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RESEARCH ARTICLE

Coordination between catch connective tissue and muscles through nerves in the spine joint of the sea urchin *Diadema setosum*

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ABSTRACT

Echinoderms have catch connective tissues that change their stiffness as a result of nervous control. The coordination between catch connective tissue and muscles was studied in the spine joint of the sea urchin *Diadema setosum*. Spine joints are equipped with two kinds of effector: spine muscles and a kind of catch connective tissue, which is called the catch apparatus (CA). The former is responsible for spine movements and the latter for maintenance of spine posture. *Diadema* show a shadow reaction in which they wave spines when a shadow falls on them, which is a reflex involving the radial nerves. Dynamic mechanical tests were performed on the CA in a joint at which the muscles were severed so as not to interfere with the mechanical measurements. The joint was on a piece of the test that contained other spines and a radial nerve. Darkening of the preparation invoked softening of the CA and spine waving (the shadow reaction). Electrical stimulation of the radial nerve invoked a similar response. These responses were abolished after the nerve pathways from the radial nerve to spines had been cut. A touch applied to the CA stiffened it and the adjacent spines inclined toward the touched CA. A touch to the base of the adjacent spine softened the CA and the spines around the touched spine inclined towards it. The softening of the CA can be interpreted as a response that reduces the resistance of the ligaments to spine movements. Our results clearly show coordination between catch connective tissue and muscles through nerves.

KEY WORDS: Catch apparatus, Mutable collagenous tissue, Nervous control, Mechanical properties, Sea urchin, Shadow reaction

INTRODUCTION

Echinoderms have special connective tissue with mechanical properties that can be altered rapidly and reversibly. This connective tissue is called catch connective tissue or mutable collagenous tissue (Wilkie, 2005). It works as an effector that is responsible for posture maintenance. It stiffens to maintain the posture of animals with little energy consumption (Takemae et al., 2009; Motokawa et al., 2012); it softens to allow muscle to change the posture. It becomes very soft in autotomy and fission to allow animals to cast off their body parts (Motokawa and Tsuchi, 2003; Wilkie, 2005). The activities of catch connective tissue are under neural control. Although ample evidence of neural control exists from studies of morphology (Wilkie, 1979; Hidaka and Takahashi, 1983; Welsch et al., 1995; Byrne, 2001), histochemistry (Inoue et al., 1999; Díaz-Balzac et al., 2007) and pharmacology (Takahashi, 1967b; Motokawa, 1987), definitive physiological evidence has been lacking. Such evidence should be based on a study in which the changes in the mechanical properties are examined when nerves are stimulated in a 'nerve–connective tissue preparation', which corresponds to the nerve–muscle preparation in classic muscle physiology. Another aspect lacking in the physiology of catch connective tissue is the coordination with muscles through nerves. Catch connective tissue and muscles are often found side by side. Although the coordination between them has been assumed (Hyman, 1955), no experimental evidence has been provided.

The present study was designed to show coordination between catch connective tissue and muscles using 'nerve–connective tissue' preparations. The material used was the catch apparatus (CA), the catch connective tissue in the spine joint of sea urchins. The joint comprises an outer cone of muscles and an inner cone of the CA (Fig. 1A). The former is responsible for spine movement and the latter is for maintenance of spine posture (von Uexküll, 1900). Von Uexküll regarded the inner cone as catch muscles similar to those of molluscs. They were, however, shown to be connective tissues with adjustable mechanical properties by Takahashi (1967a,b). The sea-urchin spine joint is the ideal material for the present purpose because the behaviour of spines (Kinosita, 1941; Bullock, 1965; Millott, 1966), the morphology of nerves innervating muscles and the CA (Millott and Takahashi, 1963; Kawaguti and Kamishima, 1965; Takahashi, 1967a; Hidaka and Takahashi, 1983; Peters, 1985) and response to stimuli of the spine muscle (Shingyoji and Yamaguchi, 1995) and CA (Takahashi, 1967b; Hidaka, 1983; Takemae and Motokawa, 2005) have all been well described. The behaviour of the spines suggests coordination between the CA and muscles. A light touch on the test surface provokes the convergence response in which spines around the stimulated location incline to cover that area (Bullock, 1965) as a result of muscle contraction. A light touch directly on a spine, however, makes the touched spine immobile in an upright position (Hyman, 1955), which we call here the 'freeze response'. Both the freeze response and the convergence response are defensive behaviours: in the former, the spine works as a spear held against the invader and in the latter, the spines make a barricade covering the surface of the test. When the frozen spine was forced to incline to a large degree by manual manipulation, the CA broke but muscles did not, whereas a forced inclination of the same degree given to the spine in the non-frozen condition caused no damage to the CA (Kinosita, 1941), which strongly suggests that it was the stiffened CA that resisted the forced deformation in the freeze response. Because the frozen spine showed a convergence response when the test surface of the adjacent area was touched, Hyman (1955) suggested that the CA must soften before spine

