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Evidence that water exudes when holothurian connective tissue stiffens

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SUMMARY

The dermis of the body wall of sea cucumbers is composed mainly of extracellular materials such as collagens, proteoglycans and water; the water content is as high as 80%. Yet it shows rapid changes in stiffness under neural control. The dermis has been proposed to assume three mechanical states, soft, standard, and stiff. We investigated the relationship between the stiffness and the dermal mass and volume. Both the mass and volume decreased by 15% when the dermis stiffened from the standard state to the stiff state by mechanical stimulation and by chemical stimulation with potassium-rich seawater. The effect of the latter was abolished by anesthesia. The mass decrease was caused largely by water exudation. Tensilin, a holothurian protein that stiffens the soft dermis to form the standard state, did not cause any changes in mass. These results suggested that the stiffening mechanisms responsible for the transition from the soft state to the standard state, and that from the standard to the stiff state, are different. The removal of water from the dermis in the standard state, by soaking in hypertonic solution, caused only slight stiffening, which suggested that water exudation was not the direct cause of the stiffening. A change of pH of the surrounding medium, either more acidic or basic, was not associated with mass changes, although it caused a large increase in stiffness. The implications of the present results for the molecular mechanisms of the stiffness changes are discussed.

Key words: catch connective tissue, mutable collagenous tissue, echinoderm, sea cucumber, water content, hydrogel.

INTRODUCTION

Echinoderms have special connective tissues with mechanical properties that can be altered rapidly and reversibly under neural control. Such connective tissues are called catch connective tissues, based on their sustained maintenance of the stiff state with little expenditure of energy (Takemae et al., 2009); they are also called mutable collagenous tissues because of the mutability of their mechanical properties and because their principal protein component is collagen (Wilkie et al., 2004). The dermis of the body wall of sea cucumbers is one of the best studied of these connective tissues. The range of stiffness that the dermis shows is extremely wide. On the one hand, the dermis becomes very stiff to create a protective mechanical ‘shell’, and on the other hand, it ‘melts’ into a near-liquid state to allow the body parts to fall apart at autotomy. Detailed mechanical measurement revealed that the dermis assumes three distinct mechanical states: soft, standard and stiff (Motokawa and Tsuchi, 2003). The three mechanical states differ not only in stiffness but also in various other mechanical parameters such as strain dependence and energy dissipation, and thus the standard state is not simply an intermediate state between the two extremes of soft and stiff states. These states could be produced in isolated dermis by various stimuli and peptides isolated from sea cucumbers (Birenheide et al., 1998). They are also produced by immersion of the isolated dermis in the following media: the standard state in natural seawater or artificial seawater with normal composition (nASW); the soft state in calcium-free artificial seawater (CaFASW); the stiff state in artificial seawater with elevated potassium concentration (KASW) (Motokawa and Tsuchi, 2003). Soft dermis in CaFASW increased in stiffness when tensilin, a cell-derived stiffening protein was applied (Trotter and Koob, 1995); the effect of KASW was not observed in dermis of anesthetized animals and in dermis in which the cells had been destroyed (Motokawa, 1984a; Motokawa, 1994). From these results, both CaFASW and KASW are thought to change the mechanical state of the dermis not by affecting the extracellular components directly but by affecting the cellular elements controlling the dermal stiffness *in vivo* (Motokawa, 1994; Trotter and Koob, 1995; Wilkie et al., 2004).

The dermis is composed mostly of extracellular components whose mechanical properties are believed to be altered by secretion from the cells in the dermis. What kinds of molecules are secreted and how they change the interaction between the extracellular macromolecules to alter the dermal stiffness is little understood. The only known molecule secreted from some cells that can change the stiffness is tensilin: a stiffening protein isolated from the body wall of sea cucumbers that causes aggregation of isolated collagen fibrils (Tipper et al., 2003). The amino acid sequence of tensilin is similar to that of the tissue inhibitor of metalloproteinase family proteins (Tipper et al., 2003). Based on these findings, Wilkie (Wilkie, 2005) presented a model of the molecular organization of catch connective tissues. He hypothesized that the tensilin and tensilin-specific protease system induces stiffening and destiffening. Our recent work, however, showed that his hypothesis can account for only half of the story at best: tensilin stiffens the soft dermis to form the standard state, but it does not cause further stiffening from the standard state to the stiff state (Tamori et al., 2006). So far, no protein has been isolated from the dermis that stiffens the dermis in the standard state to create the stiff state, and no model has been proposed for the molecular mechanism responsible for this transition. In the present study, we examined whether water movement is involved in this transition.

