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Frequency Specificity of Vibration Dependent Discharge of Nematocysts in Sea Anemones

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ABSTRACT Hair bundles on tentacles of sea anemones are similar to vertebrate hair bundles in terms of structure and function. Anemone hair bundles are involved in regulating discharge of nematocysts, "stinging capsules," used to capture prey. N-acetylated sugars from the prey including N-acetylneuraminic acid (NANA) induce hair bundles to elongate while shifting vibration dependent discharge of nematocysts to lower frequencies matching prey movements. In the present study, we find that vibration dependent discharge of nematocysts exhibits sharp frequency discrimination to within one Hz. Testing at one-Hz intervals over the range of frequencies spanning 1–75 Hz, we find that seven of these are stimulatory in seawater alone. A total of twenty-six frequencies are stimulatory in the presence of NANA. Stimulatory frequencies in NANA are lower than those in seawater alone. We find that antagonists of ryanodine receptors including ryanodine, procaine and tetracaine shift discharge to lower frequencies. Fluorescently tagged ryanodine labels numerous small loci in the apical cytoplasm of supporting cells. We propose that calcium induced calcium release (CICR) via ryanodine receptors may sharpen frequency specificity and/or cause shortening of hair bundles to shift frequency specificity to higher frequencies. *J. Exp. Zool. 281:582–593, 1998.*

Sea anemones are slow moving, predominantly sessile invertebrates that rely on prey locomotion or water currents to bring prey into contact with their tentacles. Anemones with small, fine tentacles or long, filamentous tentacles are primarily planktivorous (Shick, '91). Prey are captured by nematocysts and other cnidae, complex secretory products consisting of capsules containing eversible tubules (Mariscal, '74, '84). In response to appropriate chemical and mechanical stimuli (Pantin, '42), effector cells called cnidocytes trigger cnida discharge, rapid eversion of the tubule (Skaer and Picken, '65; Holstein and Tardent, '84). Depending on the type of cnida, the everting tubule may adhere to the surface of the prey, entangle its appendages, or penetrate its integument to inject potent toxins (Mariscal, '74, '84). In anemones, most of the published work on the regulation of cnida discharge has concerned microbasic p-mastigophore nematocyts, selected for study because this nematocyst type is abundant in the cnidom (complement of cnidae) of the tentacle. Furthermore, microbasic p-mastigophore nematocysts discharged into gelatin coated test probes are relatively easy to count using phase contrast optics (e.g., Watson and Hudson, '94).

Mechanical stimuli alone are sufficient to trigger discharge. Relevant mechanical stimulation can comprise contact alone or contact with a vibrating test probe. Contact between a tentacle and nonvibrating test probe elicits discharge of a baseline number of nematocysts. Provided the test probe is vibrating at a preferred frequency (hereafter referred to as a "key" frequency), about twice as many cnidocytes discharge nematocysts into the test probe as baseline. The number of nematocysts discharged into test probes vibrating at frequencies other than key frequencies is comparable to baseline (reviewed in Watson and Mire-Thibodeaux, '94).

Thus far, two chemoreceptors are known to affect discharge of nematocysts. These chemoreceptors bind N-acetylated sugars and amino compounds, respectively, compounds derived from prey (Thorington and Hessinger, '88). N-acetylated sugars are contained in prey mucins and other glycoproteins. Amino compounds are contained in hemolymph of prey. Chemical stimuli

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alone are insufficient to trigger discharge (Pantin, '42). However, if appropriate chemical stimuli are combined with contact with a nonvibrating test probe, discharge increases in a dose-dependent fashion to a maximum at 10^{-7} M N-acetylneuraminic acid (NANA) or 10^{-8} M proline, respectively. At optimal concentrations of these agonists, levels of discharge into test probes are approximately twice baseline (Watson and Mire-Thibodeaux, '94).

Appropriate chemical stimuli shift frequency and amplitude specificity of nematocyst discharge into vibrating test probes provided anemones are exposed to the compounds for several minutes before they are contacted by vibrating test probes. In the presence of NANA, high levels of nematocyst discharge are detected at lower frequencies and smaller amplitudes, presumably corresponding to movements produced by a small, calmly swimming prey (Watson and Hessinger, '89; Watson and Hudson, '94). This shift in key frequencies and amplitudes depends on polymerization of actin within stereocilia of certain hair bundles on the tentacle (see below), causing the hair bundles to elongate by 1–2 microns (Mire-Thibodeaux and Watson, '94a; Watson and Roberts, '95). Adding small doses of proline to seawater previously fortified with NANA shifts maximal discharge to larger amplitudes at low frequencies and to higher frequencies at small amplitudes (Watson and Hudson, '94; Watson and Hessinger, '94a). Presumably, these vibrations correspond to movements produced by small, wounded prey struggling to escape. The shift in discharge induced by proline involves depolymerization of actin within stereocilia of anemone hair bundles causing them to shorten to control lengths or shorter (Watson and Roberts, '94, '95).

Second messenger pathways detailing effects of activated chemoreceptors on hair bundles are partially resolved. It appears that the NANA receptor utilizes a cyclic AMP second messenger pathway. Agents that activate Gs proteins (cholera toxin), activate adenylate cyclase (forskolin), or otherwise elevate intracellular cyclic AMP (dibutyryl cyclic AMP), or inhibit its breakdown (caffeine) induce hair bundles on the tentacle to elongate while shifting vibration dependent discharge of nematocysts to lower frequencies (Watson and Hessinger, '92). NANA-induced lengthening of the hair bundles is inhibited in the presence of inhibitors of protein kinase A (H89) (Mire-Thibodeaux and Watson, '94b). An analysis of the time course of hair bundle elongation revealed the surprising finding that NANA induces hair bundles to elongate and shorten to control lengths in rhythmic cycles with a period of 8 min. This rhythmic elongation can be induced in the absence of NANA by photoactivation of caged cyclic AMP. Furthermore, the shortening component of the cycle can be reversed by photoactivation of caged cyclic AMP (Mire-Thibodeaux and Watson, '94b). In addition, the shortening component of the cycle is blocked by inhibitors of L-type calcium channels (verapamil or nifedipine), calmodulin (W7), or phosphodiesterase (caffeine). The elongation component of the cycle is blocked by an agonist of L-type calcium channels (Bay K-8644) (Mire-Thibodeaux and Watson, '94b). Apparently, in this system, an antagonistic relationship exists between cyclic AMP and calcium second messenger pathways.