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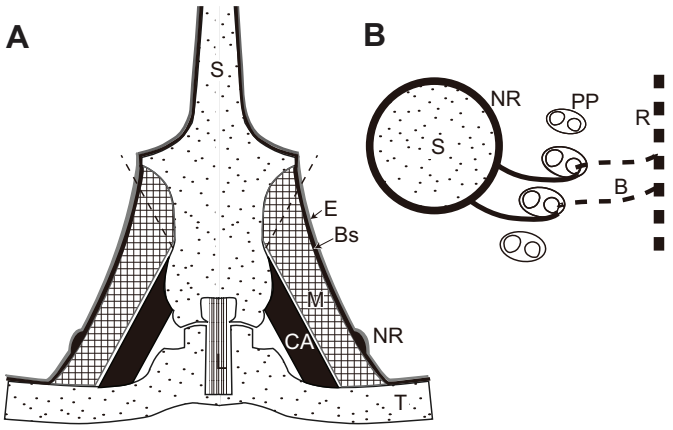

Fig. 1. Schematic drawings of the spine joint and innervation pathways in *Diadema*. (A) Spine joint cut in half along the spine axis. Dashed lines show where muscles were cut in the preparation. (B) Surface view of a test with a row of pore pairs (PP) and an interambulacral primary spine (S) with a nerve ring (NR) at its base. The top of the figure is aboral. Dashed lines indicate radial nerves (R) and its branches (B) on the inner face of the test. The parts of the branches that emerged out to the test surface through the pores are drawn with solid lines. Bs, basiepidermal nerve plexus; CA, catch apparatus; E, epidermis; L, central ligament; M, spine muscle; T, test.

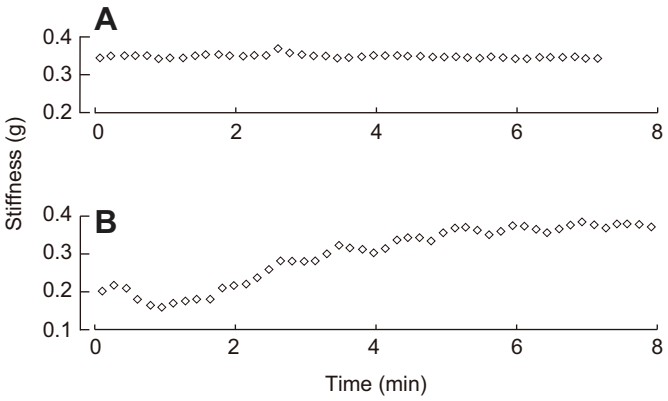

Fig. 2. Stiffness at the start of forced vibration. The vibration starts at time 0. (A) Preparation that showed steady stiffness from the start. (B) Preparation that showed a gradual increase in stiffness to reach a steady level.

muscles can move the spine, which thus suggests the presence of coordination between muscles and the CA, although the CA was believed to be catch muscle at that time.

Echinoderm nerves are difficult to investigate because they seldom produce regenerative spikes and synapses are rare (Cobb, 1989). Therefore, electrical stimulation of nerves usually causes ambiguous responses in effectors or no responses at all. The radial nerve of the sea urchin *Diadema* is exceptional: it produces regenerative spikes in response to light (Takahashi, 1964). The radial nerve of *Diadema* acts as a reflex centre for the shadow reflex in which the individual *Diadema* vigorously wave the spines when a shadow falls on it (Millott, 1966; Millott and Takahashi, 1963). It is reasonable to assume that the waving, which is caused by muscle activity, is associated with the softening of the CA because the spine movement would probably be impeded by the CA if it remained stiff. If we could show that shadows invoked not only the spine waving but also the softening of the CA in the radial nerve, spine muscle and CA preparation, we could provide evidence for coordination between the CA and muscles that is mediated by nerves.

RESULTS

Effect of forced vibration

About three quarters of preparations (48 out of 66) showed steady stiffness values from the start of vibration (Fig. 2A). Others, however, showed initial low stiffness, which increased to reach a steady value within 6 min (Fig. 2B). When the vibration frequency was increased from 0.1 to 0.16 Hz, the stiffness increased to higher steady values that were maintained for more than 10 min even after the frequency was lowered again (Fig. 3). The movement of other spines was never observed at the start of vibration or at the frequency increase. Thus the vibration applied to the CA sometimes worked as mechanical stimulation to the CA but it did not affect muscles in the adjacent spines.

Mechanical stimulation

A gentle touch applied to the CA increased the stiffness of the CA to about 1.5 times that before stimulation (Table 1). The reaction time was 10.5±1.7 s (mean ± s.d., n=4). Of four preparations, two showed transient stiffening, lasting for a few minutes and two showed longer-lasting stiffening that continued for more than 10 min (Fig. 4). The stimulus also caused the convergence response in spines around the stimulated spine and reaction time was shorter in these spines. The reaction time of the nearest spine was 0.33± 0.04 s (mean ± s.d., n=4). The reaction time of the CA stiffening and that of the spine convergence were significantly different (Mann–Whitney U-test: P<0.05).

A gentle touch applied to the base of the adjacent spine caused no movement in that spine but invoked the convergence response in the spines around it. The reaction time of the convergence response of the nearest spine was 0.36±0.07 s (mean ± s.d., n=10). The touch stimulus also caused a decrease in stiffness in the CA of the test spine. The decrease was apparent 10–20 s after stimulation and stiffness halved in 1–2 min. The low stiffness was maintained for more than 10–30 min before it returned to the value before stimulation in 7 preparations out of 10, but in others, the recovery occurred within several minutes (Fig. 5). The reaction time of the softening response was 14.4±2.2 s (mean ± s.d., n=10), which was far longer than that of the convergence response. The reaction times of these two responses were significantly different (Mann–Whitney U-test: P<0.01). Responses similar to those described above were observed in preparations free of radial nerves.

