orientation and locomotion of *Tritonia diomedea* (Audesirk, 1978a,b; Lohmann et al. 1991; Murray and Willows, 1996). Furthermore, we have reported that Pd5 and 6, and other pedal ganglion neurons that make TPePs, control pedal movement by regulating the beating of cilia on the surface of the foot.

We found that responses of both single patches and single cells of the foot epithelium of *Tritonia diomedea* to TPePs and serotonin were uniformly and vigorously excitatory. Furthermore, these ciliated cells were also excited by TPePs and serotonin when no other adhering cell types were present. This clearly implies that the observed actions of peptide and serotonin are direct and not mediated by intervening cells. Previous work with serotonin in other ciliated cell types suggests that cilioexcitation is driven by membrane hyperpolarization (Eckert, 1972). We suggest, therefore, that TPePs and serotonin act upon receptors endogenous to the ciliated cell membranes themselves, perhaps producing changes in membrane potential that increase the ciliary beat frequency.

Responses to 5-HT were similar to those elicited by TPePs. It is unclear, however, whether the mechanisms mediating these two kinds of responses are distinct. Significantly, the identified neuron pair Pd21, reported by Audesirk (1978a,b) to control pedal ciliary beating in semi-intact animal preparations, has been shown by direct chemical assay of isolated cells to contain serotonin (Audesirk et al. 1979). Furthermore, serotonin was shown to be cilioexcitatory when injected into the animal or applied to foot patches. The present results suggest that TPePs may also be involved in the same process. Clearly, experiments are needed to look for interactions
between these two neuroactive substances on their apparently common ciliated target cells.

We found differences in the basal transport rates and sensitivities of patches and isolated cells within and between animals. Size, maturity and other characteristics of different individuals may be important in the level of ciliary activity and responsiveness. In addition, there may be regional specializations of the foot in terms of the types of ciliated cells present, the level of their sensitivity to neuroactive substances, and the local density of ciliated cells. Future work is needed to determine whether the observed differences between patches are systematically related to the site from which the patch was taken. Such differences would be consistent with regional specialization of the foot for aspects of locomotion (sensory specializations of the leading and marginal edges, turning, mucus secretion, etc.).

We observed relatively small differences in the responses to the three T Pep variants (NLS, PLS, PAR), but a very large difference between the responses to all of these and the response to A Pep.

T Peps and A Pep share the same amino-acid sequence for the first four amino acids at the C terminus. At the N terminus, however, the second, fourth, fifth and sixth residues, although identical between all three T Peps, differ between the T Peps and A Pep (Lloyd et al. 1996). Given the similar responses of patches and single cells to all three T Peps and the marked lack of sensitivity to A Pep, we suggest that the N terminus may be important in receptor binding on ciliated cells of the foot of Tritonia diomedea.

Previous work with Aplysia californica (Hall and Lloyd, 1990) indicates that A Pep modulates foot muscle contractions. Specifically, it increases the amplitude and the relaxation rate of the contraction waves responsible for crawling. In Tritonia diomedea, however, crawling does not involve muscle waves (Audesirk, 1978a; Audesirk et al. 1979) and we observed no evidence of muscular waves during exposure to T Peps (or
Neuropeptide control of ciliary beating rate 1439


References


