

ANTAGONISTIC FREQUENCY TUNING OF HAIR BUNDLES BY DIFFERENT CHEMORECEPTORS REGULATES NEMATOCYST DISCHARGE

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Summary

Sea anemones capture prey by discharging nematocysts into them. Chemical and mechanical cues identify suitable prey to sensory receptor systems on the anemone. Conjugated *N*-acetylated sugars from prey bind to chemoreceptors on cnidocyte/supporting cell complexes to tune hair bundles on the complexes to lower frequencies matching prey movements. The hair bundles regulate discharge of microbasic p-mastigophore nematocysts into vibrating targets. Provided that proline receptors are activated after those for *N*-acetylated sugars, nematocyst discharge is tuned to much higher frequencies. Thus, anemone hair bundles are tuned to either higher or lower frequencies by antagonistic chemoreceptors. Chemoreceptors for proline can adapt to $10^{-8} \text{ mol l}^{-1}$ proline and yet respond to increases in proline concentration of less than $10^{-15} \text{ mol l}^{-1}$. Under these conditions, too few molecules of proline are added to activate chemoreceptors on all responding cnidocyte/supporting cell complexes. Evidence indicates that the extreme sensitivity of anemones to proline may be attributed, in part, to intercellular communication.

Introduction

Cnidarians, including jellyfish, hydra, corals and sea anemones, capture prey with nematocysts and other cnidae, intracellular capsules containing eversible tubules (Skaer and Picken, 1965). Suitable prey present chemical and mechanical stimuli to a consortium of sensory receptors on the cnidarian (Thorington and Hessinger, 1988*a,b*; Watson and Hessinger, 1987, 1989*a,b*) that trigger 'discharge', an explosive eversion of the tubule (Holstein and Tardent, 1984). The discharging cnida may inject potent toxins into the prey (Hessinger, 1988), adhere to its surface or entangle its appendages, depending on the type of nematocyst or other cnida (Mariscal, 1974).

In tentacles of sea anemones, regulation of cnida discharge has been studied most thoroughly for microbasic p-mastigophore (mpm) nematocysts. It appears that discharge

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of this cnida type is regulated by cnidocyte/supporting cell complexes (CSCCs). As suitable prey approach the tentacle, conjugated *N*-acetylated sugars from the surface of the prey activate chemoreceptors on supporting cells. These chemoreceptors regulate discharge from mpm cnidocytes by modulating two distinct mechanoreceptors: vibration-sensitive mechanoreceptors called anemone hair bundles and contact-sensitive mechanoreceptors (Thorington and Hessinger, 1988a; Watson and Hessinger, 1989a,b). At picomolar concentrations, *N*-acetylated sugars shift discharge of mpm nematocysts from maxima at 50–55, 65 and 75Hz to 5, 15, 30 and 40Hz, frequencies corresponding to the swimming movements of prey (Watson and Hessinger, 1989a, 1991). The shift in discharge to lower frequencies is accompanied by an elongation of hair bundles. In anemones, as in vertebrate systems, length correlates with frequency responsiveness such that longer hair bundles respond to lower frequencies (Frishkopf and DeRosier, 1983; Holton and Hudspeth, 1983; Watson and Hessinger, 1991). Experimentally disrupting hair bundles with cytochalasin B abolishes vibration-dependent discharge without significantly affecting discharge of mpm nematocysts into non-vibrating targets (Watson and Hessinger, 1991).

At nanomolar concentrations, *N*-acetylated sugars increase discharge of mpm nematocysts into non-vibrating targets; the effect is maximal at $10^{-7} \text{ mol l}^{-1}$. Interestingly, at higher than optimal ligand concentrations, discharge decreases to seawater control levels (Thorington and Hessinger, 1988a; Watson and Hessinger, 1989b). Time course experiments demonstrate a strong positive correlation between the presence of activated receptors at the surface of supporting cells and discharge of mpm nematocysts from cnidocytes (Watson and Hessinger, 1989b).

Massive cnida discharge is triggered immediately after prey contact the tentacle. Prey wounded by penetrant nematocysts (including mpm nematocysts) release compounds, including glutathione and the amino acid proline (Lenhoff and Lindstedt, 1974). In sea anemones, activated chemoreceptors for proline and certain other amino compounds increase discharge of mpm nematocysts into non-vibrating targets (Thorington and Hessinger, 1988a). In the anemone *Haliplanella luciae*, proline increases discharge of mpm nematocysts to a maximum at $10^{-8} \text{ mol l}^{-1}$ and, at higher concentrations decreases discharge to seawater control levels (G. M. Watson, unpublished). In many cnidarians, activated chemoreceptors for glutathione induce behaviours associated with feeding, including mouth opening and tentacle writhing (the so-called feeding response) (Loomis, 1955). Proline induces the feeding response in some cnidarians (Fulton, 1963; Mariscal and Lenhoff, 1968). Interplay between different chemoreceptor systems is known to occur for the anemone *Anthopleura elegantissima*, in which asparagine controls tentacle movements to the mouth whereas reduced glutathione controls the ingestion of food at the mouth (Lindstedt, 1971). Thus, chemical and mechanical cues from prey regulate important behaviour associated with the capture and ingestion of prey by sea anemones and other cnidarians.

Having previously characterized the effects of activating receptors for *N*-acetylated sugars on vibration-dependent discharge of mpm nematocysts, we now examine the effects of activating receptors for proline by testing discharge at specified frequencies ranging from 5 to 100Hz and by measuring the length of hair bundles. Provided that

proline receptors are activated after those for *N*-acetylated sugars, discharge of mpm nematocysts is tuned to higher frequencies while hair bundles shorten.

Materials and methods

Materials

Pertussis toxin was obtained from Calbiochem, glutaraldehyde from Polysciences, *N*-acetylneuraminic acid (NANA) type VI, proline, dimethylpolysiloxane and reagents not otherwise specified were obtained from Sigma.