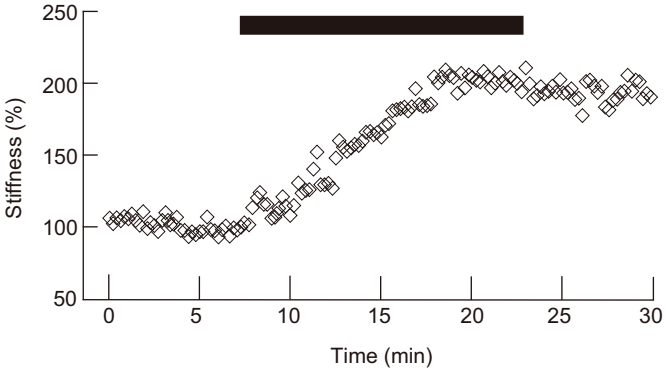

Fig. 3. Effect of higher vibration frequency. The frequency was increased from 0.1 Hz to 0.16 Hz during the period indicated by the horizontal bar. Stiffness is given as a value relative to that immediately before the frequency increase.

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Table 1. Stiffness after stimulation

Stimulus location	Mechanical	Electrical	Dark
CA	143±11 (4)	304±34 (3)	
Adjacent spine base/test surface	63±15 (10)	76±5 (3)	56±11 (10)
Radial nerve		62±9 (3)	

Data are means ± s.d. (number of experiments) of the stiffness value at the point of maximum change after stimulation expressed as a percentage of the value immediately before stimulation.

Dark stimulation

Darkening of the preparation induced spine waving and CA softening. All the spines around the test spine showed waving movements when the light was turned off. The waving started about 1 s after darkening and the reaction lasted for 20–60 s (Fig. 6). The duration of waving varied in different spines and it also differed from preparation to preparation. The reaction time of the waving response was 1.13± 0.12 s (average ± s.d., n=10). The stiffness of the CA in the test spine was halved by dark stimulation (Table 1). In most preparations (8 out of 10) the decreased value was maintained for more than 10 min before it returned to baseline, but in others the stiffness returned to baseline within several minutes. The reaction time of the CA softening was 19.1±1.4 s (average ± s.d., n=10), which was far longer than the reaction time of the spine waving. The two reaction times were significantly different by Mann–Whitney U-test (P<0.01).

The effect of severing nerve branches to spines from the radial nerve was studied. Fig. 7A shows the usual shadow response before the operation. After the operation, darkening caused no response: the CA did not soften nor did the spines around the test spine move (Fig. 7B). A touch stimulus applied to the base of the adjacent spine, however, caused both transient softening of the CA of the test spine and the convergence response in the spines around the touched spine (Fig. 7C), which implies that the operation had no adverse effects on the effectors in the spines. The same responses as those found in Fig. 7B and 7C were observed in preparations that were free of radial nerves.

Electrical stimulation

Electrical stimulation applied directly to the CA caused stiffening of the CA (Fig. 8A, Table 1) as with mechanical stimulation directly applied to the CA. The convergence response of the adjacent spines, which was always induced by mechanical stimulation, was, however, never observed. Electrical stimulation of the test surface caused both the softening of the CA and the convergence response of spines around the electrodes (Fig. 8B, Table 1). Responses similar to those described above were observed in the radial-nerve-free preparations. Electrical stimulation of the radial nerve caused softening of the CA (Fig. 8C, Table 1). The waving movement of adjacent spines was also observed, but the duration of waving was as short as a few seconds.

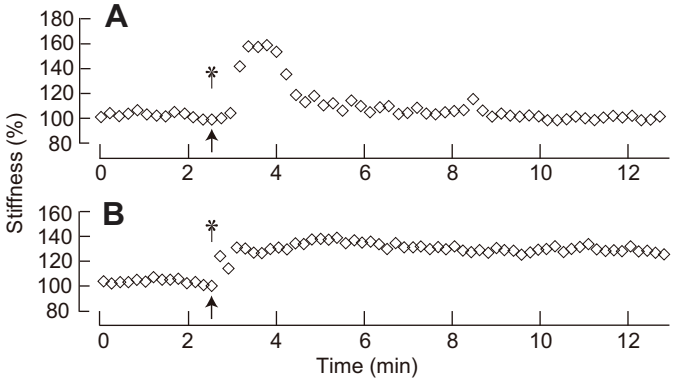

Fig. 4. Effects of mechanical stimulation applied directly on the catch apparatus. In this and the following figures arrows denote the time of stimulation and lines with asterisks denote the start of the convergence response in the spine immediately next to the stimulated spine. Stimulation invoked stiffening of the CA and the convergence response in the adjacent spines. The stiffening was either a transient one that ended within several minutes (A) or a longer lasting one that continued for more than 10 min (B).