Second messengers associated with the proline receptor are less well understood, especially with respect to effects of proline on vibration dependent discharge of nematocysts. However, regarding enhanced discharge of nematocysts into nonvibrating test probes, evidence implicates IP_3 as a second messenger to the proline receptor, apparently in association with calcium induced calcium release (CICR) through intracellular channels called ryanodine receptors (Russell and Watson, '95). Whereas baseline discharge of nematocysts into nonvibrating test probes is unaffected by known antagonists of ryanodine receptors including ryanodine and certain local anesthetics such as procaine and tetracaine, the enhancement of discharge into nonvibrating test probes attributable to proline is reduced to baseline at 10^{-4} M ryanodine or 10^{-2} M procaine (Russell and Watson, '95). In the absence of proline, photoactivation of caged IP_3 enhances discharge into nonvibrating test probes. In the presence of proline, photoactivation of caged IP_3 shifts dose sensitivity to proline. Thus, in the anemone system, IP_3 sensitive channels may be activated by proline receptors, which then release calcium to activate ryanodine receptors (Russell and Watson, '95).

In other cell systems, calcium release from internal storage vesicles can occur through channels called IP_3 receptors (because they are activated by IP_3) or through channels called ryanodine receptors (because they are activated at nanomolar concentrations of the plant alkaloid, ryanodine, and inhibited at higher concentrations of ryanodine), or through a combination of these two types of channel (Berridge et al., '96). Calcium induced calcium release (CICR) occurs through ryanodine

receptors. The basis for CICR resides in the ability of ryanodine receptors to be activated by low levels of calcium and inhibited by high levels of calcium. Additional modulation of ryanodine receptors (e.g., phosphorylation) is known with such modulation typically affecting the sensitivity of ryanodine receptors to calcium ions (Berridge et al, '96). Activation of ryanodine receptors by calcium indicates that CICR is not restricted to cell systems utilizing IP_3 second messengers. CICR amplifies calcium currents originating from cell surface channels or from IP_3 triggered release from internal stores.

Each anemone hair bundle consists of approximately 100–200 small diameter stereocilia originating from several supporting cells converging onto 5–7 large diameter stereocilia originating from a centrally located, sensory cell neuron. A single, nonmotile kinocilium extends through the center of the hair bundle. The kinocilium originates from the sensory cell (Peteya, '75; Mire-Thibodeaux and Watson, '94a; Watson and Roberts, '95; Watson et al., '97). Anemone hair bundles are structurally and functionally similar to hair bundles in vertebrates. In each case, actin-based stereocilia comprise the bundle. Furthermore, linkages interconnecting stereocilia of the hair bundle are morphologically and cytochemically similar (Watson et al., '97). The morphological similarity includes tip links, so named in vertebrate hair cells because they extend from the tips of shorter stereocilia to the adjacent longer stereocilia (Pickles et al., '84). In vertebrates, one model suggests that tip links directly, mechanically gate ion channels involved in signal transduction (reviews Hudspeth, '85, '92; Howard et al., '88; Ashmore, '91; Hackney and Furness, '95). Structural integrity of tip links is disrupted by brief exposure to calcium deleted buffers or to elastase (Assad et al., '91; Preyer et al., '95; Zhao et al., '96). The loss of tip links is correlated with a disruption of signal transduction. Signal transduction also is inhibited by agents thought to interact with the transduction ion channel. Certain aminoglycoside antibiotics including streptomycin are thought to occlude the channel in its open position (Kroese et al., '89; Hudspeth, '92). The sodium channel inhibitor, amiloride, may also inhibit the channel (Rusch et al., '94; Hackney and Furness, '95).

In anemones, vibration dependent discharge is abolished after briefly exposing animals to calcium free seawater (Watson et al., '97) or to elastase (Watson et al., '98). In addition, vibration dependent discharge is inhibited reversibly by the aminoglycoside antibiotic, streptomycin, but is unaffected by amiloride (Watson et al., '97). Electrophysiological recordings show that deflecting anemone hair bundles induces current transients in cells contributing stereocilia to the hair bundle. Responses of anemone hair cells exhibit adaptation to prolonged deflection, rapid and reversible inhibition to streptomycin, and an asymmetrically sigmoidal stimulus/response curve, properties also exhibited by vertebrate hair cells (Mire and Watson, '97).

Hair bundle mechanoreceptors respond to vibrations in a frequency specific fashion. In at least some vertebrate hair cells, frequency specificity is thought to depend on two cellular parameters: (i) length of the hair bundle (Firshkopf and DeRosier, '83; Holton and Hudspeth, '83) and (ii) gating properties of ion channels in the membrane of the hair cell (Crawford and Fettiplace, '81; Hudspeth, '85; Howard et al., '88; Roberts et al., '88; Ashmore, '91). The hair bundle pivots about its base as a passive resonator attached to the substrate at one end only (Roberts et al., '88). Inasmuch as longer bundles resonate at lower frequencies than shorter bundles, frequency specificity is determined in part by bundle length (Firshkopf and DeRosier, '83). Frequency specificity is further sharpened by an intracellular mechanism in which voltage gated calcium channels and calcium activated potassium channels act antagonistically to one another to depolarize and repolarize the membrane potential, respectively. Deflecting hair bundles in a positive direction leads to an opening of transduction channels in the stereocilia permeable to cations and membrane depolarization (Hudspeth and Corey, '77). Conversely, deflecting hair bundles in a negative direction leads to a closing of transduction channels and hyperpolarization of membrane potential (Hudspeth and Corey, '77). Apparently, as the hair bundle traverses the positive range of its motion, its membrane potential depolarizes due to the primary inward current carried by cations entering the cytoplasm through transduction channels located distally in stereocilia. Membrane depolarization induces voltage gated calcium channels to open, further depolarizing the membrane and further elevating intracellular calcium concentrations. The calcium ions activate calcium activated potassium channels (Crawford and Fettiplace, '81; Hudspeth and Lewis, '88; Roberts et al., '88; Ashmore, '91) which repolarize the cell. The model predicts that channel behavior varies among hair cells according to the resonant frequency of the hair cell. Calcium activated potas-

