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Glycerinated Catch Apparatus of Sea Urchin Spines: the Effects of Cations on its Mechanical Properties and Ultrastructure

Chizuru Ogawa[1], Tatsuo Motokawa[2], Hideki Ishida[3] and Akira Matsuno[3]*

[1]*Department of Anatomy, Kawasaki Medical School, Kurashiki 701-0192, Japan*
[2]*Basic Biology, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, O-okayama, Meguro, Tokyo 152-8551, Japan*
[3]*Department of Biological Science, Faculty of Life and Environmental Science, Shimane University, Matsue 690-0823, Japan*

ABSTRACT—Sea urchin spinal ligaments (the catch apparatus) were extracted with glycerin, and electron microscopic observations comfirmed that no cell membranes remained intact after glycerination. We studied the effects of cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) on the mechanical properties of the glycerinated ligaments. Monovalent cations decreased whereas divalent cations increased the viscosity of the ligaments. The ion dependencies were similar to previous results with detergent-extracted holothurian dermis, which suggests that the echinoid ligament shares a similar mechanism for changes in mechanical properties with other catch connective tissues. This provides evidence against the hypothesis of del Castillo *et al* (1995) that muscles in the catch apparatus are responsible for the changes in mechanical properties of the ligament. Fine projections cross-bridging collagen fibrils were observed in the glycerin-extracted ligaments as well as in the intact ligaments. They were found in all the ionic conditions studied.

INTRODUCTION

The sea urchin spine ligament or catch apparatus is the first collagenous connective tissue that was shown to change its mechanical properties rapidly by neural control (Takahashi, 1967a, b; Hidaka and Takahashi, 1983). Later, such stiffness-mutable connective tissues were named catch connective tissues (or mutable connective tissues). Examples of catch connective tissues have been found in all the classes of echinoderms studied thus far (Motokawa, 1984a, Wilkie, 1996a), and this tissue has been regarded as one of the most prominent features that characterize the phylum Echinodermata (Motokawa, 1988; Ruppert and Barnes, 1994).

The molecular mechanism of catch connective tissue has not yet been clarified. Connective tissues containing no muscle cells nonetheless show catch activities (holothurian dermis: Motokawa, 1981; echinoid central ligament: Motokawa, 1983; echinoid tooth ligament: Birenheide *et al*., 1996; crinoid cirral ligament: Wilkie, 1983; Birenheide *et al*., 1999), and thus it is evident that the changes in mechanical properties in these tissues are not produced by muscular contractions. Evidence is accumulating to support the notion that the changes derive from the mutabilities of extracellular materials of catch connective tissues (Wilkie, 1996a). There was a recent hypothesis, however, that the catch apparatus itself is an exception- that is contains a few tiny muscle cells among collagen fibril bundles, and the contraction of these muscles causes changes in the mechanical properties of the whole ligament (del Castillo *et al*, 1995). This hypothesis has been debated (Wilkie, 1996b; del Castillo and Smith, 1996). Here we report that mutability was observed even after the catch apparatus was extracted by glycerin: the glycerin-extracted ligament (glycerin model) changed its mechanical properties when cation concentrations of bathing solutions were altered. The ion dependencies were similar to those of the Triton-extracted model of the holothurian dermis, a typical catch connective tissue whose catch mechanism depends on extracellular materials rather than on muscles (Motokawa, 1994). The present study provides evidence that the catch apparatus is not an exception in the catch mechanism, contrary to the hypothesis of del Castillo *et al* (1995). The electron microscopic observations of a glycerin model under various ionic conditions are reported.

MATERIALS AND METHODS

Sea urchins of the species *Anthocidaris crassispina* were collected from the coast of Shimane Peninsula and kept in an aquarium in the laboratory. The glycerin model was prepared as follows. A spine in interambulacra was isolated with a piece of test attached. Spine muscles were gently scraped off, and the catch apparatus was dissected by a diamond cutter, leaving a piece of the catch apparatus connecting small pieces of spine and test ossicles. Most specimens relaxed at the end of the dissection. Relaxed specimens were immersed in cold sea water containing 50% glycerin for 1 month at

* Corresponding author: Tel. +81-852-32-6430;
FAX. +81-852-32-6449.
E-mail: matsuno@life.shimane-u.ac.jp

–20°C. The extraction medium was changed 3 times at 12 hr, 3 d, and 1 wk after the onset of extraction. The glycerinated samples were thoroughly washed with artificial sea water (ASW) for 2 hr at room temperature before being subjected to mechanical tests.