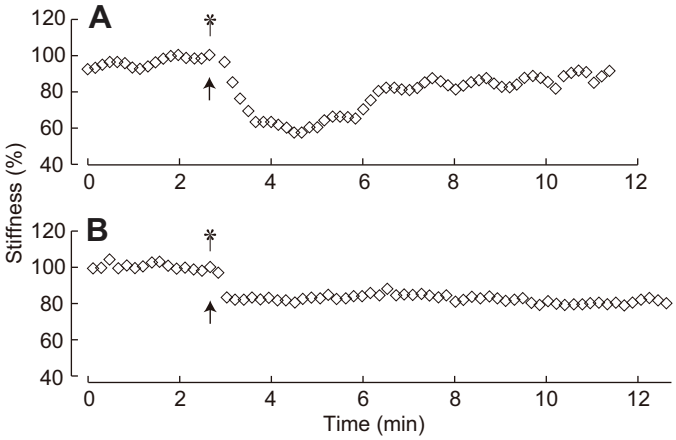

Fig. 5. Mechanical stimulation applied to the base of the adjacent spine causes both softening of the catch apparatus of the test spine and the convergence response in spines around the stimulated one. The softening was either transient (A), ending within several minutes or longer lasting and continuing for more than 10 min (B).

DISCUSSION

Coordination between the catch apparatus and muscles

Our results clearly showed coordination between the CA and spine muscles via nerves. Mechanical stimulation of the CA of the test spine invoked, on the one hand, stiffening of the CA in that spine, and on the other hand the convergence response in the adjacent

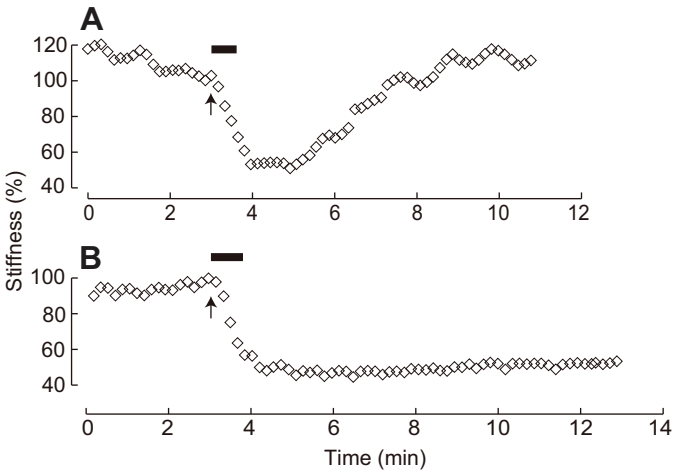

Fig. 6. Darkening induces softening of the catch apparatus and waving of spines (shadow reaction). The duration of the waving of the adjacent spine is indicated by a thick horizontal bar. The softening was either transient (A), ending within several minutes, or longer lasting and continuing for more than 10 min (B).

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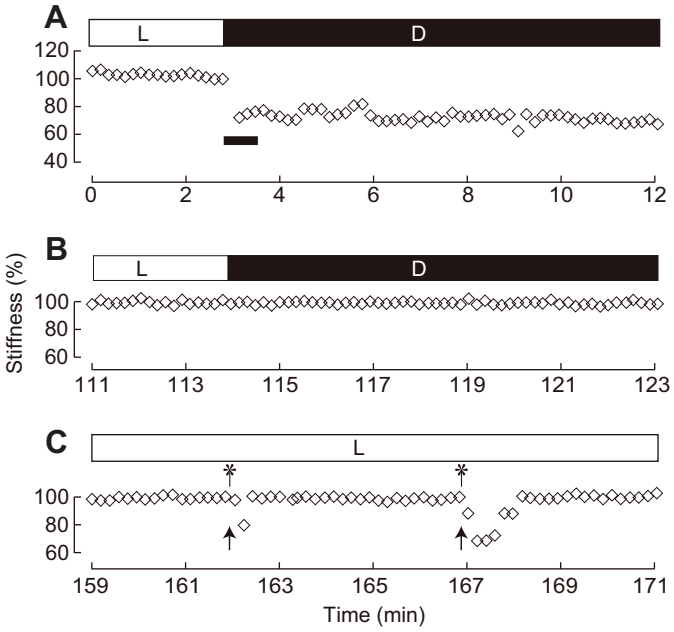

Fig. 7. Effects of cutting nerve pathways between spines and the radial nerve on the shadow reaction. The three figures are from a continuous recording from one preparation. Before the operation, both spine waving (indicated by the unlabelled horizontal bar) and CA softening were observed when the light was turned off (A). The operation to cut nerve pathways was executed at 15–25 min and the preparation was rested in seawater without forced vibration under full illumination. The vibration test was resumed at 100 min. Darkening induced neither waving nor softening (B), whereas a light touch on the base of the adjacent spine caused the softening of the test CA and the convergent response of the spines around the touched one (C). The horizontal bars denote the periods during which the light was on (L) or off (D). Stiffness is given as a relative value compared with the baseline value before darkening.