sium channels of low frequency hair cells are less abundant and exhibit slower gating properties than their counterparts in hair cells that respond to higher frequencies (Art et al., '95). Hair cells responding to the lowest frequencies have potassium channels with even slower gating properties. In these cells, the potassium channels are voltage gated channels and not activated by calcium ions (Art et al., '95). The interplay between bundle movement and gating of ion channels can account for determining frequency specificity for frequencies up to approximately 800 Hz (Ashmore, '91).

Whereas vibration dependent discharge of nematocysts is well documented in sea anemones, frequency specificity of vibration dependent discharge is poorly resolved (to a resolution of 5 Hz). The present study was initiated to better resolve frequency specificity of vibration dependent discharge of nematocysts and to investigate mechanisms responsible for determining frequency specificity.

MATERIALS AND METHODS

Animal culture

Clonal cultures of the sea anemone, *Haliplanella luciae,* were reared in natural seawater at a salinity of 32 parts per thousand and at 16–18°C according to published methods (Minasian and Mariscal, '79). The animals were fed to repletion twice weekly using freshly hatched brine shrimp nauplii.

Light microscopy

Anemones were anesthetized in high potassium seawater (KSW) consisting of 382 mM NaCl, 50 mM KCl, 24 mM $MgCl_2$-$6H_2O$, 26 mM $MgSO_4$-$7H_2O$, 12 mM $CaCl_2$, and 2 mM $NaHCO_3$. Specimens were exposed to 10^{-4} M ryanodine for 10 min or not (controls), then excised tentacles were imaged using phase contrast microscopy. Photomicrographs were viewed on a light box using a video camera. Using a blind design, measurements of bundle length were performed with Image 1-AT hardware and software (Universal Imaging, West Chester, PA).

Some hair bundles were imaged from fixed tentacles using video-enhanced differential interference contrast (DIC) optics following methods previously described (Watson and Roberts, '95).

Confocal microscopy

Specimens were anesthetized in KSW, fixed for 30 min in 2.5% glutaraldehyde and 1% paraformaldehyde in KSW, rinsed in KSW and then incubated in bodipy ryanodine (Molecular Probes, Eugene, OR) (10^{-6} M final concentration) alone or with (10^{-3} M final concentration) unlabelled ryanodine (Sigma, St. Louis, MO) as a control. Tentacles were viewed using a Biorad 600/Nikon Optiphot (Melville, NY) confocal microscope (PlanApo60/1.4 objective). Images were stored in digital form and analyzed using ImagePro-Plus software (Media Cybernetics, Silver Springs, MD).

Bioassay for testing vibration sensitivity

Anemones were transferred from mass culture to 35-mm diameter plastic Petri dishes filled with seawater where they were allowed a minimum of 1 hr recovery from handling. At this point, specimens were exposed to 10^{-7} M NANA in seawater for 10 min or not (seawater controls). Likewise, specimens exposed to antagonists of ryanodine receptors were exposed to the antagonist solution (prepared in seawater) for 10 min before their tentacles were touched with vibrating test probes.

For each experiment, four test probes were touched to tentacles of four different anemones. Probes consisted of 2-cm segments of nylon fishing line coated at one end with 25% gelatin to a final thickness of approximately 200 μm. The probes were attached to a piezo disk induced to vibrate by a function generator equipped with a frequency counter. Vibrating probes were touched to tentacles, then briefly fixed in 2.5% glutaraldehyde. They were stored hydrated in microtiter trays. Under microscopic observation using a 40× objective and phase contrast optics, discharged microbasic p-mastigophore nematocysts were counted for a single field of view for each probe.

RESULTS

Frequency specificity of nematocyst discharge

Vibration dependent discharge of nematocysts was estimated using a bioassay in which gelatin coated test probes are touched to tentacles. Each data point indicates the mean number of microbasic p-mastigophore nematocysts counted for a single field of view at 400× total magnification for four test probes in each of two replicate experiments (a total of eight test probes ± standard error of the mean). In seawater alone, transitions between stimulatory and nonstimulatory frequencies are sharply defined such that nonstimulatory frequencies are separated from stimulatory frequencies by 1 Hz or less (Fig. 1A). In seawater