The extracellular components of the holothurian dermis of the body wall include collagen fibers, microfibrils, proteoglycans, salts and water (Thurmond and Trotter, 1996). The microfibrils form a network (Thurmond and Trotter, 1996). Collagen fibers also seem

to be interwoven as a network; the interconnection between fibers is not through covalent bonds because a dermal block, even under an extremely light load, showed a continuous creep to form a thin thread before it broke (Motokawa, 1981). The water content of the dermis of most species is as high as 80–90% (Lindemann, 1900; Takemae et al., 2009), thus the dermis can be regarded as a hydrogel in which macromolecular networks are bathed in a solution with a composition similar to seawater. One possible mechanism for the changes in the stiffness in such gels is a phase transition (Tanaka, 1981; Yoshida, 2004). In hydrogels, changes in stiffness are very probably associated with changes in the number of bonds between macromolecules, which causes changes in the osmotic pressure, which then is associated with the movement of water in and out of the gels (Katayama et al., 1984; Hirotsu et al., 1987; Ilmain et al., 1991). The results of early work on sea cucumbers seem to suggest water movement during the stiffness changes. Lindemann (Lindemann, 1900) compressed the isolated sea-cucumber dermis. Fluid came out of the stiff dermis but not from the soft dermis. Serra-von Buddenbrock (Serra-von Buddenbrock, 1963) reported that the intact animal stiffened and shrank in size when the animal was stimulated. Although a stimulated animal shrinks by expelling water from its cloaca, loss of fluid from the dermis may also occur. Eylers and Greenberg (Eylers and Greenberg, 1989) studied the effect of ions on the isolated dermis, the cells of which had been killed by immersion in distilled water. The dermis swelled when it was transferred from distilled water to NaCl solution. The swollen dermis in NaCl shrank in $CaCl_2$ solution with the same tonicity. The dermis in $CaCl_2$ was stiffer than that in NaCl. Thus the dermis behaved as if it were a hydrogel, and the result suggested water movement during the stiffness changes. The ionic environment of the solutions was, however, quite different from that *in vivo*, and it is not clear whether their results are related to the stiffness change *in vivo*. Here, we report evidence that water is exuded when the dermis stiffens from the standard state to the stiff state. Both the mass and volume decreased greatly when the dermis in the standard state stiffened, which clearly showed water exudation on stiffening. The mass of the dermis, however, did not change when the soft dermis stiffened to the standard state. The present results provided evidence that the wide range in the mutability of the mechanical properties of the dermis included at least two different molecular stiffening mechanisms, one with water exudation responsible for the stiffening from the standard state to the stiff state, and the other without water exudation responsible for the stiffening from the soft state to the standard state.

MATERIALS AND METHODS

Animals and tissues

Specimens of the sea cucumbers *Actinopyga mauritiana* (Quoy et Gaimard) and *Holothuria leucospilota* Brandt were collected near the Sesoko Marine Science Center, the University of the Ryukyus, Okinawa, Japan. They were shipped to Tokyo Institute of Technology and kept in an aquarium in closed circulating seawater at 20–24°C. Two kinds of dermal samples, dermal loops and dermal blocks, were prepared from *A. mauritiana*. Only dermal blocks were prepared from *H. leucospilota*. The stiffness and mass of each dermal loop and the mass and volume of each dermal block were measured; the mass alone of some blocks was measured. The dermal loop was a 3 mm-thick cross section of a whole animal from which the outer epidermis and the inner muscle layers were removed. The dermal block (20 mm×10 mm×1 mm for *Actinopyga mauritiana* and 10 mm×10 mm×1 mm for *Holothuria leucospilota*) was dissected from the dorsal interambulacral region with a razor blade. All experiments were carried out at a constant temperature of 20°C. The data are considered to follow a normal distribution because the test of goodness of fit accepts the null hypothesis. Therefore, the statistical differences between means were tested using Student's *t*-test or, for multiple comparisons, analysis of variance and Dunnet *post-hoc* tests in most cases. When the statistical differences between the two experimental groups in solutions other than normal artificial seawater were tested for the measurements of mass or stiffness, Bonferroni *post-hoc* tests were used instead of Dunnet *post-hoc* tests.

Solutions

The composition of normal artificial seawater (nASW) was as follows: 433.7 mmol l^{-1} NaCl, 52.5 mmol l^{-1} $MgCl_2$, 10.0 mmol l^{-1} KCl, 10.1 mmol l^{-1} $CaCl_2$ and 2.5 mmol l^{-1} $NaHCO_3$. The osmolality of nASW was 1016 mOsm kg^{-1}. In calcium-free artificial seawater (CaFASW) the $CaCl_2$ was replaced with 7.2 mmol l^{-1} ethyleneglycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Artificial seawater with elevated potassium concentration (KASW) contained 100 mmol l^{-1} KCl; the concentration of NaCl was reduced to 343.7 mmol l^{-1} to retain iso-osmolality. Sucrose ASW was a hyperosmotic artificial seawater with an osmolality of either 1.19 (1.19 sucrose ASW) or 1.92 (1.92 sucrose ASW) times that of nASW on addition of sucrose. Saturated menthol in the nASW was used to anesthetize the tissue. The pH of all the above mentioned solutions was adjusted to pH 8.15.