Creep test

Mechanical properties were measured by creep tests. The ossicle of test was held by a sample holder in an experimental trough, and the spine ossicle was connected to an arm of a displacement transducer (Fig. 1). The arm was connected to a lever of a balance to stretch the catch apparatus by an applied weight. The samples rested in the trough for 30 min before mechanical tests were performed. The trough contained ASW for fresh (non-glycerinated) samples and a solution with one or two kinds of cations for glycerinated samples. Each sample elongated when a load was applied. The elongation rate became more or less constant in 10–20 min. The solution was applied some time after the 10–20 min, not during it. The inverse of the elongation rate is the measure of the normal viscosity of the sample (Motokawa, 1984b). Experiments were performed at room temperature (20–25°C).

The composition of ASW was as follows (in mM). NaCl: 433.7; KCl: 10.0; $CaCl_2$: 10.1; $MgCl_2$: 52.5 mM; $NaHCO_3$: 2.5 mM (pH 8.0-8.1). High-Ca-ASW contained 100 mM $CaCl_2$; Ca-free-ASW contained 5 mM O,O'-Bisethlene- glycol-N,N,N',N'-tetraacetic acid (EGTA) instead of $CaCl_2$.

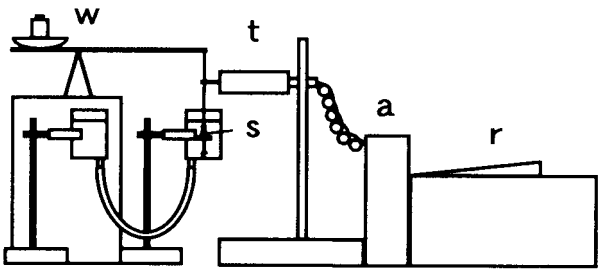

Fig. 1. Experimental set-up for creep test. a: amplifier for transducer, r: pen recorder, s: sample of ligament, t: displacement transducer, w: weight.

Electron microscopy

Both sectioned samples and negatively stained ones were observed under an electron microscope (JEM 1010, JEOL, Japan). For sectioning, excised ligaments, fresh ligaments in sea water, and glycerinated liogaments thoroughly washed by distilled water (DW) were fixed with 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 120 min at room temperature. Ligaments were washed with the buffer several times and post-fixed by ice-cold 1% osmium tetroxide in 0.1 M phosphate buffer for 120 min, followed by dehydration through a series of ethanol (80, 90, 95,100%). Resin-embedded specimens were cut into thin sections with a diamond knife and then stained with saturated aqueous uranyl acetate and lead nitrate. For negative staining, the tissues were carefully macerated into collagen fibrils in DW with a pair of needles and then negatively stained with a saturated aqueous uranyl acetate solution or a 1% phosphotungstic acid (PTA) solution.

We studied the effect of several cations. The glycerinated samples were immersed in a solution containing either 200 mM KCl, 400 mM NaCl, or 400 mM $CaCl_2$. For sectioning, the samples were processed as described above. For negative staining, the samples were macerated in the respective solution, stained, and observed by an electron microscope.

RESULTS

Effects of cations on viscosity

Fresh ligaments changed their mechanical properties when the Ca^{2+} concentration was manipulated, as reported by Hidaka (1983). When the experimental medium was changed from normal ASW to Ca-free ASW, the viscosity decreased, whereas the viscosity increased when normal ASW was changed to ASW with a high Ca^{2+} (100 mM) content. These effects were reversible: figure 2 shows the reversible viscosity changes by high and low Ca^{2+} media.

We studied the effects of major cations in sea water on the viscosity in the glycerinated catch apparatus. When the experimental solutions contained a single kind of cation, the monovalent cations K^+ and Na^+ decreased the viscosity (Fig. 3A, B). The effect was concentration dependent: in the ranges of 100–400 mM Na^+ and 100–200 mM K^+, the higher the concentration the lower the viscosity. The effect was reversible: the viscosity increased again with in a few minutes when the catch apparatus was washed in media with a lower concentration of ions. The viscosity decreasing effect of K^+ was larger than that of Na^+ when compared at the same concentration.