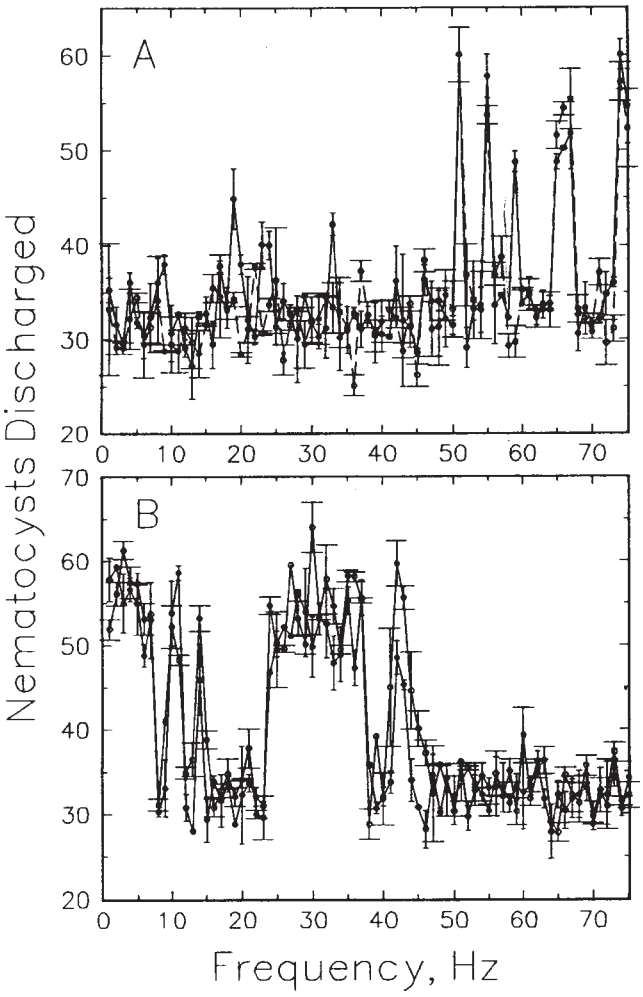

Fig. 1. Vibration dependent discharge of nematocysts. Microbasic p-mastigophore nematocysts discharged into test probes vibrating at the frequency indicated on the x-axis were counted for a single field of view (400× total magnification) for each of four test probes per experiment. Each data point plotted represents the mean number of nematocysts counted for 2 replicate experiments, each based on 4 test probes (i.e., a total of 8 probes ± SEM). Since two curves are superimposed in each panel (open circles versus closed circles), data from a total of 4 replicate experiments are shown in each panel. (**A**) Frequency response in seawater alone. (**B**) Frequency response in seawater containing 10^{-7} M NANA. Animals were touched with test probes after 10 min in the NANA solution.

alone, only seven to eight different frequencies are stimulatory over the total range from 1–75 Hz, appearing in four to five peaks. To test the stability of these "key" frequencies, experiments were repeated over a period of several weeks. In each of the four replicate experiments performed over a period of several weeks, high levels of discharge are detected at 51, 55, 65–67, and 74–75 Hz, with an additional peak appearing at 59 Hz in only one pair of the replicate experiments (compare open circles with closed circles in Fig. 1A).

In the presence of 10^{-7} M NANA, discharge tested over the range of 1–75 Hz yields high levels of discharge at 26 different frequencies, an increase in the number of key frequencies by a factor of 3.7. High levels of discharge are detected from 1–7, 10–11, 14, 24–37, and 42–43 Hz (Fig. 1B), spanning five peaks. These key frequencies are detected in each of the replicate experiments performed over several weeks (compare open circles with closed circles in Fig. 1B).

Antagonists of ryanodine receptors

Enhancement of discharge into test probes vibrating at 55 Hz is reduced to baseline at 10^{-4} ryanodine, 10^{-4} M procaine, or 10^{-4} M tetracaine (Fig. 2A). The observed reduction in discharge at 55 Hz in the presence of 10^{-4} M ryanodine appears to result from a downward shift in frequency specificity rather than from a simple inhibition of vibration sensitivity (Fig. 3A). This downward shift in frequency specificity results in discharge peaks at 44–45, 57, 61, and 73 Hz, a total of five stimulatory frequencies appearing in four peaks. In 3×10^{-5} M procaine, peaks of discharge are detected in a pattern closely resembling that obtained in 10^{-4} M ryanodine with peaks at 42–43, 56, 61 and 62 Hz (Figs. 3A,B). In 10^{-4} M procaine, a somewhat more dramatic downward shift is observed so that a single discharge peak is detected at 46–51 Hz (Fig. 3C). At this dose of procaine, a total of six stimulatory frequencies are detected. In 10^{-4} M tetracaine, a downward shift in frequency specificity occurs with a single discharge peak apparent at 45–46 Hz (Fig. 3D).

In the presence of NANA, discharge into test probes vibrating at 30 Hz (a key frequency in NANA) is reduced to baseline at 10^{-4} M ryanodine, 10^{-4} M procaine, or 10^{-4} M tetracaine (Fig. 2B). In the combined presence of 10^{-7} M NANA and 10^{-4} M procaine, a single discharge peak is detected ranging from 1–13 Hz (Fig. 3E). Compared to discharge in 10^{-7} M NANA alone (Fig. 1B), procaine appears to cause a loss of responsiveness at the highest three of five total discharge peaks and a convergence of the lower two peaks (1–7 and 10–11 Hz).

Since the downward shift in discharge normally induced by activating the receptor for N-acetylated sugars is accompanied by, and requires, an elongation of anemone hair bundles (Mire-Thibodeaux and Watson, '94a; Watson and Roberts, '95),

