Calcium Dependence of Settlement and Nematocyst Discharge in Actinulae of the Hydroid *Tubularia mesembryanthemum*

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Abstract. The influence of Ca^{2+} and Mg^{2+} ions on both *atrichous isorhiza* (AI) discharge and settlement of actinular larvae of the hydroid *Tubularia mesembryanthemum* was investigated. Mg^{2+}-supplemented artificial seawater (ASW) completely inhibited both events at a concentration of 206 mM, whereas lowered Mg^{2+} concentrations enhanced them. Ca^{2+} ions in the bathing solution highly regulated AI discharge and settlement, and Mg^{2+} ions may down-regulate these events. The effect of inorganic Ca^{2+}-channel blockers, including Gd^{3+} and La^{3+}, was also examined. Larval settlement was inhibited by Co^{2+}, Ni^{2+}, Cd^{2+}, La^{3+}, and Gd^{3+}, with half inhibitory concentrations (IC_{50}) of 5800, 260, 53, 45, and 7 μM, respectively; AI discharge was also inhibited by these ions, with IC_{50} values of 6600, 500, 78, 41, and 5 μM, respectively. These results suggest possible involvement of stretch-activated Ca^{2+} channels in the signal transmission of both AI discharge and larval settlement.

Introduction

The colonial marine hydroid *Tubularia mesembryanthemum* Allman, 1871 (Hydrozoa: Tubulariidae) is a prolific marine fouling organism that frequently dominates artificial substrata used in aquaculture. Actinula larvae, produced as

a means of dispersal, are composed of a conical manubrium, oral- and aboral tentacles, and a basal protrusion that later becomes the settlement organ. Settlement-competent actinulae initiate settlement behavior by contact of the aboral tentacle tip (ATT) with the substratum surface, which triggers discharge of *atrichous isorhiza* (AI), the agglutinant nematocysts located at the ATT.

Upon reception of appropriate stimuli, nematocysts can eject adhesive tubules (Tardent, 1988). Nematocyte excitation requires both mechanical and chemical stimuli (Pantin, 1942). Two classes of chemoreceptors were identified in the tentacle of the sea anemone *Aiptasia pallida:* one sensitive to low molecular weight amino/imino compounds, and the other specific for N-acetylated sugars (Thorington and Hessinger, 1988a). In addition, two types of mechanoreceptors are involved in triggering nematocyst discharge; one is contact-sensitive (Thorington and Hessinger, 1988b) and the other is tunable, vibration- and frequency-sensitive (Watson and Hessinger, 1989, 1991).

The necessity of external Ca^{2+} ions for nematocyst discharge has been demonstrated in *Aiptasia mutabilis* (Santoro and Salleo, 1991b), *Pelagia noctiluca* (Salleo *et al.,* 1994a), *Calliactis parasitica* (Salleo *et al.,* 1994b), *Charybdea rastonii* (Yanagita, 1973), and *Anthopleura elegantissima* (McKay and Anderson, 1988). In *Hydra vulgaris,* discharge of *stenotele* is regulated by a voltage- and Ca^{2+}-dependent mechanism, allowing Ca^{2+} influx from the bathing solution (Gitter *et al.,* 1994). The involvement of intracellular Ca^{2+} ions in the metamorphosis of hydrozoan larvae has also been suggested on the basis of studies monitoring the emission of bioluminescence from photoproteins, thereby visualizing changes in $[Ca^{2+}]_i$ during

planular metamorphosis (Freeman and Ridgway, 1987, 1990).

Previous work has shown that Ca^{2+} release from intracellular stores triggered AI discharge in *Tubularia mesembryanthemum* actinulae, followed by the inflow of Ca^{2+} ions from the bathing solution into the nematocysts (Kawaii *et al.*, 1997). Furthermore, AI discharge was usually accompanied by sinuous movement of the aboral tentacle. The involvement of Ca^{2+}-mediated signal transduction in actinular settlement and metamorphosis was suspected. To clarify the role of Ca^{2+} ions in actinular settlement, we examined AI discharge and larval settlement using intact larvae that were bathed in artificial seawater (ASW) containing various concentrations of Ca^{2+} and Mg^{2+} ions. The influence of di- and trivalent cations and Ca^{2+}-channel blockers (Yang and Sachs, 1989; Santoro and Salleo, 1991; Salleo *et al.*, 1994a, b; Gitter *et al.*, 1994) was also examined.