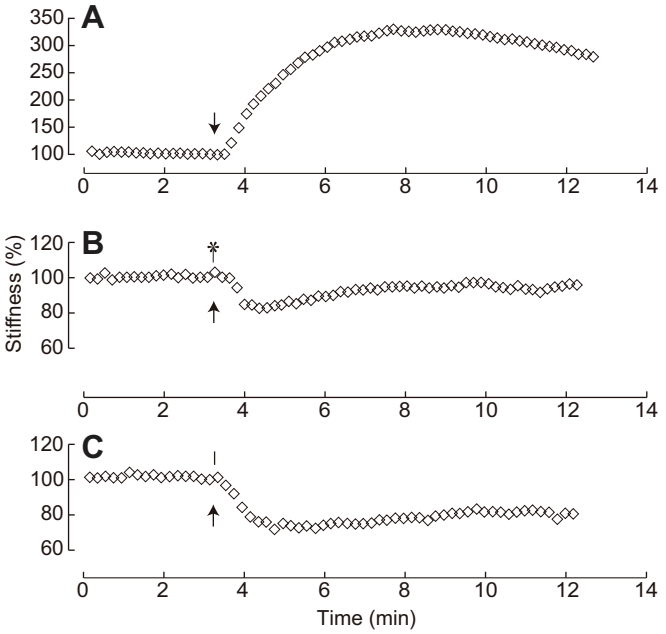

Fig. 8. Electrical stimulation applied to the catch apparatus, test surface and radial nerve. (A) CA, (B) test surface and (C) radial nerve. The short vertical line in C denotes the period of spine waving, which lasted for 2 s.

spines caused by muscle contraction. This result showed the presence of coordination between the CA in the stimulated spine and the muscles in the adjacent spines.

The coordination was not only between the effectors in the neighbouring spines but also between those in the same spinal joint, which was suggested from the results of mechanical stimulation to the base of the adjacent spines. It caused both the softening of the CA in the test spine and the convergence response in all the spines around the touched spine, which strongly suggests that the test spine also would have inclined if its muscles had been intact. Thus it is reasonable to conclude that the convergence response in the same spinal joint comprises coordinated muscle contraction and softening of the CA.

The present results whereby a touch to the CA caused both the stiffening of that CA and a convergence response in the neighbouring spine strongly suggests that the CA in the neighboring spine also changed its mechanical properties to become soft, which implies coordination between the CA of neighbouring spines.

The present results show that not only spine waving but also softening of the CA is involved in the reaction to the dark in *Diadema*. The activities of the CA and muscles are therefore very likely to be coordinated. The softening response of the CA is functionally explicable as a mechanism that enables spine muscles to work at low levels of force. The reaction to a drop in light intensity is a well-established reflex in which radial nerves are involved (Millott and Takahashi, 1963), and it was thus confirmed that the stiffness of the CA is under the control of radial nerves. Our finding that electric stimulation of the radial nerve mimicked the reaction to the dark supports this conclusion.

Coordinated role sharing between the catch apparatus and muscles

CA softening lagged ∼20 s behind waving in the response to darkening. A similar lag was also observed between softening and the inclination of spines in the convergence response. These results imply that the CA was not so stiff as to completely impede the spine movement. It is evident that the CA under the present experimental condition was neither in the stiffest state nor in the softest state because further stiffening or softening was observed when the CA was stimulated. Some catch connective tissue, including the CA, has been found to show three mechanical states: stiff, standard and soft; most, but not all, resting catch connective tissue is in the standard state, which has an intermediate stiffness value between the soft and the stiff states (Motokawa and Tsuchi, 2003; Motokawa et al., 2012). It is likely that the CA in the standard state was not stiff enough to completely immobilise the spine against spine muscle contractions that tended to incline the spine. Other interpretations might be possible. Collagen fibres in the CA are positioned along the axis of spines. In such materials with parallel fibre orientation, the deformation in the direction of fibres is met with large resisting forces whereas the deformation perpendicular to this direction produces small resisting forces (Wainwright et al., 1976). As long as the inclination angle was not large, spine waving probably causes only a small extension along the fibre axis with relatively large lateral compression of the CA (Motokawa, 1983), and thus the CA does not provide large resistive forces against the waving motion.

The reaction of the spines to darkening is a manifestation of the shadow reaction of *Diadema*, which is regarded as being a defence response to fish (Fricke, 1971). Some fish bite the tip of a spine to lift a sea urchin in order to turn it over to expose the unguarded oral surface for attack. The waving prevents spines from being caught by fish. The softening of the CA, although lagging behind muscles in

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response, would certainly permit muscles to move spines with less force during the softened period and thus with less energy expenditure. The long-lasting softening, even after the spine waving has ceased, may be in preparation for subsequent attacks by fish.

The convergence response and the freeze response are also defensive behaviours. In the convergence response, the softening of the CA also lagged behind the muscle response. Here again, the softening probably allows muscles to maintain the spine-pointing posture with less force and thus with less expenditure of energy. In the freeze response, the muscle contraction to erect the spine and to maintain that posture very likely precedes the stiffening of the CA because the reaction time of the CA stiffening is an order of magnitude longer than the reaction time of spine movement in other defensive behaviours. In the freeze response, the role of posture maintenance is probably handed over from muscles to the CA, which then complete the work with less energy expenditure. The spine joint of sea urchins is provided with two kinds of effectors. Muscles are faster in response and the CA is slower but more economical. Because defence against attacks should be initiated rapidly, the two effectors seem to be coordinated so that the muscles are recruited first to react to danger and then their role is handed over to the CA to maintain defence.