Animal maintenance

Monoclonal specimens of the sea anemone *Haliplanella luciae* (Verrill) were cultured in Pyrex dishes containing natural sea water, but tested in artificial sea water (ASW; Instant Ocean) or reagent grade ASW consisting (in mmol l⁻¹) of NaCl, 423; KCl, 9; MgCl₂, 23; MgSO₄, 26; and CaCl₂, 12; adjusted to pH8.3 with sodium bicarbonate. The animals were maintained on a 12h:12h light:dark photoperiod, cleaned daily, and fed brine shrimp nauplii (San Francisco Bay) twice weekly. Experiments were performed approximately 72h after feeding to maximize discharge of mpm nematocysts (Thorington and Hessinger, 1988b).

Measurement of hair bundles

Specimens were anaesthetized for 1h in artificial sea water containing equal parts of 0.6 mol l⁻¹ MgCl₂ and ASW (Mg/ASW). Excised tentacles were transferred to solutions of agonists prepared in Mg/ASW. Unless otherwise indicated, 10⁻⁷ mol l⁻¹ NANA and/or 10⁻⁸ mol l⁻¹ proline were used because each of these ligand concentrations induces maximal discharge of mpm nematocysts into non-vibrating test probes. After 5min in the agonist solution, tentacles were prepared as wet-mounts and the hair bundles were photographed in profile using phase-contrast optics at a final magnification of 100× (Nikon Diaphot, 40DL LWD objective) as previously described (Watson and Hessinger, 1991, 1992). Hair bundles were recognizable as triangular projections from the tentacle surface, with the kinocilium extending through the apex. For each agonist, the first 50 suitable bundles encountered by moving a 15× scale loupe around the periphery of the tentacle tip were measured directly from the photographic negatives to the nearest 1.0 μm. Suitable bundles were those appearing in focus along their total length and with clearly identifiable tips and bases. Tips of the bundles were characterized by a sharply defined abrupt termination of the stereocilium bundle from which the cilium projected. Using phase-contrast optics, the bases of the bundles were characterized by a consistent decrease in contrast at the proximal-most 1–2 μm of bundle length (Watson and Hessinger, 1991, 1992). Error caused by oblique orientations of the bundles was minimized by measuring bundles that were in focus along their total length.

Replicate experiments were performed blind with respect to treatment with live specimens photographed using differential interference contrast (DIC) optics (Olympus PlanApo100UV objective and a BH2-S DIC condensor modified to fit an Olympus IMT-

2F inverted microscope). Photographic negatives were scrambled before suitable bundles were measured using the methods described above.

Assay for testing nematocyst discharge

Animals transferred from culture dishes to 35mm diameter plastic Petri dishes (Falcon) filled with ASW were allowed 3–4h to recover normal responsiveness, at which point the ASW was replaced with test substances in ASW at specified concentrations. Unless otherwise indicated, 10^{-7} mol l⁻¹ NANA and/or 10^{-8} mol l⁻¹ proline were used for reasons outlined above. After 10min in the final test solution, anemone tentacles were touched with vibrating test probes.

Test probes consisted of 2cm segments of 0.14mm diameter nylon fishing line (Stren Brand, 2 lb test, DuPont) coated at one end with 30% (w/v) gelatin to a thickness of approximately 200 µm. The uncoated ends of vibrating test probes were inserted into glass capillary tubes (i.d. 0.27mm) that were made to oscillate using a galvanometer driven by a sine-wave generator as described previously (Watson and Hessinger, 1989a, 1991, 1992).

After contacting the tentacles, the gelatin-coated ends of the probes were fixed in 2.5 % glutaraldehyde in ASW for 1min. Probes were prepared as wet-mounts and the microbasic p-mastigophore nematocysts that had been discharged into the gelatin coating were counted in a single field of view at 400× magnification (0.16mm²) of an inverted phase contrast microscope. The field of view was placed on the region of probe that was subjectively evaluated as having the greatest number of nematocysts. For a given experiment, four replicate probes were used for each experimental condition, one probe for each of four anemones. Previously we found that averages of nematocyst counts calculated from a sample size of four test probes generate reproducible data. For presentation, data are depicted as the mean ± S.E.M. of two replicate experiments. Three other cnida types in the tentacle, spirocysts, basitrich nematocysts and small microbasic p-mastigophore nematocysts, also discharge into the test probes. Spirocyst capsules are poorly contrasted using phase-contrast microscopy, so investigations of spirocyst discharge have relied instead on a biochemical assay (Thorington and Hessinger, 1990). Because basitrich nematocysts are relatively rare, they have not yet received significant attention. The fourth type of cnida, a small ovoid microbasic p-mastigophore, was only very rarely observed on test probes. This nematocyst type is easily distinguished from the larger elongate microbasic p-mastigophore nematocysts which were more abundant. Data for nematocyst counts excluded the small mastigophore nematocysts.

Estimate of CSCC number

The number of CSCCs was estimated from serial cross sections (10 µm thick) of tentacles prepared as described in Watson and Mariscal (1983).

Analysis of data

Measurements of hair bundles

P values of less than 0.05 were considered significant. Data were subjected to one-way

analysis of variance (ANOVA; Sokal and Rohlf, 1969) to test for heterogeneity in the sample. Upon finding significant heterogeneity, multiple comparisons of the treatments *versus* a standard (seawater control) were performed by the Dunnett *t*-test (Zar, 1974), which utilizes differing critical values for each comparison in order to compensate for the loss of independence. In some instances, least-significant difference (LSD) *post-hoc* comparisons were performed between treatments after ANOVA using commercial software (CSS Statistica, Statsoft, Inc., Tulsa).

Nematocyst discharge

Mean nematocyst counts obtained at 5Hz intervals over the range 5–100Hz under different experimental conditions were subjected to one-way ANOVA (Sokal and Rohlf, 1969). Newman–Keuls tests (Sokal and Rohlf, 1969), which systematically compare pairs of mean values, were performed to identify significant maxima within the frequency responses of each treatment. Graphs were prepared using commercial software (Axum, Trimetrix, Inc.).