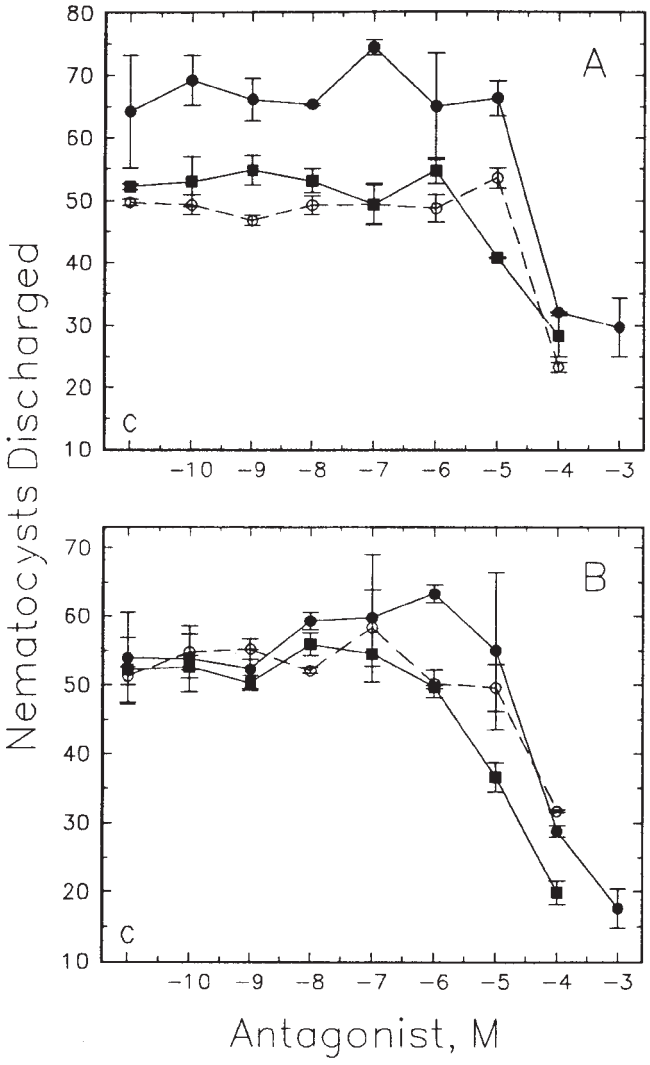

Fig. 2. Effects of antagonists of ryanodine receptors on vibration dependent discharge of nematocysts. Discharge was tested using methods described for Fig. 1 after specimens were exposed for 10 min to ryanodine (closed squares), procaine (closed circles), or tetracaine (open circles) at the log molar dose indicated on the x-axis of the graph. Data are shown for specimens tested at (**A**) 55 Hz and (**B**) 30 Hz (also in the presence of 10^{-7} M NANA).

we tested whether the downward shift in frequency specificity induced by 10^{-4} M ryanodine is accompanied by a change in the lengths of hair bundles on the tentacle. Comparisons of bundle length of specimens in seawater alone with those in seawater fortified with 10^{-4} M ryanodine indicate that ryanodine induces no significant change in length of hair bundles (Fig. 4; ANOVA followed by Tukey's honest significant difference post hoc analysis, $P = 0.868$). Comparisons of hair bundles imaged in the presence of NANA alone reveal no significant differences ($P = 0.982$) when compared to hair bundles imaged in the combined presence of 10^{-7} M NANA and 10^{-4} M ryanodine (Fig. 4). On the other hand, bundles imaged in the presence of NANA alone or in the presence of NANA and ryanodine are significantly longer than hair bundles imaged in the absence of NANA ($P < 0.0001$) regardless of whether ryanodine is present (Fig. 4).

Cytochemistry of the ryanodine receptor

Confocal microscopy of specimens labeled with fluorescently tagged ryanodine reveals punctate staining in the apical cytoplasm of the most commonly occurring epidermal cells, supporting cells (Fig. 5A). The intensity of this labeling diminishes greatly if the specimens are labeled in the presence of excess, nonfluorescent ryanodine (Fig. 5B). Most commonly, cnidocytes are not labeled (Fig. 5C) although tubules of spirocysts and some nematocyst capsules are occasionally labeled (not shown). In addition, intense labeling is apparent in the form of small rings (diameter 0.82 ± 0.11 microns) (Fig. 5C).

DISCUSSION

We find that vibration dependent discharge exhibits sharp frequency discrimination with four to five peaks of discharge detected over the range of 1–75 Hz in seawater alone (Fig. 1A) or in seawater containing 10^{-7} M NANA (Fig. 1B). Peaks of discharge in NANA are broader and at lower frequencies than in seawater alone. Replicate experiments performed several weeks apart (open circles versus closed circles) indicate that key frequencies are stable over time (Fig. 1A, B). The finding of sharp frequency discrimination associated with vibration dependent discharge of nematocysts was surprising to us because hair bundles on the tentacle vary in length approximating a normal distribution. For hair bundles in KSW alone (imaged using DIC optics), the distribution of length nicely approximates a normal distribution (Chi-Square statistic, $P = 0.81$, n = 100, data not shown). In the presence of 10^{-7} M NANA, the distribution is more bimodal because of an increased proportion of taller hair bundles, but remains statistically indistinguishable from a normal distribution ($P = 0.53$, n = 100, data not shown).

The observed "patchy" distribution of key frequencies associated with vibration dependent discharge of nematocysts can be reconciled with the normal distribution of hair bundle length if relatively few hair bundles on the tentacle respond