Neural pathways controlling the catch apparatus and muscles

A hypothetical scheme of neural control pathways for the two effectors is given in Fig. 9. The nerve ring at the spine base, which we call the outer nerve ring here, is proposed to be the control centre for the muscles. There is another nerve ring, the inner nerve ring, on the outer surface of the CA cone (Takahashi, 1967a; Peters, 1985). Cell processes between inner and outer nerve rings are present (Takahashi, 1967a). Because the processes of juxtaligamental cells, which are the neural elements controlling catch connective tissues (Wilkie, 2005), penetrate into the CA from the inner nerve ring (Peters, 1985), the inner nerve ring is proposed to be the control centre for the CA. We conjecture that mechanoreceptors are present in the CA, although nothing is known about sensory cells in the CA. We hypothesise that touch applied to the CA stimulates these receptors and they then send signals to the inner nerve ring. The inner nerve ring sends both stiffening signals to the CA and signals to the outer nerve ring from which further signals causing contraction are delivered to the spine muscles. The outer nerve ring also sends information to the adjacent spines. The stiffening of the CA is not programmed to send signals automatically to the adjacent spines because the stiffening caused by electrical stimulation or the vibration directly applied to the CA did not invoke the convergence response in adjacent spines. We hypothesise that information to the adjacent spine is sent, via the basiepithelial nerve plexus covering the test (Kinosita, 1941; Bullock, 1965) to the outer nerve ring of the adjacent spine. The outer nerve ring sends softening signals to the CA via the inner nerve ring. It also sends two kinds of signals to muscles. One causes contraction in the sector of the muscle cone facing the stimulated spine, and the other inhibits contraction in the sector opposite the stimulated spine, thus provoking the convergence response. The echinoid epidermis contains sensory cells that are sensitive to mechanical and photic stimuli (Cavey and Märkel, 1994). Touch stimuli applied to the base of the spine are received by these receptors and the information is assumed to be conveyed to the nearby spines through the common paths to those used in the convergence response invoked by touching the CA, which is

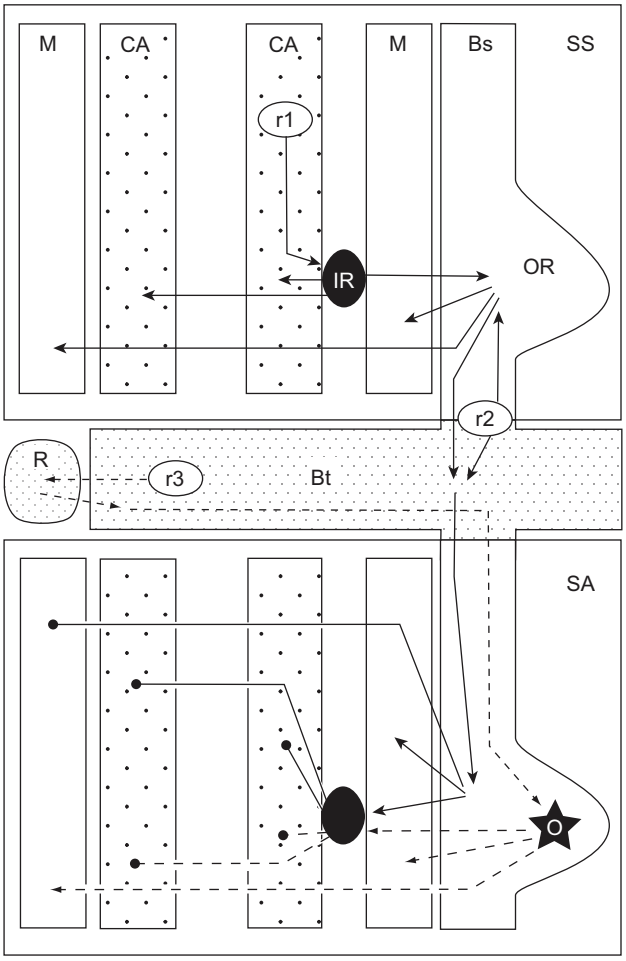

Fig. 9. Hypothetical scheme of coordination between the catch apparatus and muscles through nerves. Two spine joints, the stimulated one (SS) and the one adjacent to it (SA), are drawn. In each joint, two sets of the CA and the spine muscles (M) are shown. The two effectors are arranged in separate cones surrounding the joint, and the right ones represent the sector of the cones to which stimulus was applied in SS or to which the information from SS was delivered in SA. The left ones represent the sectors on the far side from those on the right. Lines ending with arrowheads or circles represent nerve pathways. Solid lines indicate pathways involved in the response to mechanical stimulation and dashed lines indicate those involved in the shadow reflex via radial nerves (R). Lines with arrowheads denote that the nerves provoke stiffening in the CA or contraction in muscles, whereas lines ending with solid circles denote that the nerves invoke softening in the CA or relaxation in muscles. Bs, basiepithelial nerve plexus of the spine; Bt, basiepithelial nerve plexus covering the test that is continuous with Bs; IR, inner nerve ring; O, oscillation generator for the spine waving; OR, outer nerve ring; r1, mechanoreceptor in the CA; r2, mechanoreceptor in the basiepithelial plexus; r3, photoreceptor in the basiepithelial plexus of the test. The basiepithelial plexuses of spines are omitted in the left sectors of both SS and SA. The paths involved in the freeze response in the stimulated spine are omitted, except that from r2 to OR.

supported by the finding that electrical stimulation of the test surface caused the convergence response. The information on touch is also sent to the outer nerve ring of the stimulated spine to cause the freeze response in which the stiffening and contraction in all the sectors of the CA and muscles are invoked. Photoreceptors in the epidermis covering the body surface of the sea urchins are affected by a drop in light intensity. Sensory information from them is delivered to the radial nerve from which signals to the spine are sent to cause the shadow reaction. The oscillation generator for waving is proposed to

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be in the outer nerve ring. When the generator is activated by information from the radial nerve, it sends signals to the CA that soften it. The generator also sends signals to both the opposing sectors of the muscle cone, which invokes repeated contraction–relaxation cycles in the opposing sectors that work antagonistically to produce the waving motion.