Materials and Methods

Reagents

Reagent grade artificial seawater (ASW) consisting of NaCl (460 mM), KCl (10.1 mM), $CaCl_2$ (9.2 mM), and $MgCl_2$ (42.6 mM) at pH 7.6 was adjusted by addition of 5 mM imidazole. The concentration of Ca^{2+} was reduced, without altering the osmolarity of the ASW, by adjusting Mg^{2+} accordingly. Mg^{2+}-supplemented ASW with 59, 75, 125, and 206 mM of Mg^{2+} was prepared by mixing the regular ASW with 370 mM $MgCl_2$ aqueous solution (which is isotonic to ASW) in the ratios of 1:19, 1:9, 1:3, and 1:1, respectively, to compensate for the osmolarity. In the case of Ca^{2+}-channel blockers, the osmolarity was adjusted by Na^+. All Ca^{2+}-channel blockers were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Biological materials

Mature *Tubularia mesembryanthemum* colonies were collected mainly from submerged fisheries nets and ropes in the vicinity of Nagai Port in Sagami Bay (eastern Japan, 139°30'E, 31°70'N). Colonies were divided into male and female, and were maintained separately with sand-filtered running seawater at 16° ± 2°C as described by Yamashita *et al.* (1997). After fertilization, branches of female colonies with polyps bearing many actinulae were placed in filtered seawater (FSW; pore size, 0.45 µm), and sinking actinulae were collected. A single polyp could produce up to 300 actinulae, which were maintained at 4°C prior to use in an experiment. The actinulae were transferred to an ASW-containing beaker and maintained at 21 ± 2°C for several hours to recover normal responsiveness. Larval age was defined as time following release from the maternal gono-

phore, and 24-h-old actinulae were used in the following experiments unless otherwise stated.

Settlement assay

Actinular settlement was assayed using six-well polystyrene plates (Corning Cell Wells, Corning, NY), each well containing 6 ml of ASW and 5 actinulae. The plates were placed on an orbital shaker at 21 ± 2°C. The number of settled actinulae was counted under a binocular dissection microscope after 30 h. Each experiment was performed in triplicate.

To determine the reversibility of actinular settlement upon replacement of the test ASW with regular ASW, larvae that were largely sinking or attaching to the bottom of the well were washed by several replacements of the surface of the test ASW with regular ASW (roughly half the volume of the test ASW was exchanged with each replacement).

Larval behavior and atrichous isorhiza *discharge*

Each actinula was independently examined for the effect of Ca^{2+}, Mg^{2+}, or Ca^{2+}-channel blockers on its behavior when an ATT was contacted with a clean micropipette or on AI discharge. Actinulae in regular ASW were washed and suspended in test ASWs containing various concentrations of Ca^{2+}, Mg^{2+}, or Ca^{2+}-channel blockers. Each actinula was held by suction applied through a micropipette attached to the side of the adhesive protrusion. Behavioral responses to ATT contact with a clean micropipette were examined on suction-held actinula whose ATTs did not contact any substrata.

For monitoring AI discharge, an ATT of a suction-held actinula was immobilized and attached to the bottom of a petri dish by applying gentle suction through a second micropipette attached to the "wrist," which is the part between an ATT and an aboral tentacle. AI discharge of the immobilized ATT was then triggered by the addition of 100 µl of ASW that contained 200 mM K^+ ions (K^+-ASW) and had been adjusted to the osmolarity of regular ASW by reducing the Na^+ concentration. AI discharge was observed through the 40 × objective of a microscope, and the number of AI discharged before the K^+-ASW application (usually 0–1) was subtracted from the final total.