When the effects of pH were tested, the following buffers were used instead of $NaHCO_3$. Acidic ASW (pH 4.0) contained 2.5 mmol l^{-1} sodium acetate, and basic ASW (pH 10.0) contained 10 mmol l^{-1} 3-(cyclohexylamino)-1-propanesulphonic acid.

Mass and volume measurements

The test blocks were placed in nASW or CaFASW for 3 h before the measurement of the initial mass and volume. The samples were then placed in a test solution or in the control solution. The nASW was used as a control solution except in the experiment with tensilin and in the experiment in which dermal blocks were transferred from CaFASW to nASW. In these experiments, CaFASW was the control solution. The mass (in grams) or volume (cm^3) of the samples was measured again 5, 15, 30 or 60 min after immersion in the test or control solution. In each set of experiments, control samples and experimental samples were obtained from the same animal. The experimental group was compared with the control group (Tables 1 and 2) except in the following experiments. In the experiments with tensilin and those involving transfer of *H. leucospilota* dermis from CaFASW to nASW, a common control was used. In the experiments with menthol and pH alteration, another common control was provided. The mass of the sample was measured after the medium had been removed from the surface by sandwiching it in a piece of folded laboratory wipe (Kimwipe, Nippon Paper Crecia, Tokyo, Japan) for 5 s. After measurement of the mass, the volume of the sample was determined by pycnometry.

Mechanical stimulation was applied to the dermal blocks in the following way. After weighing, the sample was wrapped in a thin plastic film and placed on the moving head of a laboratory mixer. The head vibrated at 5.5 Hz with an amplitude of 0.24 mm for 5 or 10 min. After vibration treatment, the plastic film was removed and the mass of the sample was measured again. Control samples were wrapped in the plastic film and left for 5 or 10 min without mechanical stimulation.

The mass or volume of the sample at a given time was expressed as a relative value normalized to its initial value.

Purification of *H*-tensilin

Tensilin was isolated from the dermis of *Holothuria leucospilota* (*H*-tensilin) according to the method of Tamori et al. (Tamori et al., 2006). CaFASW containing 3 μg ml^{-1} *H*-tensilin was used to determine its effect on the mass of the dermal blocks. Protein concentrations were determined with a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Dynamic mechanical test

Dynamic tests were performed on the dermal loop hooked between two holders, one stationary and the other movable. The inner surface of the loop was snugly fitted to the curved outer surface of the holders. This arrangement permitted mechanical testing without having to glue the samples to the holders and thus enabled measurement of mass before and after the test. The stationary holder was attached to the wall of the experimental chamber filled with 8.8 ml nASW. The movable holder was connected to a load cell (LTS-200GA, Kyowa, Tokyo, Japan) by which the force developed in the loop was measured. The load cell was driven by an actuator (511-A, EMIC, Tokyo, Japan) that imposed a sinusoidal cyclic stretch of 0.3 Hz and a maximal stretch of 1.25 mm on the loop along the axis corresponding to the right–left axis of the animal (Fig. 1). The maximal tensile stress was observed at the maximal tensile strain in a cycle. The former was divided by the latter to obtain the stiffness. Twenty-five minutes after the onset of a dynamic test, a test solution was introduced and its effect was measured for 50 min. In control samples the mechanical test was carried out for 75 min without changing the bathing medium. The stiffness was expressed as a relative value normalized by the value shown at the beginning of the mechanical test. When the effects of the chemicals were compared, however, the stiffness was normalized by the value just before the application of the chemical.

The mass of the loop before the mechanical test (initial mass) and after the test was measured. We estimated the change in mass induced by chemicals assuming that the mass of the sample at the application of the chemical had decreased from its initial mass by 6% which was the mean mass change in the samples 25 min after the start of the mechanical test in nASW (see Results).

RESULTS

Effects of K^+ on stiffness and mass

Both stiffness and mass were measured in the same dermal loops of *A. mauritiana.* When periodical stretches were applied to the loops in nASW, the stiffness began to increase and in about 15 min reached a peak that was maintained for about 10 min; the stiffness gradually decreased to the value at the start of the mechanical test, at 75 min (Fig. 2). The mean relative stiffness 25 min after the onset of the mechanical testing was 1.70 (s.d.=0.45; N=18) which was significantly different from the value at the start of the test (P<0.01). The cyclical stretch probably served as a mild mechanical stimulation to cause this stiffening (Shibayama et al., 1994; Tamori et al., 2006). The relative mass at 25 min was 0.94±0.02 (mean ± s.d.; N=11) which was significantly less than the value at the start of the test (P<0.01). The relative mass 75 min after the onset of testing in nASW was 0.93±0.03 (N=10), almost the same as the value at 25 min (Fig. 2).