Time lag between muscle contraction and stiffness changes of the catch apparatus

The stiffness changes of catch connective tissues are the result of the changes of passive mechanical properties of extracellular materials. Stiffening and de-stiffening proteins have been isolated from the body-wall dermis of sea cucumbers, a well-studied catch connective tissue. NSF (novel stiffening factor) stiffens the dermis in the standard state into a stiff state (Yamada et al., 2010). Tensilin stiffens the dermis in the soft state into the standard state (Tipper et al., 2003) whereas softenin softens dermis in the standard state into a soft state (Takehana et al., 2014). Tensilin and softenin have antagonistic effects on the aggregation formation of the isolated collagen fibrils. The proteins are thought to be secreted from the cells whose secretory activities are neurally controlled, although which of the cells within connective tissues are responsible is yet to be identified; the secreted proteins diffuse through the tissue extracellular matrix to reach each collagen fibril to change the cohesive forces between fibrils. This diffusion process seems to constitute the main part of the time lag found between muscle contraction and changes in stiffness of the CA.

Small numbers of thin muscles containing only several thick filaments are found in the CA (Smith et al., 1981). The time lag that we observed suggested that their contribution to the changes in stiffness was very small, if any, because if they were involved their effects would have appeared as fast as that of the spine muscle contraction. They thus seem to work in the re-shortening of the extended ligament as Hidaka and Takahashi (1983) suggested. Microfibrils found among collagen fibrils in the CA (Hidaka and Takahashi, 1983; Motokawa, 1983) may also contribute to the re-shortening because elastic microfibrils have been suggested to cause the elastic recoil in other catch connective tissues (Birenheide and Motokawa, 1994; Thurmond and Trotter, 1996). The shortening of the spine muscles probably provides the main forces for the re-shortening, and the small muscles and microfibrils in the CA may work to restore the ordered parallel array of collagen fibrils.

MATERIALS AND METHODS

Specimens of the sea urchin *Diadema setosum* Leske 1778 were collected from the subtidal area of Aburatsubo coast in front of Misaki Marine Biological Station, the University of Tokyo, Kanagawa, Japan. The test diameter at ambitus was 5.5–6.5 cm. The animals were shipped to our laboratory in Tokyo and kept in an aquarium of circulating seawater at 20°C.

Nerve–CA preparation

A sea-urchin spine forms a ball-and-socket-like joint with a projection on the test which is called the tubercle. The spine base is connected to the tubercle by four layers of conical tissues that encapsulate the joint (Fig. 1A). The superficial layer is a thin epidermal layer under which lies a thin layer of the subepidermal nerve plexus. Beneath the plexal layer is a layer of spine muscles, and the innermost layer is the CA. The subepidermal nerve plexus thickens at the spine base to form a nerve ring from which nerve branches penetrate into the cones of muscles and the CA (Takahashi, 1967a; Peters, 1985). The radial nerves run meridionally on the inner face of the centre of each ambulacram. The branches of a radial nerve that extend to the primary spines emerge to the outer surface of the test through pore-pairs that contained the ampullary canals of the podia (Fig. 1B). A primary spine is innervated by two nerve branches, each from the adjacent pore-pairs (Millott and Takahashi, 1963).

A piece of test with two interambulacra and an ambulacram in between was dissected. The inner face of the test was cleaned of the viscera leaving a radial nerve at least 3 cm long in the ambulacrum. An aboral primary spine in one interambulacrum immediately next to the ambulacrum was used in the mechanical measurements of the CA. This spine or the test spine was left intact and other spines on this interambulacrum and the adjacent ambulacrum were trimmed ~2 mm above the milled ring. All the spines were removed from the other interambulacrum that was used for clamping to the experimental trough. An incision was made on the upper end of the muscle cone to disconnect muscles from the spine (Fig. 1A). This procedure caused muscles to contract toward the base of the tubercle, revealing the CA underneath. In the radial-nerve-free preparations, a test piece containing a single interambulacrum was used.

The effects of disconnecting from the radial nerve were examined in three preparations. The test was scratched with a scalpel between the interambulacral spinal row and the row of pore pairs on the adjacent ambulacrum in order to remove the covering soft tissues to expose the test surface. The scratch extended from the oral end to the aboral end of the test piece so that the nerve branches from the radial nerve to the test spine and other possible pathways through the basiepithelial nerve plexus were severed. The preparation was rested for more than 1 h after the operation before the vibration test was resumed.