To avoid larval disintegration in Ca^{2+}-free ASW, a micropipette-held actinula in regular ASW was quickly and thoroughly washed and suspended in Ca^{2+}-free ASW. Immediately afterward, an ATT of the actinula was immobilized as described above and AI discharge was triggered.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured in whole mounts of living actinulae ATT in which AI discharge was induced. The actinulae had been treated with the Ca^{2+}-chelating fluorescent indi-

cator fura-2 (Kawaii *et al.,* 1997). The fluorescence intensities of ATTs from fura-2-loaded actinulae were approximately 100 times stronger than those from non-labeled actinulae, and were strong enough for $[Ca^{2+}]_i$ measurement. The value of the fluorescence ratio between excitation at 340 nm and 380 nm ($R_{340/380}$) was used to indicate $[Ca^{2+}]_i$. The minimum interval was 1.9 s for ratiometric imaging.

Results

Ca²⁺ dependence of actinular settlement

Reducing the external Ca^{2+} concentration had a significant effect on actinular settlement, and the effect was competitively antagonized by Mg^{2+} (Fig. 1A). Settlement was comparable to normal in 4.6–9.2 mM Ca^{2+}, but it was inhibited at lower concentrations (70% ± 31% at 2.3 mM Ca^{2+}, 8% ± 10% at 1.1 mM Ca^{2+}).

Raising the Mg^{2+} concentration inhibited actinular settlement; 100% settlement was obtained at 75 mM Mg^{2+}, 50% at 125 mM Mg^{2+}, and 6.7% ± 11.6% at 206 mM.

The relative inhibitory effect of Mg^{2+} on actinular settlement was magnified at lower Ca^{2+}. In ASW containing 2.3 mM Ca^{2+}, settlement was reduced to 53% ± 12% at 59 mM Mg^{2+} and completely inhibited at 206 mM Mg^{2+}. The effects of Ca^{2+} and Mg^{2+} concentration on settlement were statistically significant as assessed by two-way ANOVA (Table I). Furthermore, there was a significant interaction between Ca^{2+} and Mg^{2+}. In each case the dose-dependent reduction of actinular settlement was reversed by rinsing the larvae with regular ASW 9 h after the experiment.

Antagonistic effect of Mg²⁺

Raising the Mg^{2+} concentration in an otherwise regular ASW had an inhibitory effect on AI discharge (Fig. 1B); the number of discharged AI was 4.0 ± 4.2 at 125 mM Mg^{2+}, and AI discharge was completely arrested at 206 mM Mg^{2+}. The inhibitory effect of Mg^{2+} increased when the external Ca^{2+} ion concentration was lowered. In ASW containing 4.6 mM Ca^{2+}, AI discharge was reduced to 5.7 ± 3.8 at 59 mM Mg^{2+} and 4.4 ± 3.1 at 75 mM Mg^{2+}. In ASW containing 2.3 mM Ca^{2+}, discharge was lowered to 4.3 ± 1.4 at 59 mM Mg^{2+}, and was completely inhibited at 125 mM Mg^{2+}. Two-way ANOVA revealed that the effects of Ca^{2+} and Mg^{2+} concentration on AI discharge were statistically significant, and that there was a significant interaction between Ca^{2+} and Mg^{2+} (Table I). Again, this dose-dependent reduction of AI discharge was reversed by rinsing the larvae with regular ASW 3 h after the experiment.