The divalent cations Ca^{2+} and Mg^{2+} increased the viscosity (Fig. 3C, D). The elongation of the sample was hardly discernible in the media containing Ca^{2+} above 200 mM. The reduced elongation was maintained even after the catch apparatus was washed with media containing 50 mM Ca^{2+} for more than 30 min. Elongation was more discernible in the media containing 200 mM Mg^{2+} than in media with high Ca^{2+}, and the reduced elongation rate in high Mg^{2+} was recovered by washing with media containing 50 mM Mg^{2+}.

Because the monovalent cations and divalent cations exerted opposite effects on viscosity, we studied the effects of using a combination of monovalent and divalent cations. The increasing effect of Ca^{2+} was observed in the presence of 400 mM Na^+ (Fig. 3E), while the effect was not observed in the presence of 200 mM K^+ (Fig. 3G). The viscosity-increas-

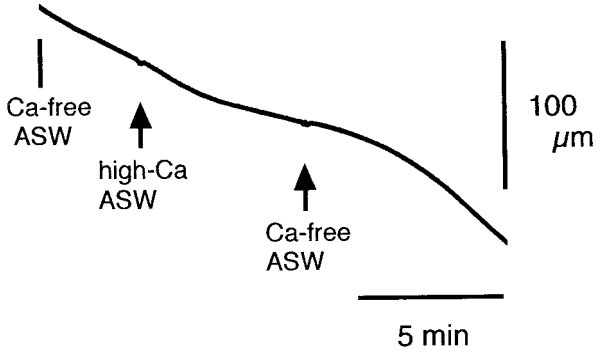

Fig. 2. Creep curve of fresh (non-glycerinated) ligament. In this and the following figures, elongation is shown as downward deflection. The vertical bar on the far left with a solution name denotes that the experiment started in that medium. The medium was changed at the time indicated by an arrow. The addition of Ca^{2+} (100 mM) was followed by a decrease of elongation rate, which implies an increase in viscosity. The response was reversed by Ca^{2+} removal.