Experimental setup

The mechanical testing machine is shown schematically in Fig. 10. The sea-urchin test piece with the test spine in the upright position was fixed to the experimental trough so that the test spine was held parallel to the plane of the forced vibration that was tilted a little so that the distal half of the spine emerged out of seawater when seawater was filled up to cover the spine joint. The spine was attached to the force gauge that was mounted on the moving arm of the testing machine. The arm was driven by the vibrator. Because the pivot of the arm was adjusted so that its position was just beneath the spine joint, the spine was forced to wave in an arc similar to the natural waving of the spine. The angle of the forced waving was ±1.5° when the upright spinal position was taken as 0°. Although *Diadema* has a central ligament, which is

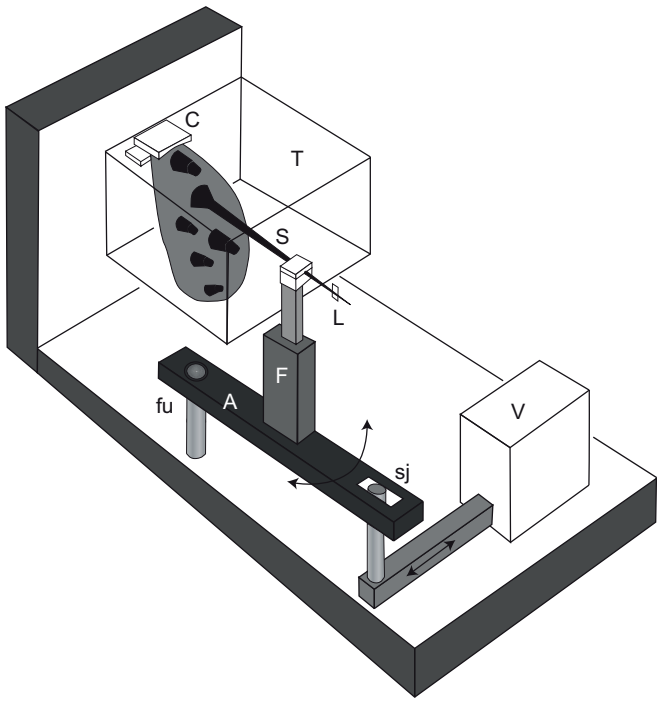

Fig. 10. Schematic drawing of testing machine. A piece of a test with a test spine (S) was held in a trough (T). The spine we fixed to the head of force gauge (F) mounted on the moving arm (A) that was driven by a vibrator (V). C, clamp; fu, fulcrum of arm; L, reflector for laser displacement sensor; sj, sliding joint between arm and vibrator head.

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housed in depressions in the spine base and the tubercle connecting these structures, it does not contribute to the resistive forces within this range of inclination angles (Motokawa, 1983). The vibrator was driven by an electromagnet that was fed with isosceles triangular waves of 0.1 Hz. Although higher frequencies gave better time resolution of responses, such frequencies worked as mechanical stimulation to the CA (see the Results). A frequency of 0.1 Hz was the highest frequency that maintained the preparation at a steady stiffness that was soft enough to respond to stiffening stimuli. This steady stiffness was maintained for more than 1 h at this frequency. The forced movement of the test spine was monitored with a laser displacement sensor. Signals from this and other sensors were recorded with the digital data recorder (NR-2000, Keyence, Osaka, Japan). The trimmed spines around the test spine moved when stimulated just like the intact spines did. Their movement was recorded with a video camera (HDR-SR7, Sony, Tokyo, Japan) that was sensitive to red light. The experiment was performed in a darkroom that had two lights: a darkroom red light that was on constantly because sea urchins show little sensitivity to light of this wavelength (Millott and Yoshida, 1960) and a 50 W halogen spotlight (~19,000 lx) that illuminated the preparation. The preparation was rested in the seawater-filled trough for at least 15 min before the vibration tests started. The temperature of the darkroom was controlled to 20±1°C.

Stiffness

Forced vibration produced maximum and minimum force values in a cycle. The difference between these values was regarded as 'stiffness' of the CA at the middle of the time period between the times when the maximum and minimum forces were recorded. The stiffness was expressed as a relative value that was normalised against that just before stimulation. Time resolution of the stiffness was 7–20 s depending on the phase of the vibration cycle in which stimulation was given. Stimulation was applied at the timing that gave the best time resolution of the reaction time of stiffness changes. Because the stiffness values fluctuated a little from cycle to cycle and sometimes showed a very slow steady increase or decrease, the reaction time of stiffness change was determined in the following way. The regression line of the stiffness values from 5 min before stimulation and immediately before stimulation was calculated and the line was extended to time after stimulation. The stiffness values after stimulation successively appeared above the line when the CA stiffened and below the line when it softened. The earliest time that such changes were detected was taken as the reaction time.

Stimulation

Stimuli were applied after the stiffness had reached steady values (see the Results). Darkening stimulation was given by the extinction of the spotlight. Mechanical stimulation was given by a gentle touch with a blunt forceps tip. It was given directly to the CA of the test spine or to the base of the tubercle of the interambulacral primary spine immediately adjacent to the test spine. Electrical stimulation was given through a pair of Ag–AgCl electrodes that was fed with 50 Hz AC of 2 V and 5 s duration from a stimulator. When the CA was directly stimulated the electrodes were placed on the opposite sectors of the CA cone. When the test surface was stimulated, one electrode was placed near the base of the test spine and the other was placed on the far side of the adjacent spinal base from the test spine. When a radial nerve was stimulated, the electrodes were placed on the part of the radial nerve that was at least 10 mm distant from the test spine. The distance between electrode tips was about 2 mm. Seawater in the experimental trough was drained when electrical stimulation was applied. Three preparations were used in each type of electrical stimulation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.M. conceived and designed the experiments and wrote the paper. Y.F. performed the experiments.

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