KASW was applied to the loops at 25 min. It caused a further increase in stiffness (Fig. 2). The stiffness increased rapidly in the first 10 min, and later it continued to increase gradually. The mean relative stiffness of the dermal loops in KASW for 50 min was 3.19±0.41 (N=8), and their relative mass was 0.81±0.02 (N=8). The relative stiffness and relative mass 50 min after the application of KASW in the mechanical test were normalized to the values just before the application of KASW. The statistical analysis showed that the samples treated with KASW were significantly stiffer and lighter (Fig. 3).

Effects of K^+ on mass and volume

Both mass and volume were measured for the same dermal blocks of *A. mauritiana.* These two parameters decreased in exactly the same manner in KASW (Fig. 4). The decrease was apparent within 5 min and reached a plateau within 30 min. The mean relative mass and volume 60 min after the application of KASW was 0.84±0.06 (N=9) and 0.85±0.07 (N=9), respectively. The maximally reduced sample had a relative mass value of 0.75. Very little change in mass or volume was observed in the control dermis left in nASW for up to 60 min (Fig. 4).

The response to KASW was abolished by anesthesia. When the test pieces were anesthetized with menthol for 3 h, the samples showed no significant mass change after incubation for 15 min in KASW (N=6; Table 1).

The response to KASW was partially reversible. The test blocks that had been immersed in KASW for 60 min were weighed and then immersed in nASW for 60 min and weighed again. The mean relative mass in KASW was 0.87±0.06 (N=12), which increased to 0.90±0.03 (N=12) in nASW. The increase was observed in 11 out of 12 samples. Sign tests showed that the increase was statistically significant (P<0.01).

The decrease in mass caused by KASW was also observed in the dermis of *Holothuria leucospilota* (Table 2).

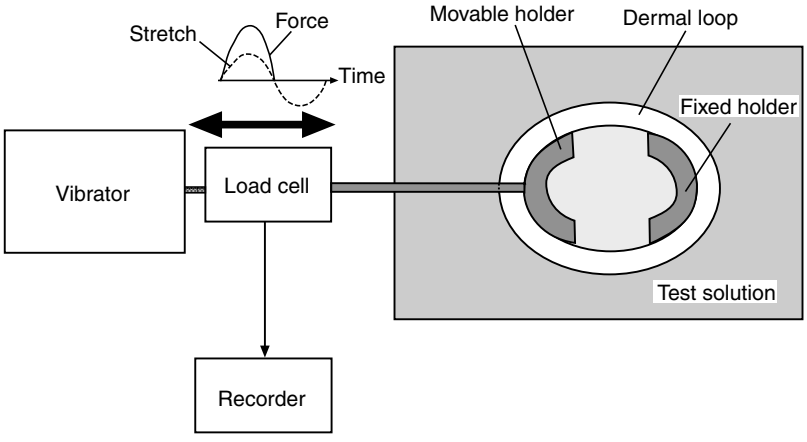

Fig. 1. Experimental apparatus for dynamic mechanical tests (viewed from the top of the trough).

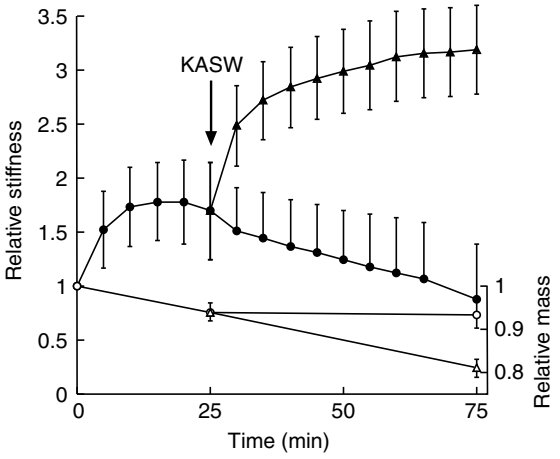

Fig. 2. Stiffness (closed symbols) and mass (open symbols) of the dermal loops of *Actinopyga mauritiana* in nASW (circles) and in KASW (triangles). The abscissa is the time after the onset of mechanical testing. KASW was introduced at 25 min. Values are means ± s.d. The number of experiments before 25 min is 18 and after 25 min, 10 and 8 for samples in nASW and in KASW, respectively.