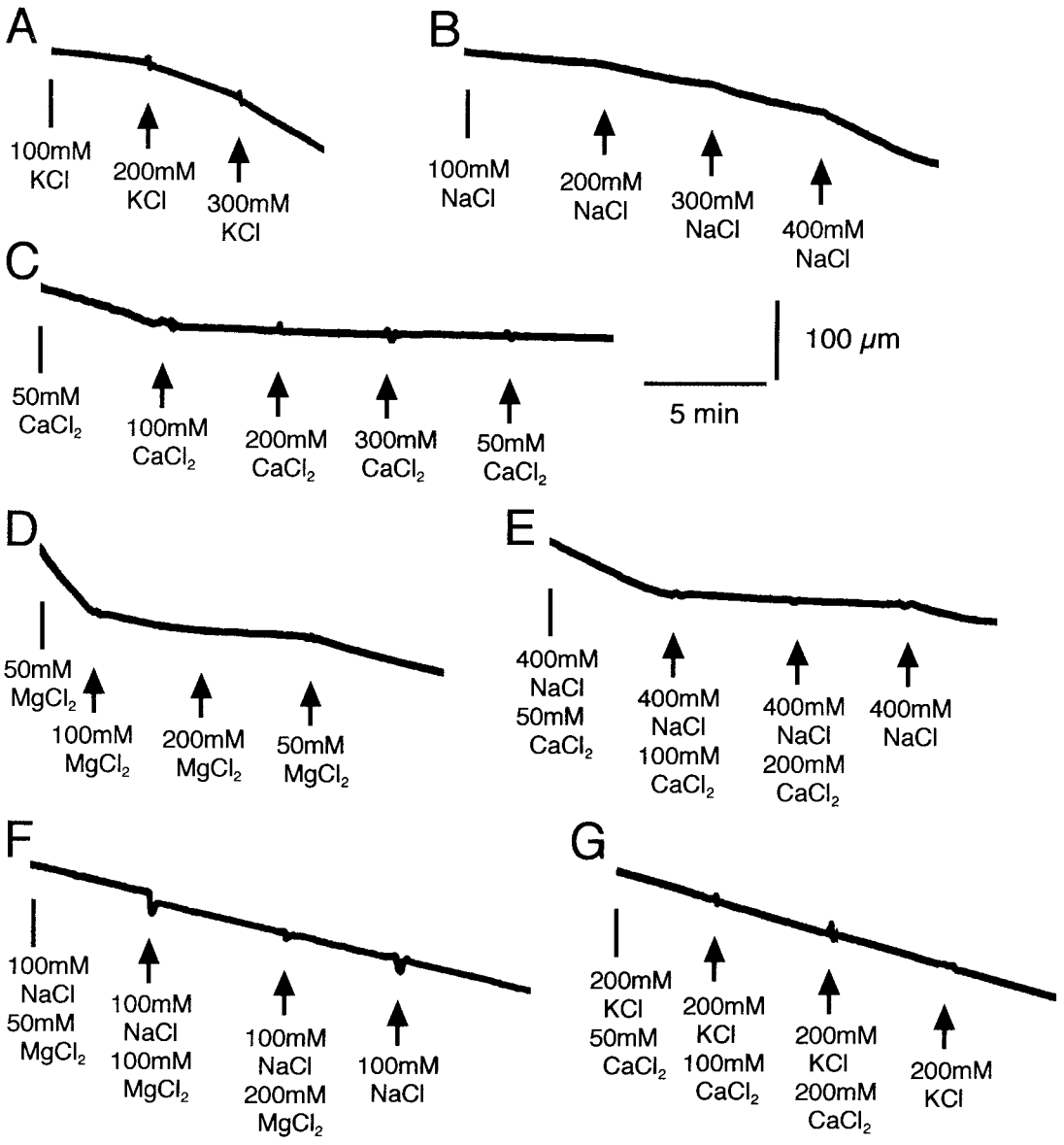

Fig. 3. Effects of cations on glycerinated ligaments. A: excess K^+ decreased the viscosity, B: excess Na^+ decreased the viscosity, C: excess Ca^{2+} increased the viscosity, D: excess Mg^{2+} increased the viscosity, E: excess Ca^{2+} increased the viscosity in the presence of 400 mM Na^+, F: The effect of excess Mg^{2+} was suppressed by the presence of 100 mM Na^+, G: the effect of excess Ca^{2+} was suppressed by the presence of 200 mM K^+.

ing effect of Mg^{2+} was not observed in the presence of 100 mM Na^+ (Fig. 3F) or in the presence of 200 mM K^+.

Electron microscopy

The most conspicuous element in the glycerinated catch apparatus was, as in the intact ligaments, the bundles of collagen fibrils. Cellular debris was sparsely scattered among the fibrils (Fig. 4A). We could not find cells with an intact cell membrane (Fig. 4B), nor could we find any debris of muscle cells with discernible myofibrillar bundles, although we searched for them extensively.

Fine projections cross-bridging the collagen fibrils, similar to the projections reported previously (Smith *et al*, 1981; del Castillo *et al*, 1995), were observed in an intact catch apparatus. These collagen-fibril bridging filaments (CBFs) were also present in all of the glycerinated ligaments in thin sections (Fig. 5). We did not find differences in the appearance of CBFs regardless of whether the bathing solutions contained 200 mM KCl, 400 mM NaCl, or 400 mM $CaCl_2$ (Fig. 7). Negative staining did not show CBFs (Figs. 6, 8).

The collagen fibrils from both intact and glycerinated specimens showed a characteristic banding pattern in longitudinal sections and in negative staining. The D periods measured in negative-stained glycerinated samples were as follows: 63 nm in 200 mM KCl; 65 nm in 400 mM NaCl; 64 nm in 400 mM $CaCl_2$.

