BENDING TESTS ON CUCUMARIA FRONDOSA DERMIS SHOW THAT EXTRACELLULAR Ca^{2+} AFFECTS THE CELLULAR CONTROL OF TISSUE VISCOSITY, AND THAT CELL LYSIS PRODUCES A SOLUBLE STIFFENING FACTOR

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In the sea cucumber Cucumaria frondosa, as in all other echinoderms thus far examined, cellular activity controls the reversible changes in the tensile properties of its collagenous tissues (Motokawa, in "Echinoderm Biology", eds. Burke et al., Balkema, Rotterdam, pp. 39-54, 1988). The dermis of C. frondosa consists of:

- discontinuous, fusiform collagen fibrils to which proteoglycans are tightly bound;
- proteoglycans which are not tightly bound to fibrils;
- microfibrils that surround bundles of collagen fibrils;
- other (non-collagenous) proteins;
- nerve processes, morula cells, and granular cells.

The nerve processes, the granular cells, and the morula cells, are thought to be responsible for regulating those molecular interactions that determine whether or not stress is effectively transferred between collagen fibrils. The most frequently cited hypothesis is that the granular cells stiffen the matrix by secreting calcium ions, and soften it either by secreting a calcium chelator, or by active uptake of Ca^{2+} . That is, the extracellular Ca^{2+} concentration directly affects the matrix. Published data, showing that the stiffness or viscosity of freshly excised echinoderm tissues can be modulated by chelation and restoration of Ca^{2+} to the bathing medium, are consistent with this "extracellular calcium hypothesis" (Motokawa, ibid; Wilkie, J. Zool., London 228:5-26, 1992; Shadwick and Pollock, in "Echinoderm Biology", eds. Burke et al., Balkema, Rotterdam, pp. 635-640, 1988). However, they are also consistent with a "cellular calcium hypothesis", in which only the cellular events that increase the viscosity of the matrix are calcium-dependent. The experiments reported here were undertaken to test two specific predictions of the "extracellular calcium hypothesis:"

1. that the viscosity of the matrix should be calcium-dependent in the absence of active cells; and
2. that pharmacological agents that affect cellular calcium-dependent processes ought not to affect the viscosity of the matrix when normal Ca^{2+} concentrations are maintained.

The results are clearly inconsistent with the "extracellular calcium hypothesis" and support the "cellular calcium hypothesis." In addition, extracts of tissues in which cells were lysed caused freshly excised tissues to become stiff in the presence of a calcium chelator. These results confirm and extend those previously reported in the Bulletin (Trotter and Koob, Bull. MDIBL 33:2-4, 1994).

C. frondosa adults were obtained from commercial dredgers during the month of September and were maintained in live cars in the float at MDIBL. Unless otherwise indicated, all incubations and experiments were conducted at the temperature of the flowing sea water

system at MDIBL, 12 - 15°C. Test specimens 0.9x1.7x30 mm were prepared from the inner dermis of the ventral interambulacra, as previously described (Trotter and Koob, ibid). The specimens were incubated in test solutions for at least 90 min before testing. Mechanical tests were performed by mounting specimens as cantilever beams with 15mm of free extension and recording the time required for them to bend through a constant distance of 4 mm under the influence of gravity. Times were rounded up to the nearest second. Very plastic specimens therefore had a uniform deflection time of one second. Most tests were completed in less than 2 minutes, and no tests took longer than 5 minutes.

The gravity-bending of a viscoelastic cantilever beam is associated with complex tensile, compressive, and shear stresses. The magnitude and distribution of the stresses are determined by the geometry and density of the specimens. Since all the specimens had uniform dimensions and there were no noticeably consistent changes in specimen size during pre-test incubations, the stresses within all of the specimens were assumed to be identical. Comparisons of the times required for similar stresses to produce similar deformations are therefore directly related to the viscous component(s) of the tissue. This is because, for a Newtonion fluid, $\eta = \sigma/\varepsilon$, where η is viscosity, σ is stress, and ε is strain rate. Hence for specimens loaded with identical stresses, $\eta \propto 1/\varepsilon$. Because $1/\varepsilon = t/\varepsilon$, where t = time and ε = strain, and the strain was the same for every specimen, therefore $\eta \propto t$.

The principal test solutions used in these studies were MOPS-buffered artificial sea water (ASW), which consisted of 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, 0.01 M KCl, and 0.01 M 3-(N-morpholino)propane sulfonic acid (MOPS), pH 7.8 - 8.0; and EGTA-ASW in which the $CaCl_2$ was replaced by 0.0072 M ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA). Standard specimens were incubated in the test solutions at sea water temperature, with intermittent gentle agitation. Specimens from a single animal were used for each set of tests within a given experiment. Approximately 30 specimens could be obtained from a each animal. Five replicates were used for each experimental variation. Free Ca^{2+} concentrations were estimated using the B&S stability constants in the computer program Maxchelator v6.5, obtained from Dr. Chris Patton, Stanford University.