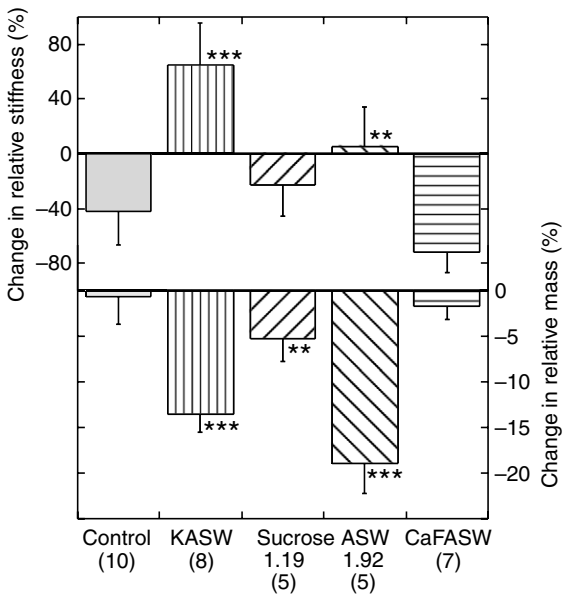

Fig. 3. Stiffness (upper figure) and mass (lower figure) of dermal loops of *Actinopyga mauritiana* immersed in test solutions for 50 min. Values are the relative values normalized to those just before the introduction of test solutions. The mass value just before the chemical stimulation was assumed to be 0.94-fold of the value before the mechanical test. Values are means with bars indicating the s.d. The numbers of experiments are given in parentheses below the test solutions. Asterisks indicate a statistical difference from the control (**P<0.01 and ***P<0.001).

Effects of mechanical stimulation and pH on mass

The effects of mechanical vibration (Shibayama et al., 1994) and ASW with acidic or basic pH (Hayashi and Motokawa, 1986) which are known to stiffen the standard dermis to the stiff state were investigated on dermal blocks of *A. mauritiana*. Vibration from a laboratory mixer caused a mass decrease. The means of the relative mass 5 and 10 min after the mechanical stimulation were 0.92±0.04 (N=13) and 0.90±0.03 (N=13), respectively. The difference in these values from those of the control samples to which no mechanical stimulation was applied was highly significant (P<0.001; Table 1).

When the dermis was transferred from nASW (pH 8.15) to acidic ASW (pH 4.0), the mass decreased slightly; the mean decrease was at most 3% (Table 1). Although the decreased was slight, the mean was significantly less than that of the control. The relative mass of the dermis in basic ASW (pH 10.0) was not different from the control value (Table 1).

Effects of osmolality on stiffness and mass

The dermal loops of *A. mauritiana* were made hyperosmotic to study the relationship between dermal stiffness and mass (Fig. 3). The relative mass of the samples immersed for 50 min in the hyperosmotic ASW containing sucrose was significantly less than that of control samples (P<0.01). The mean relative mass normalized to the initial mass was 0.89±0.02 (N=5) for 1.19 sucrose ASW and 0.76±0.03 (N=5) for 1.92 sucrose ASW. The latter value was significantly less than the value in KASW (P<0.05; Fig. 3). The mean relative stiffness of the samples immersed for 50 min in 1.19 sucrose ASW was not significantly different from the value of the control samples (P>0.05). Although 1.92 sucrose ASW caused a large decrease in mass in all the samples, its effect on the stiffness was variable. Three samples out of five became stiff but two showed a slight decrease in stiffness; the average stiffness on immersion in this solution for 50 min was 1.06±0.29 (N=5). This value was significantly greater than that of the control (P<0.01), whereas it was significantly less than the stiffness in KASW (P<0.01; Fig. 3).

Soft state and effect of tensilin

The effects of Ca^{2+} removal on mass and stiffness were measured in the same dermal loops of *A. mauritiana*. When transferred from nASW to CaFASW, the stiffness continued decreasing for 50 min but the mass remained the same (Figs 3 and 5).

Whether the mass might change when the stiffness increased from the soft state to the standard state was investigated in dermal blocks from both *A. mauritiana* and *H. leucospilota.* The samples were soaked in CaFASW for 3 h and the mass was measured for reference. Normal ASW that contained a normal concentration of Ca^{2+} was then introduced. The addition of Ca^{2+} did not alter the dermal mass (Tables 1 and 2).

H-tensilin is a protein that causes stiffening of the soft dermis to the standard state in *H. leucospilota* (Tamori et al., 2006). *H. leucospilota* dermal blocks were soaked in CaFASW for 3 h to produce the soft state. The introduction of 3 μg ml^{-1} *H*-tensilin into the CaFASW caused no significant changes in mass compared with the control samples treated with CaFASW without *H*-tensilin (P>0.05; Table 2). However the introduction of *H*-tensilin at this concentration did result in stiffening of the dermal blocks from the same animal that was used in the mass measurement. The stiffness of the blocks had increased approximately tenfold by 30 min after the addition of *H*-tensilin. *H*-tensilin at this concentration always resulted in stiffening of the dermal blocks of *H. leucospilota* in CaFASW (data not shown).