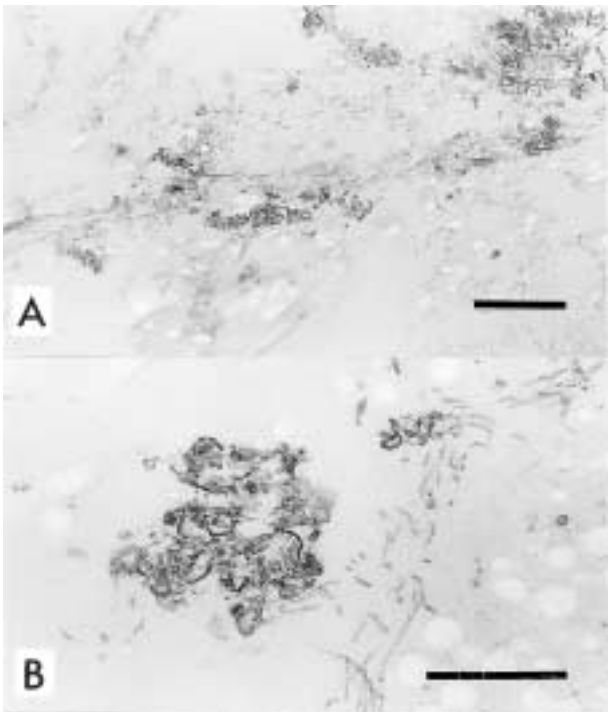

Fig. 4. Electron micrographs of glycerinated ligaments. A: Collagen fibril bundles and a broken cellular element. Scale bar 2 μm. B: High magnification of cellular elements with disrupted cell membranes. Scale bar 1 μm.

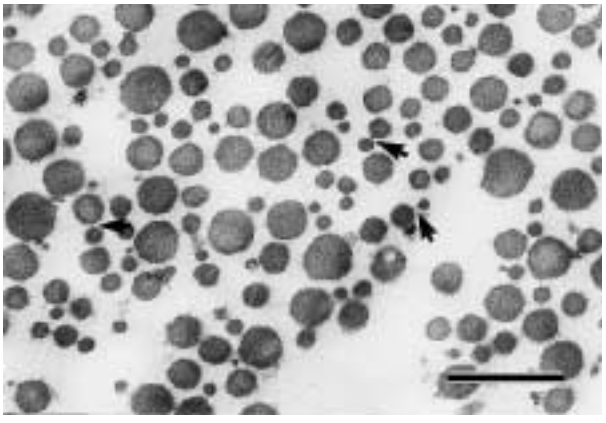

Fig. 5. Cross-section of collagen fibrils of a glycerinated ligament in ASW. Arrows denote CBFs. Scale bar 0.5 μm.

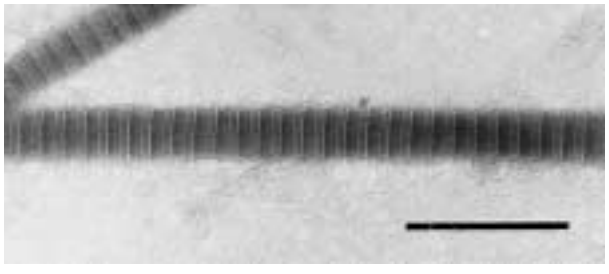

Fig. 6. Negatively stained collagen fibrils from a glycerinated sample in DW. Scale bar 0.5 μm.