Figure 2 indicates significant enhancement of AI discharge in Mg^{2+}-reduced ASW (one-way ANOVA; $F = 11.9759, P < 0.0001$). In this experiment, AI discharge was triggered by K⁺-ASW (100 mM). The number of discharged AI was 2.1 ± 0.6 at 50 mM Mg^{2+} (regular ASW)
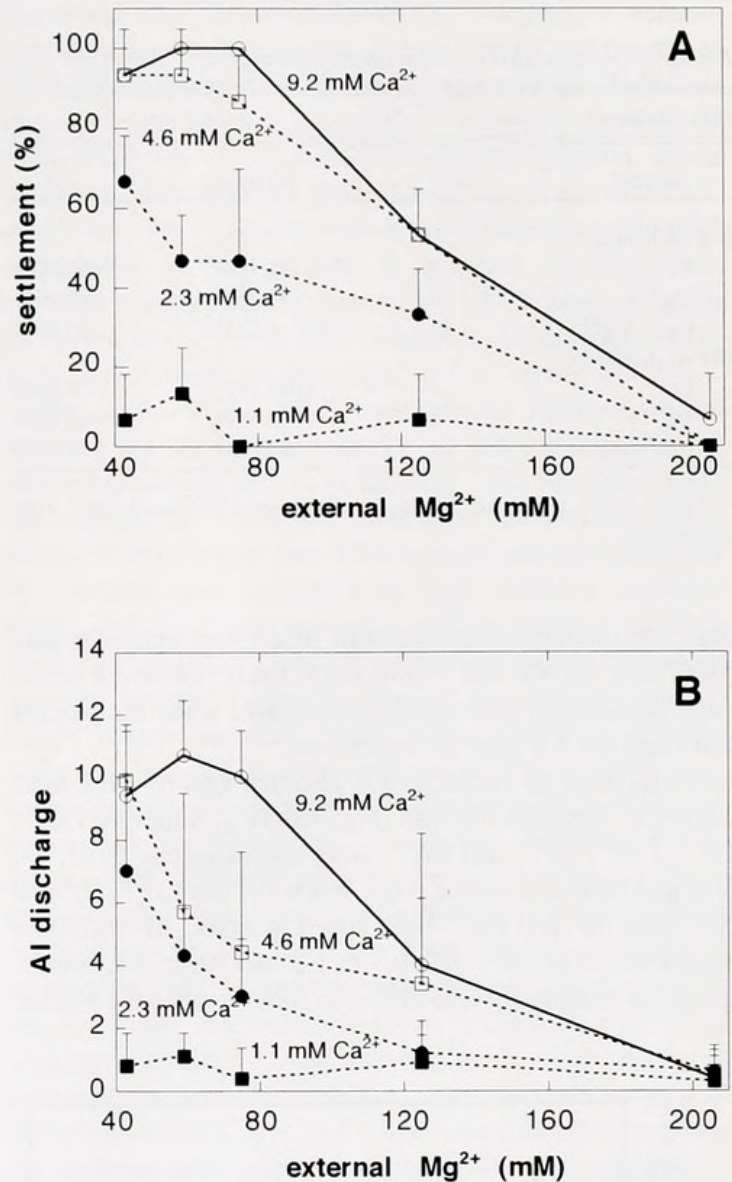

Figure 1. (A) Actinula settlement in artificial seawater with different concentrations of calcium and magnesium. Five larvae were incubated at specified concentrations of external Ca^{2+}. Vertical bars indicate standard deviations ($n = 5$). (B) K⁺-ASW triggered *atrichous isorhiza* (AI) discharge in artificial seawater containing different concentrations of Ca^{2+} and Mg^{2+} ions. Means were obtained by averaging the number of discharged AI in each aboral tentacle tip ($n = 10$). Vertical bars indicate standard deviations.

and increased to 7.6 ± 1.6 and 9.2 ± 1.9 at 25 and 10 mM, respectively. Addition of Mg^{2+}-reduced ASW (5 mM) also induced sinuous movement of the aboral tentacles.

Effects of Ca²⁺-channel blockers on actinular settlement

The effects of inorganic Ca^{2+}-channel blockers on actinular settlement were examined in various concentrations of chloride salt cations added to regular ASW. In each case, a dose-dependent reduction of actinular settlement was observed (Fig. 3A). The following ions are listed in order of increasing inhibition efficiency: $Co^{2+} < Ni^{2+} < Cd^{2+} < La^{3+} < Gd^{3+}$.

Table I

Results of two-way ANOVA to assess the effects of Ca^{2+} and Mg^{2+} concentrations on (A) actinular settlement and (B) atrichous isorhiza (AI) discharge

Source*	df	F-ratio	P
(A) settlement			
Ca^{3+}	3	118.4902	<0.0001
Mg^{2+}	4	75.7353	<0.0001
Ca^{2+}-Mg^{2+}	12	9.2255	<0.0001
(B) AI discharge			
Ca^{2+}	3	1022.1822	<0.0001
Mg^{3+}	4	1012.5841	<0.0001
Ca^{2+}-Mg^{2+}	12	534.8241	<0.0001

* Ca^{2+}-Mg^{2+} represents the interaction between Ca^{2+} and Mg^{2+} concentrations.