DISCUSSION

We showed that the mass and volume decreased in response to stimuli that stiffened dermis that was in the standard state. The mass of the dermis decreased to as low as 75% of the mass before stimulation in *Actinopyga mauritiana*. Because the water content

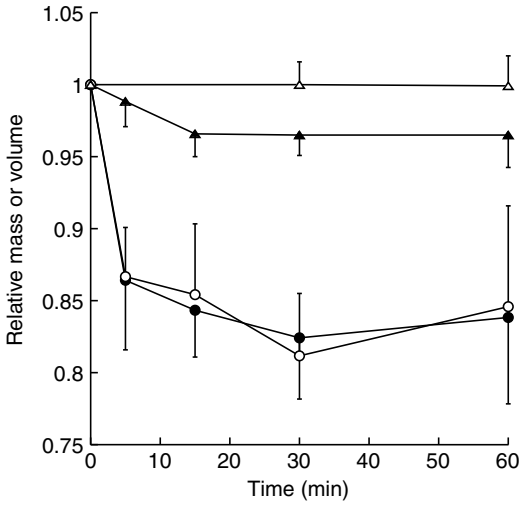

Fig. 4. The effects of KASW on mass (closed circles) and volume (open circles) of dermal blocks of *Actinopyga mauritiana*. Control values are shown as closed triangles (mass) and open triangles (volume). Values are means +s.d. or –s.d. (*N*=9–13). All the values of KASW-treated samples are significantly different from the control (*P*<0.001).

of this sea cucumber is 83% (Takemae et al., 2009), such a large decrease was impossible without exudation of water from the dermis. The recovery experiment provided evidence for water movement. It is known that the KASW-induced high stiffness returns to the value before stimulation by re-immersion in nASW (Motokawa, 1984b), and we found recovery of mass, although partial, in nASW. The nASW contained only water and dissolved inorganic salts and thus the mass recovery was no doubt caused by the re-entry of water with salts into the dermis. There is a possibility that, other than water and salts, the components of the dermis which had already dissolved or became dissolved or suspended at the stiffening may be lost from the dermis. However, the amount of such materials, if any, seems to be small. The finding that the mass and volume followed almost identical curves (Fig. 4) implied that the specific gravity of the lost materials was nearly 1. This suggests that the materials lost were in a quite diluted form and thus the bulk of the lost materials was water. The reversible nature of the stiffness changes also suggests this. If we assume no water loss, a 6% decrease in mass, for example, which accounts for only one-third of the mass loss in KASW, implies a loss of 30% of such materials other than water. It seems unlikely that such a large amount of materials moves out and in every time the tissue changes in stiffness. Therefore, we concluded that it is most likely that the material which moved out of the dermis at stiffening was mainly water.

Mechanical stimulation such as a mild touch or light repetitive patting on the intact sea cucumbers causes stiffening of the body wall (Motokawa, 1988; Takemae et al., 2009). Similar stimulation is experienced by the animals in daily life from, for example, waves and the impact of sand grains. The touch was probably sensed through nerve endings. The isolated dermis was repetitively stretched to mimic this natural stimulation. Stiffening and decrease in mass were also observed as a result. This response was assumed to be also mediated by cellular elements, possibly nerves in the dermis, as in the intact sea cucumbers (Shibayama et al., 1994). Another mechanism is possible, however, in which the stretch directly drives water out: uniaxial stretching of the dermis causes a lateral compression that might squeeze water out. This possibility seems

Table 1. The effect of mechanical stimulation or chemical stimulation on the relative mass of dermal blocks of *Actinopyga mauritiana*

	Means ± s.d.	*N*
Vibration	0.90±0.03***	13
No vibration (control)	0.98±0.01	13
KASW	0.84±0.03***	9
nASW (control)	0.97±0.02	12
KASW + menthol	1.00±0.01	6
pH 4	0.97±0.02***	10
pH 10	0.99±0.01	10
nASW (pH 8.15; control)†	1.00±0.01	6
CaFASW to nASW	0.99±0.01	6
CaFASW to CaFASW (control)	1.00±0.01	6

Duration of chemical stimulation was 15 min and 10 min vibration for the mechanical stimulation.
***Statistically different from control (*P*<0.001).
†Control for above three experimental groups.

to be small because the stiffness returned to the level before stimulation at the end of a period of sustained repetitive stretch for 75 min, which could best be explained by adaptation in the cellular elements to sustained stimulation. The decrease in mass in the dermis that was just laid on a vibrating platform, without stretching, also supported this view. The deformation imposed by vibration was much smaller than that produced by uniaxial stretching. Yet the stiffening effect of the vibration was as large as that of KASW, whereas that of the uniaxial stretch was much smaller than that of KASW. Uniaxial stretch very probably weakly stimulated the cells controlling dermal stiffness. KASW is known to stimulate such a mechanism rather than to directly affect macromolecular interactions (Motokawa, 1994), which was confirmed by the result in which anesthetized dermis did not change mass in response to KASW. Two different stimulations, KASW and mechanical stimulation, resulted in both stiffening and mass loss. This result strongly suggested a common stiffening mechanism. Because stiffening, as a result of mechanical stimulation, is a naturally observed response in intact animals, the stiffening mechanism, which, in the isolated dermis was found to be associated with water movement, is expected to function also in the intact body wall. Lindemann (Lindemann, 1900) compressed isolated dermis. Water came out of stiff dermis but not from unstiffened dermis. In the stiff state, water flow seems to become easier, and thus less pressure is needed to drive water out of the dermis. In the intact body wall, water is likely to be lost from the dermis during stiffening to the adjacent regions such as the body coelom or the outside of the animal because the body wall is permeable to water (Koizumi, 1932). When the sea cucumber is stimulated under natural conditions, dermis stiffens and