Results

Proline effects on bundle length

Hair bundles induced to elongate significantly by NANA were induced by subsequent proline exposure to shorten significantly to lengths comparable to, or less than, Mg/ASW controls (Fig. 1A,D). Proline alone had no significant effect on length of the hair bundles (Fig. 1B,E). Several other treatment regimes produced more variable results. For example, with simultaneous exposure to NANA and proline (Fig. 1C), proline blocked the elongation of hair bundles induced by NANA (cf. Fig. 1A). This block did not occur in the blind replicates (Fig. 1F). Exposure to NANA and proline after exposure to proline alone resulted in an elongation of bundles (compared with untreated controls) in one trial (Fig. 1E), but not in the other (Fig. 1B). Exposure to NANA alone after exposure to proline did not result in bundle elongation (Fig. 1E).

Proline effects on vibration-dependent discharge of nematocysts

Discharge of mpm nematocysts was assayed for the most consistent treatment regimes. With pre-exposure to NANA, proline shifted maximal discharge to frequencies that were 60Hz higher (65, 75, 90 and 100Hz; Fig. 2A) than those in NANA alone (5, 15, 30 and 40Hz; Fig. 2B). Proline alone did not significantly shift discharge maxima (50, 55, 60 and 75Hz; Fig. 2C) from those recorded in sea water (50, 55 and 75Hz; Fig. 2D). These maxima were determined by Newman–Keuls analyses of the data (Sokal and Rohlf, 1969).

Receptors for NANA and proline are antagonistic

Activating receptors for NANA induces hair bundles to elongate while tuning discharge of mpm nematocysts to lower frequencies (Figs 1A,D, 2B; Watson and Hessinger, 1991). Subsequently activating receptors for proline induces hair bundles to shorten while tuning discharge to higher frequencies (Figs 1A,D, 2A). Thus, receptors for

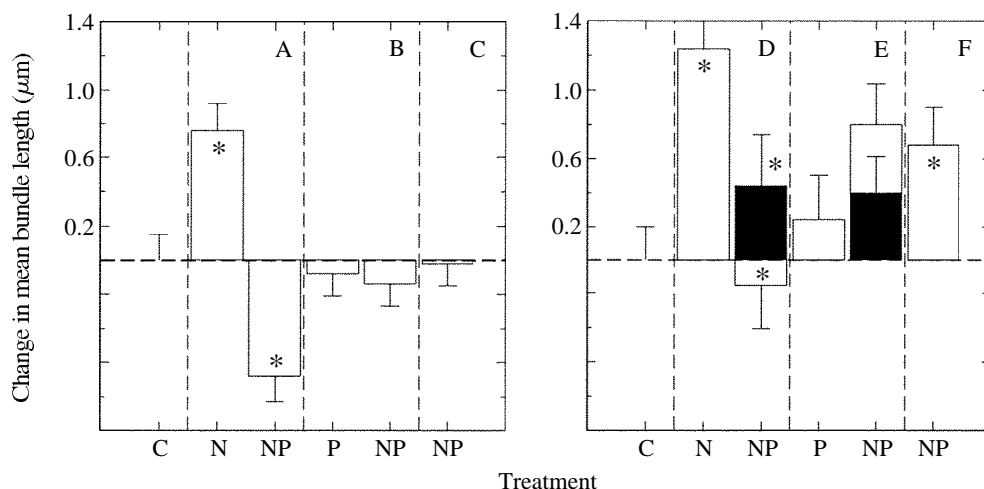


Fig. 1. Changes in hair bundle lengths following serial transfer to solutions of different agonists. Mean lengths of hair bundles on tentacles in magnesium/artificial seawater (Mg/ASW) controls (designated C on the x-axis of each panel) are compared with those either transferred directly to a mixed solution of $10^{-7} \text{ mol l}^{-1}$ *N*-acetylneuraminic acid (NANA) and $10^{-8} \text{ mol l}^{-1}$ proline (NP) or transferred first to $10^{-7} \text{ mol l}^{-1}$ NANA (N) or $10^{-8} \text{ mol l}^{-1}$ proline (P) and then to $10^{-7} \text{ mol l}^{-1}$ NANA and $10^{-8} \text{ mol l}^{-1}$ proline (NP) in Mg/ASW. Mean lengths of bundles in Mg/ASW controls were normalized to zero and the effects on bundle length of subsequent treatment are plotted as changes in mean length in μm (\pm S.E.M.). A–C represent the original data set, whereas D–F represent data from blind replicate experiments ($N=50$ bundles per treatment). The solid bar in D indicates bundle length for a specimen transferred from the NANA solution to proline alone. The solid bar in E indicates bundle length for a specimen transferred from the proline solution to NANA alone. Significant changes in bundle length compared with the previous treatment are indicated by asterisks.

NANA and proline exhibit antagonistic activities. The common point of interaction for these receptors may be G-protein regulation of adenylyl cyclase, since cholera toxin, forskolin and dibutyryl cyclic AMP induce bundles to elongate while tuning discharge to the same frequencies as NANA (Watson and Hessinger, 1992) and pertussis toxin blocks the effects of proline on vibration-dependent discharge (Fig. 3).

Dose-dependent effects of proline on nematocyst discharge

Discharge of mpm nematocysts was tested over a range of proline concentrations at frequency maxima determined previously for specimens treated with NANA (5, 15, 30 and 40 Hz; Fig. 2B). At these frequencies, proline had no effect at concentrations below $10^{-18} \text{ mol l}^{-1}$, but at higher concentrations it decreased discharge to control levels (filled circles in Fig. 4). However, at 15 Hz and at 40 Hz, second peaks of discharge appeared at $10^{-16} \text{ mol l}^{-1}$ and $10^{-12} \text{ mol l}^{-1}$ proline, respectively (Fig. 4B,D). These surprising results suggested that bundles induced to shorten by proline may become tuned to progressively higher frequencies as the proline concentration is increased. Thus, the second peak of discharge observed at $10^{-16} \text{ mol l}^{-1}$ proline for the 15 Hz probes (Fig. 4B) may have corresponded to the peak seen in NANA at 5 Hz (Fig. 2B). Similarly, the second peak of