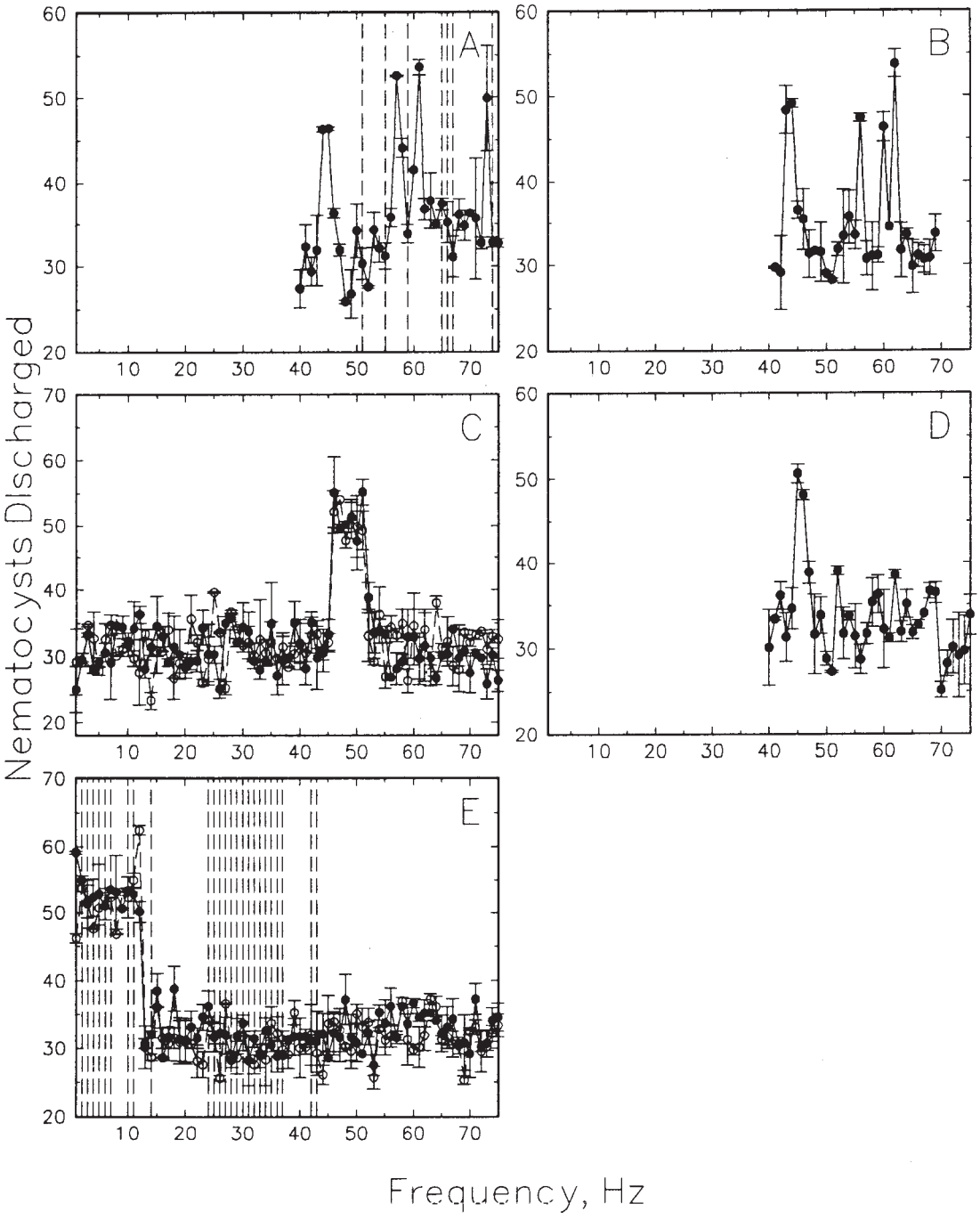

Fig. 3. Effects of antagonists of ryanodine receptors on frequency specificity of vibration dependent discharge. Specimens were touched with test probes vibrating at frequencies shown on the x-axis after 10 min in seawater containing (**A**) 10^{-4} M ryanodine, (**B**) 3×10^{-5} M procaine, (**C**) 10^{-4} M procaine, (**D**) 10^{-4} M tetracaine, or (**E**) 10^{-7} M NANA and 10^{-4} M procaine. Dashed lines appearing in panels A and E depict peaks of discharge (A) in seawater alone or (E) in seawater containing 10^{-7} M NANA, from Fig. 1. Data from a total of four replicate experiments are shown in panels C and E. Nematocysts were counted and data tabulated as described for Fig. 1.

to vibrations by generating biologically meaningful signals that sensitize cnidocytes to discharge nematocysts. Mechanistically, this would be possible if anemone hair bundles, like vertebrate hair bundles, determine frequency specificity by means of a dual system in which two periodic events must coincide in order to generate a meaningful biological signal.

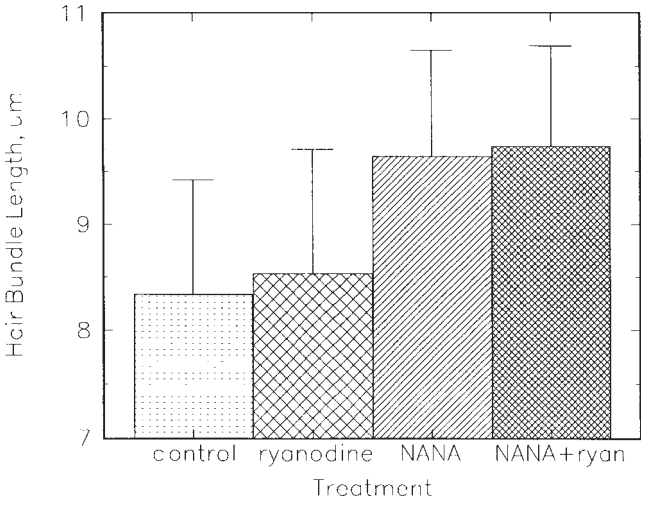

Fig. 4. Effects of antagonists of ryanodine receptors on mean hair bundle length. Hair bundles were measured from photomicrographs using Image 1-AT software and a blind experimental design. Bars indicate the mean length of hair bundles (n = 35) ± standard deviation. Live specimens were exposed to 10^{-4} M ryanodine with or without 10^{-7} M NANA for 10 min before they were imaged. Specific treatments are indicated on the x-axis.

Recall that voltage gated calcium channels are important in determining frequency specificity of hair cells in vertebrates (see introductory section). Apparently, such channels are unlikely to function in determining frequency specificity in the anemone system because antagonists of L-type, voltage gated calcium channels do not significantly affect vibration dependent discharge of nematocysts (Watson and Hessinger, '94b). Interestingly, L-type calcium channels appear to be involved in the NANA-induced enhancement in discharge of nematocysts into nonvibrating test probes (Watson and Hessinger, '94b). Thus, while L-type, voltage gated calcium channels evidently are present in anemones, they do not appear to be involved in determining frequency specificity of anemone hair bundles. Searching for an alternative mechanism to one involving voltage gated ion channels, we wondered if CICR might act in concert with the receptor current to determine frequency specificity of anemone hair bundles. CICR is an attractive possibility because it serves to amplify relatively small calcium currents in other cell systems, and the receptor current is likely to be small in magnitude. Furthermore, previously we found that ryanodine receptors are involved in the proline-induced enhancement in discharge of nematocysts into nonvibrating test probes (Russell and Watson, '95), indicating that ryanodine receptors are present in anemones.