The 50% inhibition concentrations (IC_{50}) were approximately 5800, 260, 53, 45, and 7 μM, respectively. Observed settlement percentages were similar to controls when the blocker was removed 5 h after the experiment.

K^+-induced AI discharge was also inhibited by these inorganic Ca^{2+}-channel blockers (Fig. 3B). IC_{50} values for Gd^{3+}, La^{3+}, Cd^{2+}, Ni^{2+}, and Co^{2+}, were approximately 5, 41, 78, 500, and 6600 μM, respectively. When 10^4 μM Co^{2+}, 10^3 μM Ni^{2+}, and 10^2 μM Cd^{2+} were added to ASW, AI discharges were lowered to 1.0 ± 0.9, 0.8 ± 1.2, and 0.6 ± 1.1, respectively. AI discharges were similar to controls when the blocker
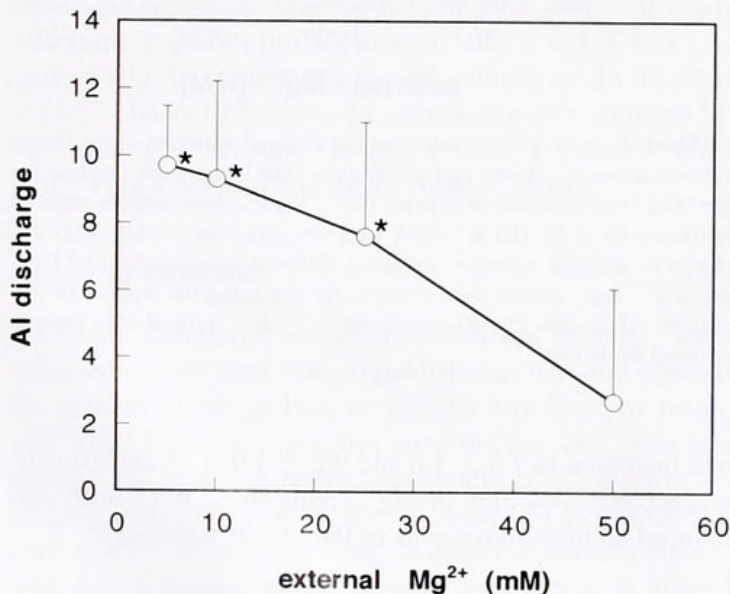

Figure 2. Enhancement of *atrichous isorhiza* (AI) discharge and settlement by low Mg^{2+} artificial seawater (ASW). Actinulae were bathed in ASW containing various concentrations of Mg^{2+} (regular ASW = 42.6 mM Mg^{2+}) for 1 h, and AI discharge was triggered by K^+-ASW (50, 100, or 200 mM) treatment. Means were obtained by averaging the number of discharged AI in each ATT ($n = 10$). Vertical bars indicate standard deviations. Asterisks (*) indicate significant differences relative to the untreated controls at $P < 0.05$ by the Tukey-Kramer honestly significant difference test.
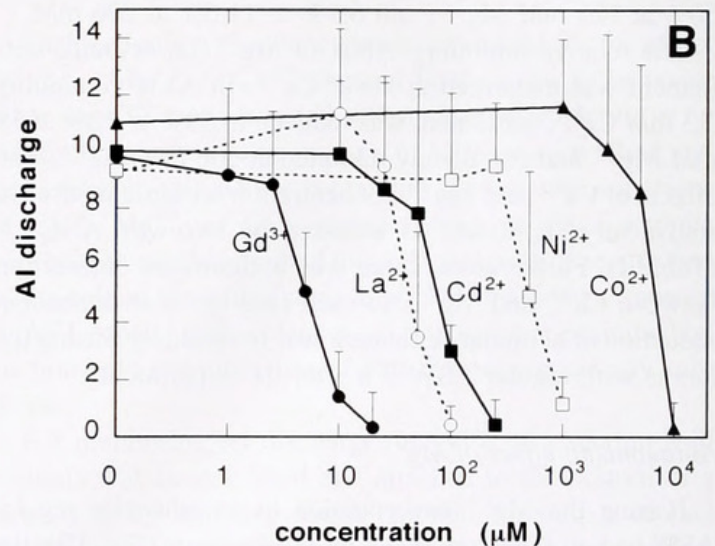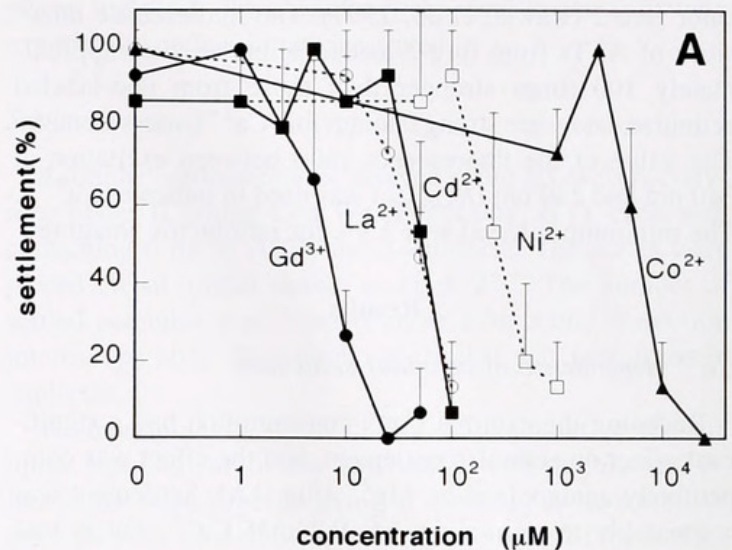