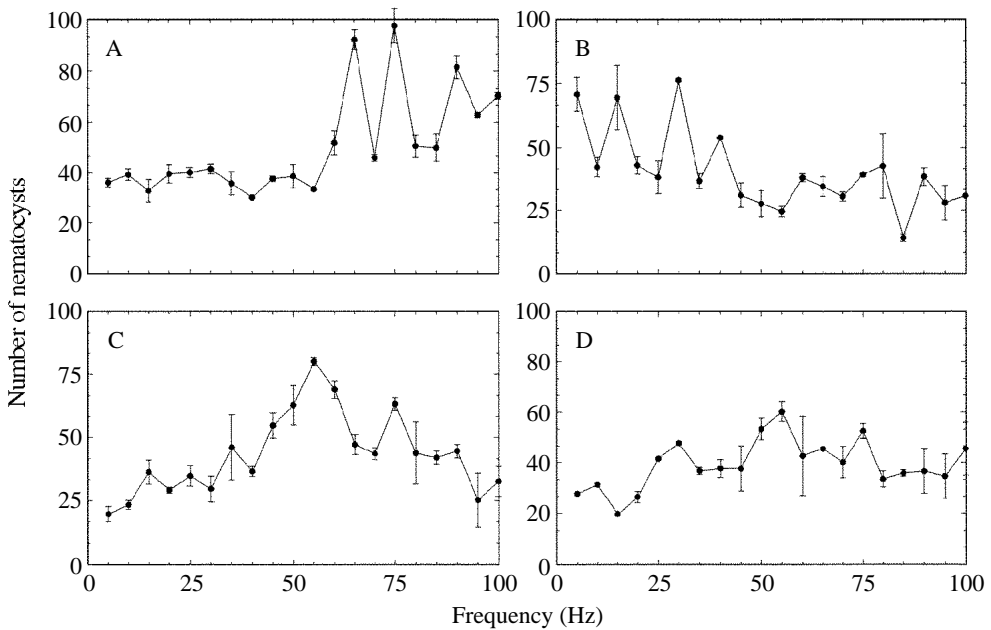


Fig. 2. Frequency-dependent discharge of microbasic p-mastigophore nematocysts. Tentacles were touched with gelatin-coated test probes after (A) 5min of exposure to artificial sea water (ASW) containing $10^{-7} \text{ mol l}^{-1}$ *N*-acetylneuraminic acid (NANA) followed by 10min of exposure to ASW containing $10^{-7} \text{ mol l}^{-1}$ NANA and $10^{-8} \text{ mol l}^{-1}$ proline, or after 10min of exposure to ASW containing (B) $10^{-7} \text{ mol l}^{-1}$ NANA, (C) $10^{-8} \text{ mol l}^{-1}$ proline or (D) ASW alone. Microbasic p-mastigophore (mpm) nematocysts that had been discharged into the probes were counted within a single microscopic field at $400\times$ total magnification. The field of view was chosen to maximize the number of nematocysts counted. Four probes were used for each experiment for each frequency. Points represent the mean maximum number of mpm nematocysts counted for two replicate experiments (\pm S.E.M.). Frequency optima described in the text were ascertained by Newman–Keuls analyses at the 0.05 level.

discharge observed at $10^{-12} \text{ mol l}^{-1}$ proline for the 40Hz probes (Fig. 4D) may have corresponded to the peak seen in NANA at 30Hz (Fig. 2B). To examine this possibility, discharge was tested at specific frequency minima (10, 23, 35 and 47Hz) intermediate to the frequency maxima described above. At these frequencies, NANA did not induce large increases in discharge. Proline had no effect on discharge at concentrations below $10^{-18} \text{ mol l}^{-1}$, but at higher concentrations it induced one (10 and 35Hz) or two (23 and 47Hz) peaks of discharge (open circles in Fig. 4). The peak of discharge at 10Hz occurred at $10^{-18} \text{ mol l}^{-1}$ proline, coinciding with the concentration that induced a decrease in discharge at 5Hz, but being lower than that required ($10^{-16} \text{ mol l}^{-1}$) to generate the second peak of discharge at 15Hz (Fig. 4A,B). At 23Hz, peaks of discharge were detected at $10^{-17} \text{ mol l}^{-1}$ and $10^{-12} \text{ mol l}^{-1}$ proline, concentrations higher than those required to generate peaks of discharge at 15Hz ($10^{-19} \text{ mol l}^{-1}$ or lower and $10^{-16} \text{ mol l}^{-1}$, respectively; Fig. 4B). The single peak of discharge at 35Hz occurred at $10^{-16} \text{ mol l}^{-1}$ proline (Fig. 4C), a concentration of proline lower than that required to

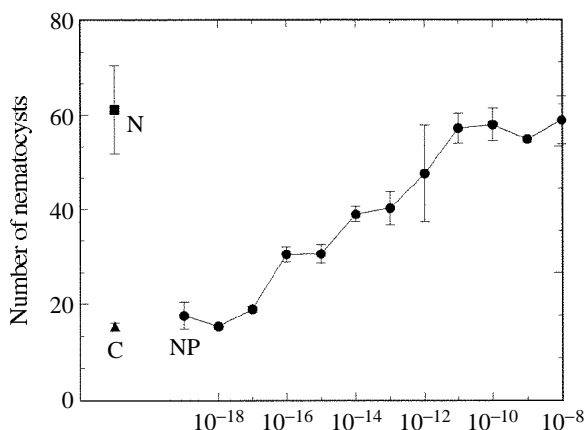


Fig. 3. Dose-response curve for discharge of microbasic p-mastigophore nematocysts in response to pertussis toxin (PTX). Test probes vibrating at 30Hz were touched to tentacles after 15min in ASW alone (designated C), after 15min in ASW containing $10^{-7} \text{ mol l}^{-1}$ *N*-acetylneuraminic acid (NANA, N), or after 5min in ASW containing $10^{-7} \text{ mol l}^{-1}$ NANA followed by 10min in $10^{-7} \text{ mol l}^{-1}$ NANA and $10^{-8} \text{ mol l}^{-1}$ proline (NP). The remaining points indicate data for specimens exposed for 5min to $10^{-7} \text{ mol l}^{-1}$ NANA followed by 10min to $10^{-7} \text{ mol l}^{-1}$ NANA, $10^{-8} \text{ mol l}^{-1}$ proline and PTX at the concentrations indicated. Microbasic p-mastigophore (mpm) nematocysts were counted as described in Fig. 2. Data points depict the mean maximum number of mpm nematocysts counted for two replicate experiments (\pm S.E.M.). Each experiment was based on four test probes per data point, one probe for each of four anemones.

produce the second peak of discharge at 40Hz ($10^{-12} \text{ mol l}^{-1}$; Fig. 4D). At 47Hz, peaks of discharge were detected at $10^{-17} \text{ mol l}^{-1}$ and $10^{-9} \text{ mol l}^{-1}$ proline, concentrations higher than those required to generate peaks of discharge at 40Hz ($10^{-19} \text{ mol l}^{-1}$ or lower and $10^{-12} \text{ mol l}^{-1}$; Fig. 4D). Thus, as the proline concentration is increased, discharge shifts to progressively higher frequencies over a range spanning tens of hertz.