Cytochemistry suggests the presence of ryanodine receptors in cells contributing stereocilia to anemone hair bundles. We find that fluorescently tagged ryanodine labels numerous punctate loci in the apical cytoplasm of supporting cells. Labeling is inhibited in the presence of excess, nonfluorescent ryanodine. Thus, supporting cells likely have internal stores of calcium ions released into the cytoplasm through activated ryanodine receptors. Strong labeling also is detected in the pattern of small rings having a diameter of approximately 0.8 microns. This labeling corresponds in size to a group of small vesicles located apically in sensory cell neurons just inside the rootlets of large diameter stereocilia. The vesicles can be seen in transmission electron micrographs of sensory cells (e.g., Watson et al., '97).

With respect to vibration dependent discharge of nematocysts, antagonists of ryanodine receptors cause a downward shift and a convergence of key frequencies. It appears that antagonists of ryanodine receptors do not inhibit discharge per se but specifically alter frequency specificity of vibration dependent discharge. The downward "tuning" of vibration dependent discharge induced by antagonists of ryanodine receptors suggests that these channels may be involved in regulating frequency specificity of vibration dependent discharge of nematocysts. At present, we cannot identify the mechanism by which these antagonists of ryanodine receptors "tune" vibration dependent discharge. Two possible mechanisms seem likely. The downward shift and convergence of frequency specificity may be caused by an elongation of hair bundles stemming from actin polymerization. Actin polymerization, in turn, would result from a decrease in resting levels of intracellular calcium ions caused by antagonists of ryanodine receptors. On the other hand, the downward shift of frequency specificity may be unrelated to changes in bundle length and instead result from drug effects on a CICR and sequestration cycle that may normally accompany stimulation of anemone hair bundles.

Considering first the possibility of bundle elongation, the elongated bundles would resonate at lower frequencies than at their resting lengths. In this way, frequency specificity could be shifted to lower frequencies. This relationship between hair bundle length and frequency specificity of hair bundles is well established for vertebrate hair bundles (Frishkopf and DeRosier, '83) and for anemone hair bundles (Mire-Thibodeaux and Watson, '94a,b; Watson and Roberts, '95). In the

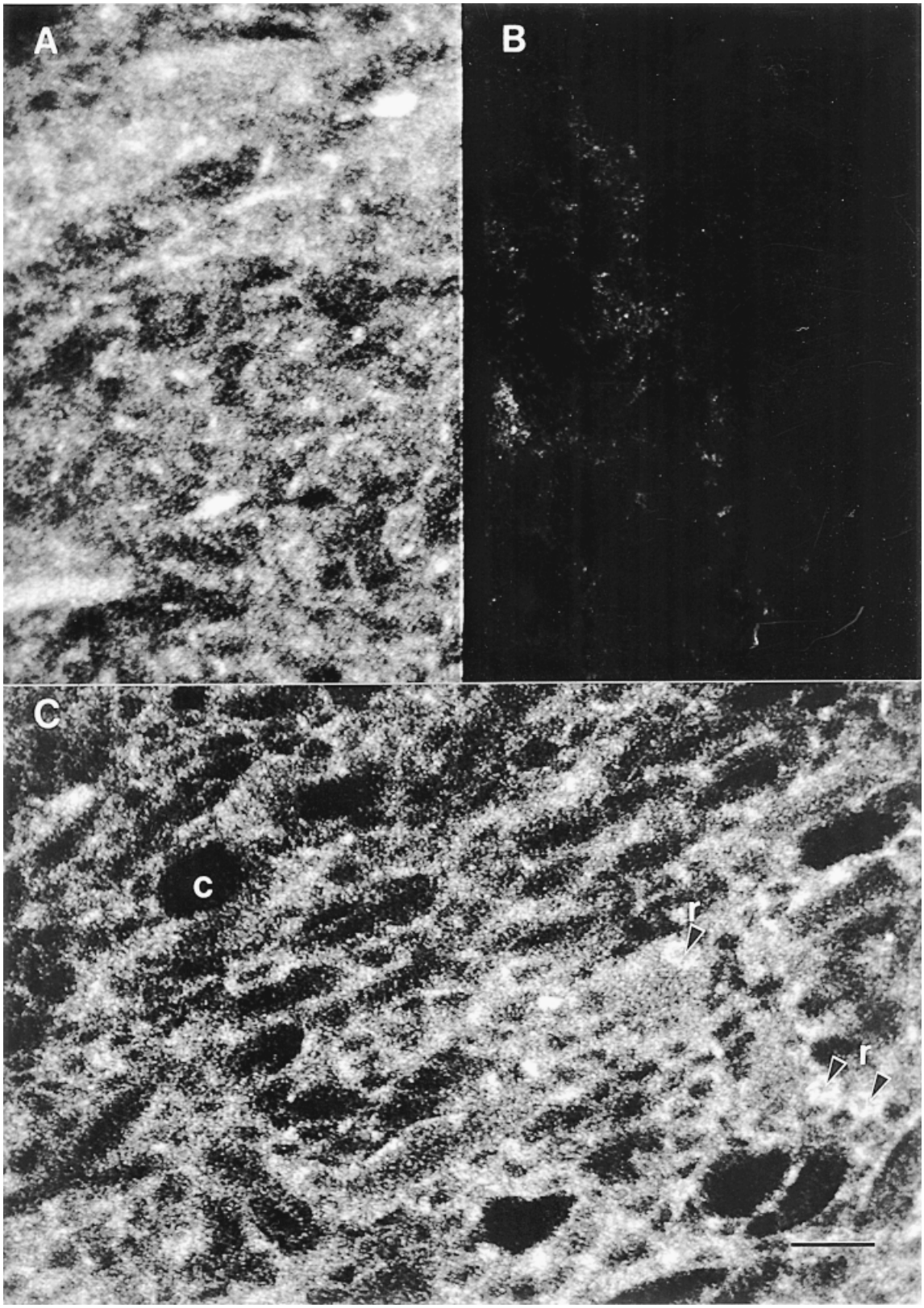

Figure 5.