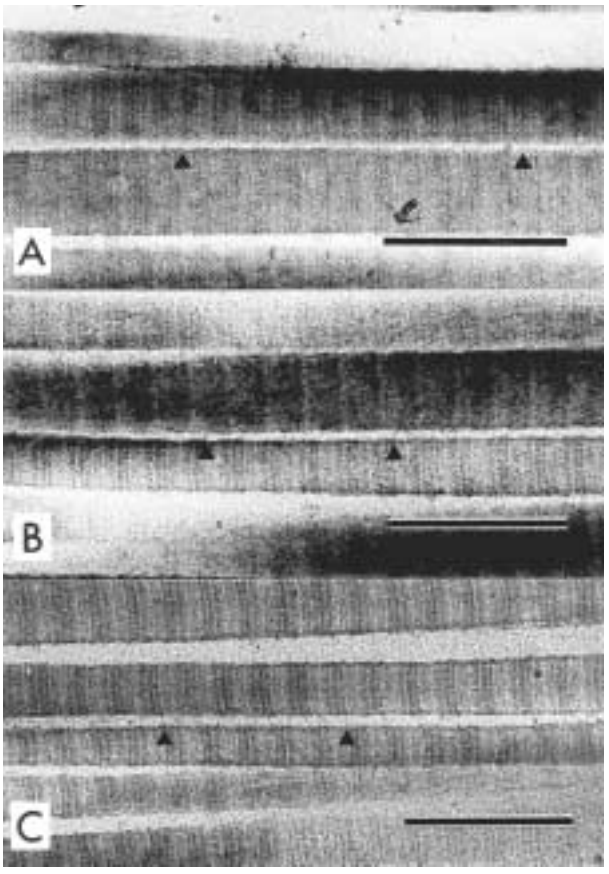

Fig. 7. Longitudinal sections of collagen fibrils in glycerinated samples. CBFs were recognized in all micrographs (arrowheads). A: 200 mM KCl, B: 400 mM NaCl, C: 400 mM $CaCl_2$. Scale bar 0.3 μm.

DISCUSSION

Ion dependency of mechanical properties

The present study clearly showed that the catch apparatus after glycerin extraction maintained the capacity to change its mechanical properties in response to ionic manipulation. The electron micrographs of the extracted catch apparatus revealed that the cell membranes were destroyed; we could not identify the debris of any muscles containing bundles of myofibrils. It is thus evident that the viscosity changes observed in the glycerin model were not caused by muscle contraction. The present method of preparing models included a month-long extraction with thorough washing. Materials extracted by glycerin seem to be fully treated, so any materials that defused from the cells through the perforated cell membranes are very likely to have been washed away from the tissue. Because only sparsely scattered cellular debris was found, the effects of ions on mechanical properties observed in the glycerin model no doubt derive from their effects on extracellular materials.

The four kinds of cations tested here exerted different effects on the mechanical properties of the glycerin model. Divalent cations increased the viscosity, whereas monovalent cations decreased it. The strength of the effects was not

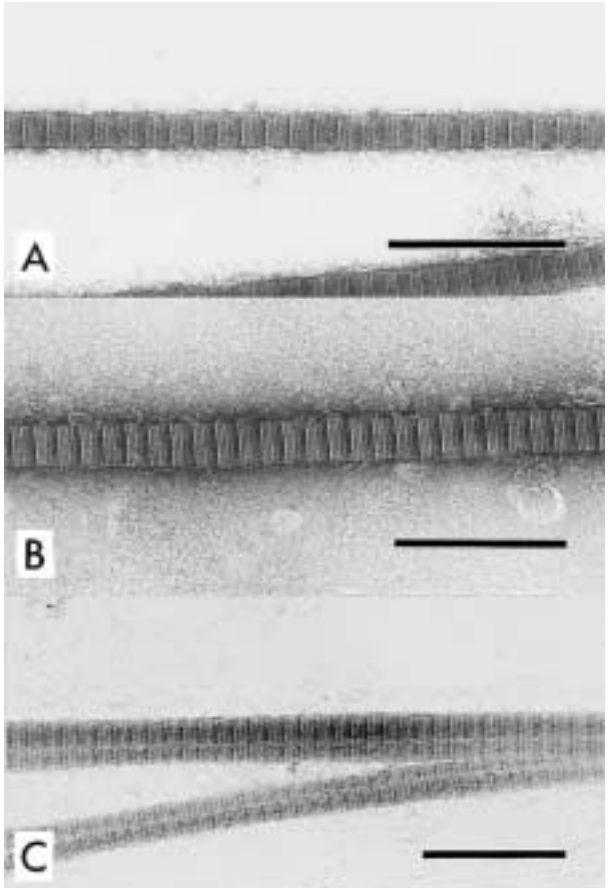

Fig. 8. Electron micrographs of negatively stained collagen fibrils from glycerinated samples in solutions with different ions. A: 200 mM KCl, B: 400 mM NaCl, C: 400 mM $CaCl_2$. Scale bar 0.5 μm.

equal among the cations; the viscosity decrease was more prominent in K^+ than in Na^+ at the same concentration. The viscosity increase was more prominent in 200 mM Ca^{2+} than in 200 mM Mg^{2+}, and the effect of the former was not recoverable by washing but that of the latter was recoverable.