Adaptation of proline-induced tuning

The extreme sensitivity of anemones to proline after pretreatment in NANA, as depicted in Fig. 4, raises the question of whether the cells involved respond to absolute concentrations of proline or to changes in concentrations such as would occur in natural sea water containing natural organic compounds. We incubated specimens for 3–4 h in natural sea water or in reagent grade ASW containing 10^{-12} – $10^{-8} \text{ mol l}^{-1}$ proline (adaptation medium), then tested discharge of mpm nematocysts at 10Hz after 5 min of exposure to $10^{-7} \text{ mol l}^{-1}$ NANA (prepared in adaptation medium) followed by 10 min of exposure to $10^{-7} \text{ mol l}^{-1}$ NANA and proline ranging in concentration from 10^{-20} to $10^{-16} \text{ mol l}^{-1}$ (added to adaptation medium). As was found for 10Hz stimulation in Instant Ocean ASW (Fig. 4A), a single peak of discharge occurred at $10^{-18} \text{ mol l}^{-1}$ added proline regardless of the initial proline concentration (Fig. 5). Therefore, cnidocyte/supporting cell

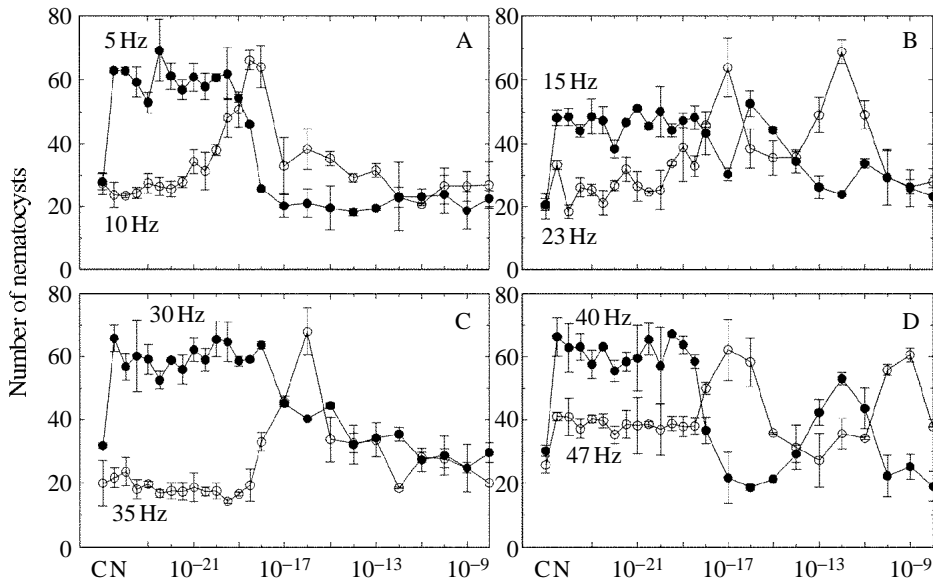


Fig. 4. Dose-dependent effects of proline on discharge of microbasic p-mastigophore nematocysts. Tentacles were touched with probes 15min after exposure to ASW alone (designated C on the x-axis of each panel), after 15min of exposure to 10^{-7} mol l $^{-1}$ *N*-acetylneuraminic acid (NANA, N), after 5min of exposure to ASW containing 10^{-7} mol l $^{-1}$ NANA followed by 10min of exposure to ASW containing 10^{-7} mol l $^{-1}$ NANA and proline at the specified concentrations ranging from 10^{-24} to 10^{-8} mol l $^{-1}$. Discharged microbasic p-mastigophore (mpm) nematocysts were counted for probes vibrating at (A) 5 and 10Hz, (B) 15 and 23Hz, (C) 30 and 35Hz and (D) 40 and 47Hz (filled and open circles, respectively). Four probes were used for each experimental condition (dose and frequency). Data points depict the mean maximum number of mpm nematocysts counted for two replicate experiments (\pm S.E.M.).

complexes appear to adapt to proline concentrations in the range 10^{-12} – 10^{-8} mol l $^{-1}$ and yet respond to increases in proline levels in the range 10^{-18} – 10^{-16} mol l $^{-1}$.

Cellular communication related to tuning

A second question raised by the sensitivity of anemones to proline concerns whether CSCCs respond independently to such minor changes in proline concentration. We estimate that each tentacle has approximately 41000 CSCCs, of which 12300 contain large microbasic p-mastigophore nematocysts (Watson and Mariscal, 1983). Assuming 50 tentacles per animal and four animals per dish, 8200×10^3 CSCCs are available to compete for the approximately 3000 molecules of proline in the dish (at 10^{-18} mol l $^{-1}$ proline); i.e. 2733 CSCCs for each proline molecule. Assuming 25 tentacles per animal and including only CSCCs containing large mastigophore nematocysts, 410 CSCCs are available to compete for each proline molecule. This estimate of the number of CSCCs available to bind proline suggests that it is unlikely that CSCCs respond independently