Table 2. The effect of chemical stimulation on the relative mass of dermal blocks of *Holothuria leucospilota*

	Means ± s.d.	*N*
KASW	0.90±0.06***	7
nASW (control)	1.03±0.01	7
H-tensilin	1.08±0.02	9
CaFASW to nASW	1.06±0.04	9
CaFASW to CaFASW (control)†	1.08±0.02	9

Duration of stimulation was 1 h. The effect of *H*-tensilin was tested in the absence of Ca^{2+}.
***Statistically different from control (*P*<0.001).
†Control for above two experimental groups.

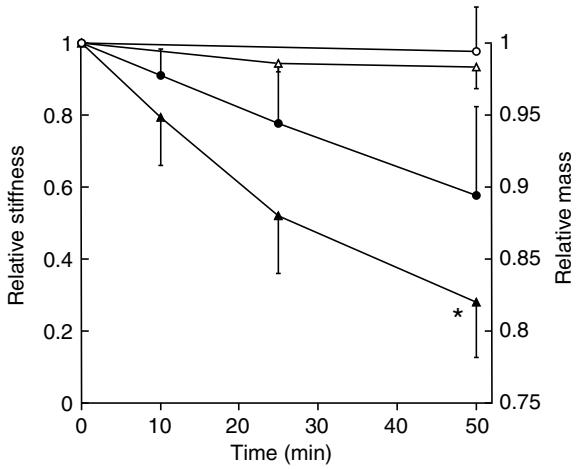

Fig. 5. Stiffness (closed symbols) and mass (open symbols) of the dermal loops of *Actinopyga mauritiana* in CaFASW (triangles) and in nASW (circles). Sinusoidal stretch was applied for 25 min in nASW, and then CaFASW or new nASW solution was introduced. In the first 25 min, stiffness increased slightly and then began to decrease slowly; stiffness continued decreasing in nASW and by 75 min it returned to the initial value (see Fig. 2). Only the response after 25 min, the time of the introduction of CaFASW, is shown in this figure. A faster decrease was observed in CaFASW (closed triangles) than that observed in ASW (closed circles). The stiffness value at 50 min in CaASW (*) was significantly smaller than the value before the introduction of CaFASW (P<0.05). Mass (open triangles), however, remained the same. Values are means +s.d. or –s.d. (N=10 for nASW and N=7 for CaFASW).

the body wall muscle contracts. The contraction of the muscle results in an increase in the pressure in the dermis. Such an increase may drive out water which is released by the stiffening. We speculate, however, that the water loss in the intact animals may be less than that in the isolated dermis because the cut ends of the isolated dermis provided little barrier to water movement.

It may be possible that the volume decrease caused by water loss shortens the distance between macromolecules in the dermis, which increases the interaction between them and thus causes stiffening. Such a simple mechanism was, however, not responsible for the dermal stiffening mechanism of sea cucumbers, because the hypertonic solutions containing sucrose induced little or no stiffening of the dermis, although they caused a large decrease in mass. Thus water loss was not the direct cause of dermal stiffening.

The effects of pH were studied because pH is expected to affect molecular interactions and the extent of hydration through altering the extent of dissociation of charged groups in the macromolecules. There are biological examples in which both pH and water content of extracellular materials are altered during neurally controlled stiffness changes. In insect cuticle, both the shift of pH to a more basic value and water loss have been observed during cuticle stiffening (Reynolds, 1975; Wappner and Quesada-Allué, 1996). In sea cucumbers, pH 4 ASW and pH 10 ASW are also reported to stiffen the dermis to an extent as large as that of KASW (Hayashi and Motokawa, 1986). The water content was, however, was affected little by pH, and thus pH changes are not involved in the water movement in the present study.