Figure 3. Effects of Ca^{2+}-channel blockers on actinular settlement (A) and *atrichous isorhiza* (AI) discharge (B). All experiments were performed using sulfate-free artificial seawater (ASW) at normal pH. Five larvae were incubated for 30 h in ASW containing various concentrations of Ca^{2+}-channel blockers.

was removed prior to K^+-ASW stimulation 1 h after onset of the experiment. ASW containing 25 μM Gd^{3+} or 100 μM La^{3+} decreased the AI discharge to 1.2 ± 0.8 and 0.8 ± 1.1, respectively, but the discharge was not restored completely when the blockers were removed.

Squashing an ATT with a micropipette caused AI discharge even in the presence of 20 μM Gd^{3+}, but sinuous tentacle movement, which was usually observed in regular ASW, was inhibited.

$[Ca^{2+}]_i$ transients during atrichous isorhiza *discharge*

Figure 4 compares representative changes of $R_{340/380}$ of ATT during AI discharge in Ca^{2+}-free ASW, Mg^{2+}-supple-
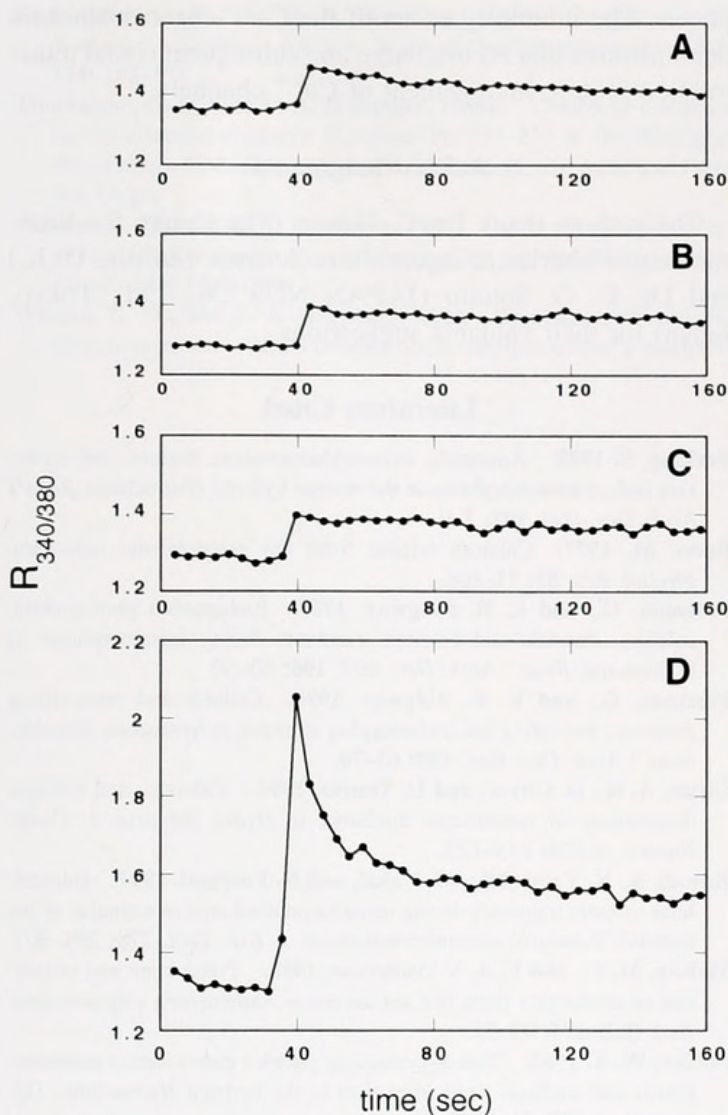
Figure 4. [Ca²⁺]ᵢ transients during *atrichous isorhiza* discharge in (A) Ca²⁺-free artificial seawater (ASW), (B) Mg²⁺-supplemented ASW (200 mM Mg²⁺), (C) 20 μM Gd³⁺-ASW, and (D) regular ASW.