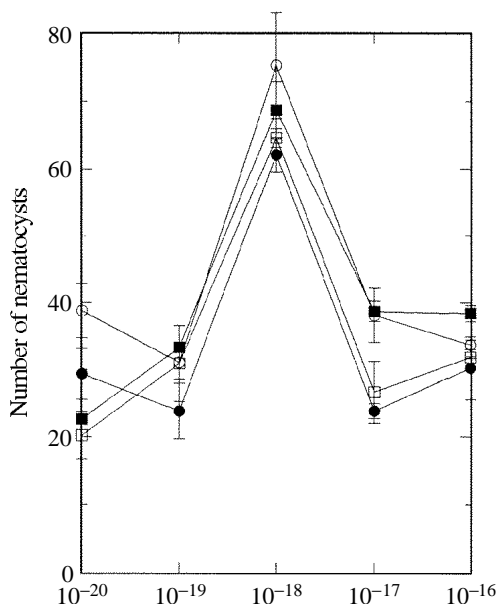


Fig. 5. Adaptation to proline of discharge of microbasic p-mastigophore nematocysts at 10Hz. Specimens were pre-incubated for 3–4h in natural sea water (filled circles) or in reagent grade ASW containing $10^{-8} \text{ mol l}^{-1}$ (open circles), $10^{-10} \text{ mol l}^{-1}$ (filled squares) or $10^{-12} \text{ mol l}^{-1}$ (open squares) proline. Discharge of microbasic p-mastigophore (mpm) nematocysts was tested at 10Hz after 5min of exposure to $10^{-7} \text{ mol l}^{-1}$ *N*-acetylneuraminic acid (NANA) followed by 10min of exposure to $10^{-7} \text{ mol l}^{-1}$ NANA and added 10^{-16} – $10^{-20} \text{ mol l}^{-1}$ proline. Nematocysts were counted as described above. Data points depict the mean of two replicate experiments (\pm S.E.M.), each based on the mean maximum number of mpm nematocysts counted for each of four probes for each experiment.

because 400–2700 CSCCs are available for each added proline molecule (at $10^{-18} \text{ mol l}^{-1}$).

To test for intercellular communication related to tuning of hair bundles, the oral disc and tentacles from three anemones were placed in a drop of Mg/ASW on a glass slide divided into two compartments by a 1 mm strip of liquid silicone (Fig. 6). NANA was added to one side, designated N, followed by proline to the other side, designated P. Only the central specimen, specimen 2, had tentacles extending through the barrier. Portions of this specimen were thus exposed to NANA or to proline (Fig. 6). Hair bundles of specimen 1 (N) and of both sides of specimen 2 elongated after NANA treatment, but only those on both sides of specimen 2 significantly shortened after proline treatment (Fig. 7A,B). Although, in the blind replicate experiment, the extent of bundle shortening induced by proline was less on the side of the anemone not directly exposed to NANA (Fig. 7B), it still was statistically significant. The hair bundles of specimen 3, which was located on side P, neither elongated significantly after NANA had been added to side N nor shortened significantly after proline had been added to side P (Fig. 7A,B), findings consistent with results for specimens exposed to proline without prior exposure to NANA (Fig. 1B,E).

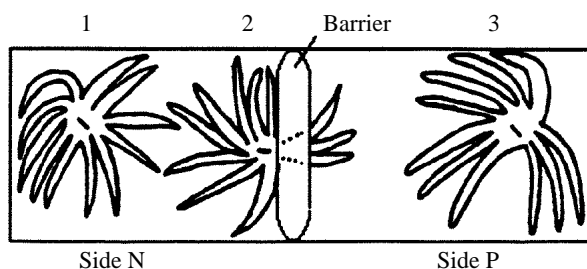


Fig. 6. Experimental design used to test for intercellular communication. As shown diagrammatically, the tips of the tentacles of specimen 2 extended through a barrier of liquid silicone (dimethylpolysiloxane, $0.6\text{ m}^2\text{ s}^{-1}$; Sigma). Specimens 1 and 3 were located on sides N (to which *N*-acetylneuraminic acid was added) and P (to which proline was added), respectively of the barrier.

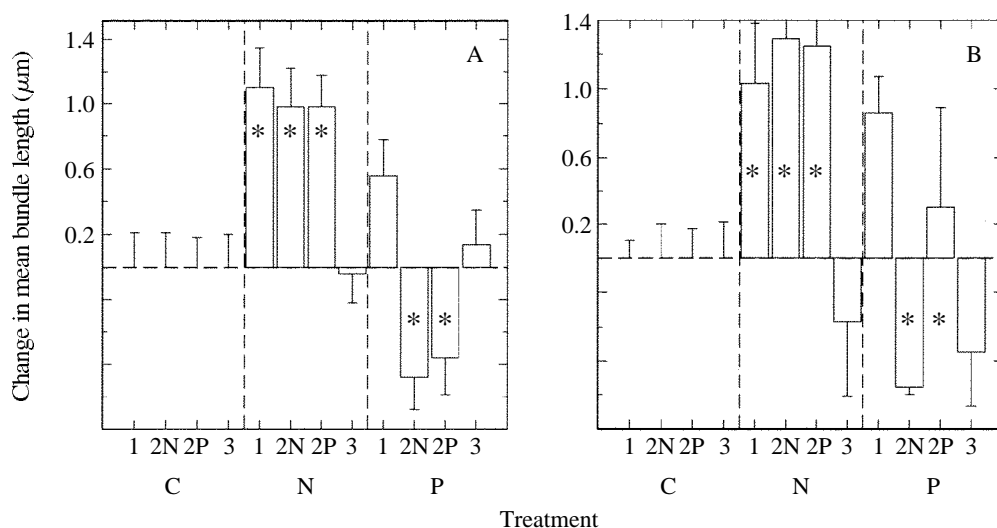


Fig. 7. Changes in hair bundle length. Refer to Fig. 6 for experimental design. (A) Original data set. (B) Blind replicate experiments. Fifty suitable hair bundles were measured from photomicrographs. Bar graphs depict changes in mean length of the bundles in relation to the control length (designated C on the x-axis of each panel) in Mg/ASW (\pm S.E.M.) for specimens 1, 2N, 2P and 3; (N) 15min after adding $10^{-7}\text{ mol l}^{-1}$ NANA to side N (final concentration); and (P) followed by 15min after adding $10^{-8}\text{ mol l}^{-1}$ proline to side P (final concentration). Least significant difference *post-hoc* comparisons were performed after ANOVA-determined significant effects at the 0.05 level or less. Significant changes in bundle length, compared with the previous treatment, are indicated by asterisks.