The stiffness changes in the dermis are probably derived from changes in the strength of the interaction between macromolecules in the dermis that are woven into networks. The water content of the dermis is high and thus we might regard the dermis as a hydrogel. One possible mechanism for the stiffening of a hydrogel is the formation of covalent bonds between the macromolecules, although the involvement of these bonds in the stiffening of the dermis is unlikely. This is because the dermis, even in the stiff state, showed complete relaxation in stress-relaxation tests (Motokawa, 1984b). Eylers and Greenberg (Eylers and Greenberg, 1989) also suggested that covalent bonds do not regulate dermal stiffness. It is probable that the macromolecular interaction responsible for stiffening depends on weak bonds. The kinds of weak bonds that contribute to the interaction between macromolecules in hydrogels are hydrophobic, ionic, van der Waals, and hydrogen bonds (Katayama et al., 1984; Hirotsu et al., 1987; Ilmain et al., 1991). Bond formation between adjacent macromolecules through any of these interactions will result in water exudation: water between the macromolecules will be expelled if the facing portions of adjacent macromolecules increased hydrophobicity to form hydrophobic bonds. If the charged groups on adjacent macromolecules formed ionic bonds, the water that had been attracted to them will be freed; if helices were formed between macromolecules of the holothurian dermis through van der Waals or hydrogen bonds, the macromolecular network would shrink and water would be exuded. Any bonding that causes shrinkage of the network will expel water from the network, which suggests that some of these bonds are involved in the water movement observed on stiffening in the present study. It is known that all of these bonds cause a phase transition in gels (Yoshida, 2004) and thus phase transition may be a mechanism of stiffness change in the dermis between the standard state and the stiff state. A shift in pH invokes phase transition in ionic gels (Tanaka et al., 1980). Although sea-cucumber dermis could be regarded as an ionic gel, the present results showed that pH had little effect. Thus phase transition driven by pH shift seems not to be involved in the dermal stiffening mechanism. Further studies are required to identify the bonding responsible for this mechanism.

In contrast to the stiffening from the standard state to the stiff state, stiffening of soft dermis to the standard state was not associated with mass loss under the present conditions. We consider that the condition induced by CaFASW represented the soft state *in vivo* based on the evidence below. The soft state does not simply mean low stiffness; it shows characteristic strain dependence. The soft state induced by CaFASW showed a strain dependence quite similar to that of an intact soft animal (Motokawa and Tsuchi, 2003). Tensilin, the protein isolated from sea cucumbers, has been regarded as the stiffener of soft dermis *in vivo*; tensilin changes soft dermis to the standard state. The effect of tensilin has usually been tested in CaFASW because it is the easiest and most reliable way to produce the soft state. The effect of tensilin is, however, not limited to the calcium-free condition, which is of course not physiological. The isolated dermis in nASW is sometimes found to be in the soft state, and such dermis stiffened in response to tensilin (Tamori et al., 2006). Based on these results, the tensilin-induced response in CaFASW has been believed to represent the transition from the soft state to the standard state *in vivo*. Although calcium ions have some direct effect on the extracellular materials (Eylers and Greenberg, 1989; Motokawa, 1994), the prominent effect of calcium in the live dermis has been attributed to an effect on Ca^{2+}-dependent cellular activities; CaFASW very likely inhibited the secretory activity of tensilin-containing cells, which resulted in the soft state because of a low concentration of tensilin in the dermis (Trotter and Koob, 1995; Tipper et al., 2003; Wilkie, 2004). The procedure of changing the bathing solution from CaFASW to nASW is best interpreted as similar to a stimulation of the cellular control system *in vivo*, and the results obtained seem to reflect the *in vivo* molecular mechanism

of stiffness changes. Changing the bathing solution from nASW to KASW can also be interpreted as a stimulation of the cellular control system *in vivo* (Motokawa, 1994). Menthol anesthesia abolished the effect of KASW. Detailed mechanical measurements showed that the stiff states induced by KASW, acetylcholine and mechanical stimulation have similar mechanical parameters such as stiffness and energy dissipation (Motokawa and Tsuchi, 2003). The evidence above strongly suggests that the stiff state induced by KASW represents the *in vivo* physiological state. In conclusion, the three states we studied in the present work very likely represent the states *in vivo*, and the two procedures used, the change of medium from CaFASW to nASW and that from nASW to KASW, both stimulated the *in vivo* stiffness control mechanisms.

The mass of the dermis did not change when CaFASW was changed to nASW and when nASW was changed to CaFASW. This may suggest that neither hydrophobicity nor the extent of hydration of macromolecules in the dermis changed greatly at this transition. Tensilin has been regarded as the protein responsible for this transition. The present results showed that tensilin did not induce mass changes. Wilkie (Wilkie, 2005) proposed a model in which water movement is not involved in stiffness changes. In that model, tensilin forms cross bridges between collagens and a tensilin-specific protease that cleaves the tensilin cross bridges.

We showed that water movement was associated with the transition from the standard state to the stiff state, whereas it was not associated with the transition from the soft state to the standard state. The results strongly suggest that the two stiffening responses were based on different molecular mechanisms. We have little information on these mechanisms especially on the mechanism responsible for the changes from the standard state to the stiff state in which water movement seems to be associated.

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