The experiments with a combination of two cations also showed differences among the responses. The effect of Mg^{2+} was canceled by Na^+ but that of Ca^{2+} was not. A similar result has been reported for the intact catch apparatus of *Anthocidaris crassispina* (Hidaka, 1983). The effects of both Mg^{2+} and Ca^{2+} were canceled by K^+. The results suggest that Ca^{2+} is more potent in viscosity-raising activity than is Mg^{2+}, and K^+ is more potent in viscosity-reducing activity than is Na^+. Similar results have been reported in the more quantitative study using the model of holothurian dermis whose cells were disrupted by the non-ionic detergent Triton X-100 (Motokawa, 1994). The present study thus showed that the ion dependence of the mechanical properties of the extracellular materials of the catch apparatus is not different from that of other catch connective tissues.

Szulgit and Shadwick (1994) studied the effects of Ca^{2+} on intact and Triton X-100-treated catch apparatus of the sea urchin *Eucidaris tribuloides*. They obtained results similar to ours in the intact samples: high Ca^{2+} stiffened while Ca-free softened the ligament. Their results in the Triton-treated samples, however, differed from ours: their sample became stiff in Ca-free media. Similar differences have been found in previous studies on the dermis of sea cucumbers. The fresh holothurian dermis was stiff in the media containing a high concentration of Ca^{2+} and soft in Ca-free media (Hayashi and Motokawa, 1986; Motokawa and Hayashi, 1987). The Triton model of Motokawa (1994) behaved similarly to the intact ones in response to the changes in Ca^{2+} concentration including Ca-free media, which decreased the viscosity. Trotter and Koob (1995) observed, however, that the dermis of a Triton-extracted model was stiff in Ca-free media. These discrepancies could be the results of differences in the methods of model preparation. Our present glycerin model and Motokawa's Triton model were prepared with a long extraction period followed by thorough wash, whereas the models of Szulgit and Shadwick and of Trotter and Koob were prepared with a brief extraction time. Szulgit and Shadwick, and Trotter and Koob observed the effects of Triton just after its application to the tissue, and thus the extraction time was on the order of minutes. They also observed the effect of Triton without washing or after a brief period of washing. It is very likely that these models retained materials that had diffused from the perforated cells into the extracellular spaces in the tissues (Trotter and Koob, 1995). Since cell-derived factors that modulate the stiffness were reported for the holothurian catch connective tissue (Koob *et al*, 1999), it is very probable that the presence of such factors in the briefly extracted and non-washed models ('shallow' models) affected the mechanical properties and thus gave different results than those of the fully extracted and thoroughly washed models ('deep' models).

The present study revealed the common features of catch connective tissues. From the results of studing deep models we conclude that the extracellular materials of catch connective tissues are affected by the concentration of cations; low Ca^{2+} condition definitely makes the tissue soft. The presence of cells has been reported which may control the concentration of Ca^{2+} in extracellular materials (Matsuno and Motokawa, 1992; Wilkie, 1996a). A comparison of the results of studing deep models with results of studing shallow models seems to reveal the presence of some factor with stiffening abilities that is liberated to extracellular spaces from some cells by Triton treatment. There is also a possibility that Triton activated a stiffening system in the extracellular materials in some unknown way. The common features of ion dependence and the presence of some factor liberated or activated by Triton suggest that the catch apparatus is no exception among the echinoderm catch connective tissues. Therefore we disagree with the hypothesis of del Castillo *et al* (1995) that the catch apparatus has a muscle-based mechanism that is totally different from the mechanism of other catch connective tissues.

Collagen binding fibrils

We observed CBFs in *Anthocidaris crassispina*, the species of the subclass Euechinoidea. They were reported in *Eucidaris tribuloides*, the species of the subclass Peri-

schoechinoidea (del Castillo *et al*, 1995), and thus CBFs seem to be a common features of collagens in the echinoid catch apparatus. The present study showed that CBFs and the D period of collagens did not change by glycerination and ion manipulation. CBFs seem to add mechanical stability to collagen-fibril bundles, as del Castillo *et al*.(1995) speculated. We do not know, however, whether they are involved in the mechanism of connective tissue catching.

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