The effects of proline on tuning are reversible

The oral disc of a single specimen was placed across a silicone barrier as described above. Hair bundles on both sides of the barrier elongated significantly after NANA had been added to side N and shortened after proline had been added to side P (Fig. 8). Hair bundles on side N elongated significantly again after the portion of the specimen extending into side P had been killed by the addition of glutaraldehyde to side P (Fig. 8).

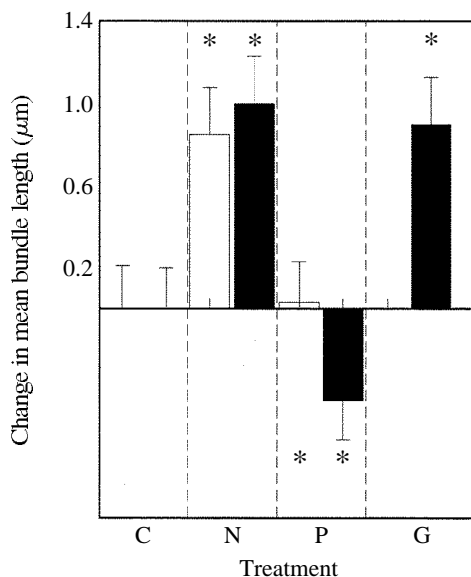


Fig. 8. Reversibility of proline effects on bundle length. A specimen was prepared and subsequently processed as described for specimen 2 in Fig. 6, with the exception that glutaraldehyde (2.5% final concentration) was added to side P 15min after the addition of proline to side P. Changes in the mean bundle length (\pm S.E.M.) relative to the control in Mg/ASW (designated C on the x -axis) are shown for side N (filled bars) and side P (open bars) for a single specimen 15min after *N*-acetylneuraminic acid (NANA) had been added to side N (10^{-7} mol l $^{-1}$ final concentration) (N), 15min after subsequent addition of proline to side P (10^{-8} mol l $^{-1}$ final concentration) (P) and 15min after subsequent addition of glutaraldehyde to side P (G). Least significant difference *post-hoc* comparisons were performed after ANOVA-determined significant effects at the 0.05 level or less. Significant changes in bundle length, compared with the previous treatment, are indicated by asterisks.

Proline effects on tuning are time-dependent

At 10Hz, maximum discharge of mpm nematocysts is detected at 10^{-18} mol l $^{-1}$ proline (after 10min exposure) (Figs 4A, 5, 9). The time course of tuning, from 5Hz in 10^{-7} mol l $^{-1}$ NANA to 10Hz upon subsequent exposure to added proline, is dependent on the dose of proline, occurring sooner at higher concentrations (Fig. 9). Thus, proline-induced tuning of nematocyst discharge to higher frequencies is transient.

Discussion

Antagonistic frequency tuning of anemone hair bundles

Perhaps the most striking and possibly unique attribute of anemone hair bundles is that they are tunable; that is, the frequency responsiveness of these bundles can change. By stimulating chemoreceptors for *N*-acetylated sugars, frequency responses of nematocyst discharge shift to lower frequencies as the bundles elongate (Watson and Hessinger, 1989a, 1991). We now find that, by stimulating chemoreceptors for proline after

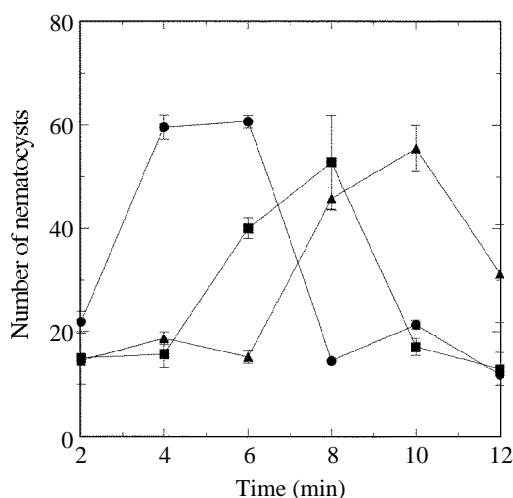


Fig. 9. Time course of proline effects on vibration-dependent discharge of microbasic p-mastigophore nematocysts. Discharge of microbasic p-mastigophore (mpm) nematocysts was tested in Instant Ocean ASW at 10Hz for specimens exposed for a total of 15min to 10^{-7} mol l⁻¹ *N*-acetylneuraminic acid, including 2–12min of exposure to 10^{-18} mol l⁻¹ (triangles), 10^{-17} mol l⁻¹ (squares) or 10^{-16} mol l⁻¹ (circles) proline. Nematocysts were counted as for Fig. 2. Data points represent the mean maximum number of mpm nematocysts counted for two replicate experiments (\pm S.E.M.). Each experiment employed four test probes per data point, one probe for each of four anemones.

stimulating receptors for NANA, frequency responses of discharge shift to higher frequencies as the bundles shorten.

Evidence suggests a common point of interaction between NANA and proline receptors. On the one hand, cholera toxin, forskolin and dibutyryl cyclic AMP tune anemone hair bundles to the same lower frequencies as NANA. Furthermore, in the presence of NANA, adenylyl cyclase activity is detectable cytochemically on the apical membranes of supporting cells (Watson and Hessinger, 1992). On the other hand, the mixed effects of GTP- γ -S on tuning first suggested the possibility of antagonistic G-proteins (Watson and Hessinger, 1992). This possibility is strengthened by the observation that pertussis toxin blocks the effects of proline on vibration-dependent discharge (Fig. 3). Thus, receptors for *N*-acetylated sugars may stimulate adenylyl cyclase *via* G_s-proteins whereas receptors for proline may inhibit adenylyl cyclase *via* G_i-proteins, suggesting that the frequency-specificity of hair bundles varies with intracellular levels of cyclic AMP. According to this model, hair bundles respond to specific frequencies within a range of frequencies depending on the level of intracellular cyclic AMP. The finding that discharge was tuned to progressively higher frequencies under conditions in which ambient levels of proline were increased while the level of NANA was held constant (Fig. 4) is consistent with this model. More recently, the relationship between bundle length and intracellular cyclic AMP concentration was confirmed and extended by using photoactivated caged derivatives of cyclic AMP (Thibodeaux and Watson, 1992). Furthermore, changes in bundle length induced by NANA (elongation)

and by proline (shortening) are accompanied by a mobilization of actin into and out of bundles, respectively (Watson *et al.* 1992).