case of anemone hair bundles, activating chemoreceptors for N-acetylated sugars causes hair bundles to elongate by 1–2 microns (e.g., Fig. 4) while shifting discharge to lower frequencies (e.g., Fig. 1). Averaging each of the key frequencies shown in Fig. 1 gives a mean value of 22.1 ± 13.7 Hz (mean ± standard deviation) in NANA as compared to 64.7 ± 8.9 Hz in seawater alone. This represents a mean shift of 42.6 Hz per 1–2 microns mean increase in bundle length. Thus, each 1 Hz downward shift in responsiveness may require that the bundles elongate on average by 0.023 to 0.047 microns. In 10^{-4} M ryanodine, mean discharge occurs at 56.0 ± 12 Hz, a downward shift of 8.7 Hz. In the presence of 10^{-4} M ryanodine, bundles would be expected to elongate by from 0.2 to 0.4 microns. We find that ryanodine induces bundles to elongate by 0.19 microns (Fig. 4). While these data nearly fit the prediction, the error associated with these measurements precludes us from reaching statistical significance. Some of our previous results suggest an antagonistic relationship between elevated levels of intracellular calcium and bundles elongated by NANA (Mire-Thibodeaux and Watson, '94b). Elongation of hair bundles by NANA is blocked by Bay K-8644, an agonist of L-type voltage gated calcium channels. Furthermore, shortening of elongated bundles is blocked by nifedipine, an antagonist of L-type calcium channels, by W7, an antagonist of calmodulin, and by cypermethrin, an inhibitor of protein phosphatase 2B (Mire-Thibodeaux and Watson, '94b). Thus, the effects of ryanodine on vibration dependent discharge may be attributable, at least in part, to a subtle increase in hair bundle length. Unfortunately, this line of reasoning cannot account for sharp frequency discrimination associated with vibration dependent discharge, only a downward tuning of discharge to lower frequencies. Likewise, this line of reasoning cannot account for the patchy distribution of key frequencies observed for each treatment (Fig. 1A, B).

We next consider the possibility that antagonists of ryanodine receptors "tune" vibration dependent discharge via a mechanism unrelated to bundle length. Perhaps CICR and sequestration sharpens frequency specificity of anemone hair bundles. According to this model, vibrations at a given frequency induce certain hair bundles to pivot about their bases. As is the case for vertebrate hair bundles, the extent to which the hair bundle pivots depends on its length. As a hair bundle pivots about its base, a supporting cell on one side of the bundle alternately depolarizes and hyperpolarizes (Mire and Watson, '97). During one-half of its range of motion when the stereocilia of that supporting cell are moving in a positive direction (i.e., toward taller stereocilia), transduction channels permeable to cations open leading to membrane depolarization. Since the extracellular calcium concentration in seawater is 10–12 mM, calcium ions likely comprise a portion of the receptor current. As intracellular calcium concentrations increase, ryanodine receptors open to further increase intracellular calcium concentrations. This step may amplify the signal carried by the receptor current and, presumably, is necessary for downstream signalling events to occur. At higher intracellular calcium concentrations ryanodine receptors are inhibited, preventing a further release of calcium ions from intracellular storage sites. Meanwhile, the hair bundle pivots so that the stereocilia of the supporting cell enter the negative half of their range of motion. At this point, transduction channels associated with the stereocilia close so that the inward receptor current ceases. Calcium ATPases in the membrane of intracellular storage sites sequester calcium to reduce intracellular calcium concentrations to resting levels. The ryanodine receptors return to their naive state. This component of the cycle would be completed before the stereocilia pivot into the positive range of motion. Thus, two cyclical events may be associated with signal transduction: (i) the receptor current flowing through channels in stereocilia and (ii) calcium induced calcium release and sequestration into intracellular storage vesicles. Presumably, in seawater alone, relatively few hair bundles exhibit agreement between these two cyclical events, thereby accounting for sharp frequency discrimination associated with key frequencies. In the presence of

Fig. 5. Cytochemistry of fluorescently tagged ryanodine. A single specimen was fixed and then divided into two parts, one of which was exposed to (**A**) 10^{-6} M bodipy ryanodine alone, and the other part to (**B**) 10^{-6} M bodipy ryanodine with 10^{-3} M unlabeled ryanodine. Confocal microscopy was performed as described in the Methods section. The gain was held constant, contrast was adjusted equally and then the images were paired on the computer screen for final photography. They were printed from the same negative. Numerous, punctate fluorescent loci are present in the apical cytoplasm of epidermal (supporting) cells in panel A that are not apparent in panel B. (**C**) In this preparation, comparable to panel A, diffuse punctate labeling is present in most epidermal cells (supporting cells) but not in cnidocytes (c). In addition, bright fluorescent rings (r, arrowheads) are visible. Scale bar = 2 microns.

antagonists of ryanodine receptors, the calcium release component of this cycle would be partially inhibited, in effect slowing the cycle to a lower frequency. This may shift responsiveness of these hair bundles to lower frequencies.

In anemones, the situation is even more interesting because anemone hair bundles naturally change their length and frequency specificity in the presence of N-acetylated sugars. Lengthening of hair bundles by NANA depends on an activation of protein kinase A (Mire-Thibodeaux and Watson, '94b). Perhaps certain cation channels in the hair cell complex (i.e., a subset of the ryanodine receptors) are modulated through phosphorylation by protein kinase A so that they are inactivated or, at least, are less sensitive to activation by calcium ions. Inactivating some of these channels, or decreasing their sensitivity to calcium, would slow the calcium release component of the CICR and sequestration cycle (because of a slower propagation of calcium induced channel activation across the membrane) shifting the cycle to a lower frequency. At the same time, protein kinase A is essential to the actin dependent elongation of anemone hair bundles (Mire-Thibodeaux and Watson, '94b). The elongated bundles would resonate at lower frequencies than at their resting length. Thus, NANA may cause elongation of hair bundles and a slowing of the CICR and sequestration cycle. In this context, it is interesting to note that key frequencies are 3.7 times as common in NANA as in seawater alone. Perhaps some hair bundles that are inactive in seawater because of a mismatch between bundle length and the CICR cycle are activated in NANA as the bundles elongate and the CICR cycle slows.

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