mented ASW (200 mM Mg²⁺), 20 μM Gd³⁺-ASW, and regular ASW. Despite the discharge inhibition by Gd³⁺ ions, $R_{340/380}$ transients were induced by the addition of K⁺-ASW (200 mM). The rise of $R_{340/380}$ measured in the presence of Gd³⁺ ions was similar to that observed in Ca²⁺-free ASW and considerably lower than that observed in regular ASW [$\Delta R_{340/380}$ = 0.23 ± 0.11, 0.18 ± 0.17, and 0.62 ± 0.24, respectively]. No significant difference was observed between Ca²⁺-free ASW ($\Delta[Ca^{2+}]_i$ = 0.31 ± 0.09, n = 6) and Mg²⁺-supplemented ASW (0.28 ± 0.11, n = 6).

Discussion

Ca²⁺-dependence of settlement and atrichous isorhiza discharge

Although external Ca²⁺ ions have been reported as being required for nematocyst discharge in several species of cnidaria (Salleo *et al.*, 1993; Santoro and Salleo, 1991a, b),

this is the first demonstration of Ca²⁺-dependent nematocyst discharge in larvae. We also confirmed and quantified the Ca²⁺-dependence of actinular settlement in *Tubularia mesembryanthemum*. Reduced-Ca²⁺ seawater also inhibits metamorphosis of *Hydractinia echinata* planula larvae (Berking, 1988; Müller, 1985). The EC₅₀ of external Ca²⁺ was 3 mM for *T. mesembryanthemum* actinular settlement, comparable to that of both *H. echinata* settlement and actinular AI discharge (Kawaii *et al.*, 1997). Since actinulae immersed in Ca²⁺-free ASW tended to disintegrate, settlement assays were not performed in Ca²⁺-free ASW.

The action of K⁺-ASW was strongly inhibited by increasing the Mg²⁺ concentration of the bathing solution, and the inhibitory effects of the Mg²⁺ ion increased when the external Ca²⁺ concentrations were lowered. Similarly, Müller (1985) found that TPA-induced metamorphosis of *H. echinata* was promoted in Mg²⁺-reduced seawater. Mg²⁺ and Ca²⁺ ions compete with each other in many biological processes; consequently, we expected that an increase of Mg²⁺ concentration in the bathing solution would reduce the Ca²⁺ influx, thus inhibiting actinular settlement and AI discharge. However, the rise of $R_{340/380}$ values measured in Mg²⁺-supplemented ASW was equivalent to that observed in Ca²⁺-free ASW, and there was no influx of Ca²⁺ ions from the Mg²⁺-supplemented ASW to nematocytes. These observations indicate that inhibition of AI discharge by Mg²⁺ did not result from the lowering of nematocyte [Ca²⁺]ᵢ level by competitive influx of Mg²⁺ and Ca²⁺ ions. The enhancement of AI discharge and larval settlement by Mg²⁺ reduction may be a result of cation-mediated alteration of membrane-associated cell function in signal transduction, and Mg²⁺ ions would therefore regulate AI discharge and settlement of the hydroid as an inhibitory element.