Ultrasensitivity of tuning

We find it sufficiently difficult to explain the finding of subfemtomolar sensitivity to proline of the hair bundle tuning response that we are compelled to consider either an undetected experimental artefact or an unknown mechanism of chemosensory amplification.

Experimental artefact

Experimental artefact seems to be an inadequate explanation for these findings, for data were collected by G.M.W. over 2 years from two geographically separated laboratories using different sources of purified water and natural sea water, and two batches of proline (88F-0023 and 92F-3205) prepared by different primary suppliers. Nevertheless, experiments performed with natural sea water, Instant Ocean ASW and reagent grade ASW confirm the effects at $10^{-18} \text{ mol l}^{-1}$ proline (Figs 4, 5, 9). Thus, it is unlikely that these effects are caused by the presence of a water- or reagent-borne contaminant. Furthermore, tuning of nematocyst discharge occurs consistently upon the addition of $10^{-18} \text{ mol l}^{-1}$ proline to specimens pre-incubated in reagent grade ASW fortified with proline at 10^{-12} – $10^{-8} \text{ mol l}^{-1}$ (Fig. 5). Thus, any attempt to explain the ultrasensitive mechanism of proline tuning must recognize that anemones do not respond to absolute levels of proline, but to low-level increases in proline concentration.

Kinetics of binding

It may be questioned whether the association between proline and its receptor is feasible within periods that are biologically relevant at $10^{-18} \text{ mol l}^{-1}$ proline, concentrations at which tuning of discharge to higher frequencies occurred (Figs 4, 5, 9). To explore this question, it was assumed (i) that the diffusion coefficient in water of a small molecule such as proline is $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Berg, 1983); (ii) that 10^{12} proline receptors are present in the 5 ml bath (i.e. 10^7 receptors per supporting cell and 10^5 CSCCs); (iii) that each receptor occupies a mean volume of $5 \mu\text{m}^3$ and that the volume associated with each receptor is spherical. Thus, each receptor may occupy a space having a mean radius of $1.06 \times 10^{-4} \text{ cm}$. On the basis of these assumptions, the mean time for a proline molecule to move randomly round the average distance of this radius was calculated to be $1.86 \times 10^{-4} \text{ s}$, a time sufficiently brief to be of biological relevance and well within the time span of $6 \times 10^2 \text{ s}$ over which the experiments were conducted. The conditions set forth above would permit several thousand binding events to occur at each receptor during an experiment.

Amplification

Perhaps the most difficult challenge in attempting to explain the ultrasensitivity of proline-induced tuning is understanding the nature of the amplifier that can detect low-level increases in proline against high background levels of proline. Powerful amplifiers are known to operate in the rhodopsin system, but such amplifiers require low

background noise to avoid being driven by frequent false signals. Proline levels in sea water are not precisely known, but best estimates are between 10^{-9} and $10^{-8} \text{ mol l}^{-1}$ (Siegel and Degens, 1966; Lee and Bada, 1975). With a background concentration of 10^{-12} – $10^{-8} \text{ mol l}^{-1}$ proline (Fig. 5), the anemone system is responding to changes in concentration of only 1 part in 10^6 to 1 in 10^{10} , respectively. Because noise in measuring a small number of events will vary with the square root of the number (MacDonald, 1962), to resolve a change with a minimal signal to noise ratio of 1 would require measuring approximately 10^{12} – 10^{20} events; either binding of that many molecules or averaging of that many repeated bindings.

Assuming 10^7 receptors per supporting cell, two supporting cells per CSCC, 4.1×10^4 CSCCs per tentacle and 50 tentacles per anemone, the rate of binding (I) for different concentrations of proline is calculated from:

$$I = 4DNsC_0$$

for a spherical cell (Berg, 1983), where D is the diffusion coefficient of proline in water, N is the number of receptors per supporting cell, s is the assumed diameter of the proline binding site (1nm) and C_0 is the proline concentration. At $10^{-12} \text{ mol l}^{-1}$ proline, approximately 10s is required for 10^{12} binding events to occur at the 4.1×10^6 supporting cells present. At $10^{-8} \text{ mol l}^{-1}$ proline, approximately 167min is required for the predicted 10^{20} binding events. Thus, existing paradigms for receptor activity can adequately account for some, but not all, of our experimental observations. Under the most challenging conditions (i.e. detecting increases of $10^{-18} \text{ mol l}^{-1}$ proline against a background of $10^{-8} \text{ mol l}^{-1}$ proline), such predictors of receptor performance fall short of explaining our results by more than an order of magnitude. We speculate that a biological amplifier improves the performance of these cells in detecting proline by at least an order of magnitude. We do not know, nor can we speculate on, how this amplifier functions. We suggest, however, that it operates in concert with neuronal or non-neuronal cellular communication, possibly involving gap junctions (Hessinger *et al.* 1992; Boothby and McFarlane, 1986; Dunlap *et al.* 1987) to coordinate the response of the entire anemone.

Role of frequency tuning in the capture of prey

Conjugated *N*-acetylated sugars from the prey may stimulate receptors on the tentacle so that hair bundles are tuned to lower frequencies, which match prey movements (Watson and Hessinger, 1989a). Following contact and subsequent discharge of penetrant nematocysts, proline leaks (Gilles, 1979) from nematocyst-inflicted wounds while the prey gradually succumbs to the injected nematocyst toxins. Because sugar receptors have been activated, small increases in proline concentration are sufficient to tune hair bundles to frequencies higher than those produced by the prey (Fig. 4). This information is communicated to other CSCCs to prevent additional discharge into wounded 'captured' prey, thereby ensuring that the minimum adequate number of nematocysts is discharged.

During feeding, several prey may be captured and ingested. We suggest that ingestion of prey is accompanied by a decrease in ambient proline concentration. Because the

effects of proline on hair bundles are reversible and/or transient (Figs 8, 9), new prey approaching the tentacles can stimulate receptors for *N*-acetylated sugars to tune the bundles to low frequencies again. Hence, bundles may undergo several cycles of tuning during feeding.

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