Involvement of stretch-activated (SA) channels

It is natural for us to speculate about involvement of stretch-activated (SA) channels in actinular settlement, because the inhibitory effect of gadolinium ions at low concentrations in our study is comparable to that of opening SA channels in patch-clamped *Xenopus* oocytes (Yang and Sachs, 1989). Gd³⁺ is the most effective blocker of SA channels found to date, although it also has some effect on Ca²⁺ channels (Sadoshima *et al.*, 1992).

We previously demonstrated that both ATT contact with substrata and chemical stimuli are necessary to induce AI discharge (Kawaii *et al.*, 1997). By itself, a mechanical stimulus, such as immobilization of ATT by suction and vibration applied through a clean micropipette, did not trigger AI discharge. However, contact with a bacterial-film-coated micropipette did trigger discharge and also induced sinuous movement of the tentacles. Moreover, K⁺-ASW treatment did not result in AI discharge from ATTs

that were not in contact with any substrata. These results suggest that K^+-ASW treatment replaced sensory input of chemical stimuli, but did not mimic physical stimuli from ATT contact. Therefore, the observation that the stretching caused by immobilization of ATT did not trigger AI discharge does not rule out the involvement of SA channels in the discharge mechanism.

Effect of Ca^{2+}-channel blockers

All the Ca^{2+}-channel blockers tested for inhibitory effects on K^+-ASW triggered AI discharge and larval settlement demonstrated similar dose-response curves and similar IC_{50} values. This consistency suggests that similar Ca^{2+}-channel types were involved in these events. Previously, we showed close relationships between discharge of AIs and sinuous movement of aboral tentacles (Kawaii *et al.*, 1997). Aboral tentacles that had discharged AIs usually initiated sinuous movements, resulting in settlement behavior. Thus AI discharge can be considered as the first step in the settlement process, and inhibition of AI discharge by Ca^{2+}-channel blockers interrupts the subsequent processes. Squashing of ATTs caused AI discharge, but no sinuous movement was observed when ASW was treated with a Ca^{2+}-channel blocker. These results suggest that these cations inhibited not only the AI discharge but also the signal transmission system that follows discharge and leads to the induction of sinuous movement or the aboral tentacle sinuous movement itself.

Direct monitoring of intracellular Ca^{2+} ions also indicated that AI discharge required both Ca^{2+}-release from intracellular stores and Ca^{2+}-influx from bathing solution, and that Ca^{2+}-channel blockers inhibited the large Ca^{2+} influx. Taking into consideration that K^+-ASW treatment triggered limited AI discharge in Ca^{2+}-free ASW (Kawaii *et al.*, 1997) or in ASW containing Ca^{2+}-channel blocker, a Ca^{2+} release from intracellular stores functioned as the signal transmission system in AI discharge. In the sea anemone *Haliplanella luciae,* a precise pharmacological study demonstrated that Ca^{2+} acts as a second messenger, and intracellular Ca^{2+} stores play an important role in the regulation of nematocyst discharge (Russell and Watson, 1995), via a mechanism of "calcium-induced calcium release" (Endo, 1977). It is conceivable that similar mechanisms for regulating nematocyst discharge are present in different types of nematocytes. In Ca^{2+}-free ASW or ASW containing 10 μM Gd^{3+}, K^+-ASW treatment triggered limited AI discharge and caused slight elevation of $R_{340/380}$. These small $[Ca^{2+}]_i$ transients could be interpreted as a Ca^{2+} release from intracellular stores to nematocyte cytosol.

In conclusion, AI discharge was the primary action in actinular settlement, and signals from this discharge were spread throughout the larva, initiating actinular metamor-

phosis. The inhibitory effect of the Ca^{2+}-channel blockers demonstrated that AI discharge and subsequent signal transmission require involvement of Ca^{2+} channels.

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