Tissue extracts were prepared from the same region of the dermis used for mechanical tests. To prepare a freeze-thaw (FT) extract, the dermis was minced into pieces about 1 mm on a side, and the mince was then extracted at sea water temp. in 5 volumes of EGTA-ASW for 90 min., after which it was frozen at -60°C for at least two hours, followed by incubation at sea water temp. until the liquid was completely thawed. These steps were repeated for a total of five freeze-thaw cycles. An EGTA-ASW extract (without freezing) was made by separating the tissue from the liquid after 180 min. of incubation at sea water temp. A freeze-thaw extract was also made from the longitudinal muscles of the body wall. In all cases the extracts were clarified by centrifugation at 27,000 x g for 30 min. and were stored frozen.

Results are summarized in Table 1. Freshly excised specimens incubated for at least 90 min in EGTA-ASW, which was calculated to have a free Ca^{2+} concentration no greater than 52 micromolar, were very plastic. Those incubated in ASW, or in EGTA-ASW followed by ASW,

were much stiffer. Specimens incubated sequentially in EGTA-ASW, 1% Triton X-100 in EGTA-ASW, and EGTA-ASW were as stiff as those transferred from EGTA-ASW to ASW. Dermis specimens incubated sequentially in EGTA-ASW, deionized water, and EGTA-ASW were as stiff as or stiffer than those transferred from EGTA-ASW to ASW. Specimens incubated in EGTA-ASW for 90 min, followed by five cycles of freezing and thawing in EGTA-ASW, were stiffer than those transferred from EGTA-ASW to ASW. Electron microscopy verified that Triton X-100, water, and freeze-thaw treatments all lysed the cells. Cell lysis by three physically distinct mechanisms thus caused the specimens to become stiff in the continued presence of EGTA. These results showed that Ca^{2+} is not required for matrix stiffening in the absence of viable cells.

TABLE 1. Effects of experimental interventions on dermis bending times. Means and standard deviations are from replicates of five specimens. For those treatments that were done in more than one experiment the median values for the means and the associated standard deviations are given as representative values. NA: not applicable.

TREATMENT (Experiments)	FINAL $[Ca^{2+}]$	TIME (Mean)	SD	COMMENT
ASW (12)	NORMAL	63	20	STIFF
EGTA/ASW (20)	LOW	<2	NA	PLASTIC
EGTA-ASW >ASW (7)	NORMAL	50	18	STIFF
TX-100 (3)	LOW	31	16	STIFF
WATER (3)	LOW	95	38	STIFF
FREEZE/THAW (3)	LOW	201	59	STIFF
VERAPAMIL (3)	NORMAL	1	NA	PLASTIC
TMB-8 (3)	NORMAL	1	NA	PLASTIC
F/T EXTRACT (4)	LOW	33	31	STIFF
EGTA-ASW EXTRACT (1)	LOW	3	1	PLASTIC
MUSCLE F/T EXTRACT (1)	LOW	5	3	PLASTIC

Fresh specimens incubated in EGTA-ASW, ASW, or verapamil in ASW, showed a dose-dependent inhibition by verapamil of the stiffening response to the Ca^{2+} in ASW. The concentration of verapamil required to completely block the stiffening effect of Ca^{2+} was about 1 mM, which is much greater than that required to block voltage-gated Ca^{2+} channels in mammals, but is similar to the concentration of organic channel blockers needed to block channels in echinoderm nerves (Berrios et al., Comp. Biochem. Physiol. 81A:15-23, 1985).

Fresh specimens incubated in EGTA-ASW, in ASW, or in 3,4,5 Trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) in ASW also showed a dose-dependent inhibition by TMB-8 of the Ca^{2+}-dependent stiffening response, with the maximal effect attained at about 1 mM. TMB-8 has pleotropic effects on intracellular signalling pathways. Therefore, although the

observed inhibition is likely to be on one or more pathways within cells, the exact mechanism of inhibition cannot be specified from this experiment.

The effects of both verapamil and TMB-8 were fully reversed by washing out the drug. Neither verapamil nor TMB-8 had a plasticizing effect on specimens in which the cells had been lysed by freezing and thawing. The results with these two agents therefore indicate that the calcium-dependent stiffening response of fresh tissues reflects the presence of one or more calcium-dependent steps in the cellular mechanism of tissue stiffening.

These results confirm those previously obtained on specimens tested in creep (Trotter and Koob, ibid). The observations made using both test modalities that specimens become stiff when their cells are lysed, even in the presence of a calcium-chelator, suggest that cell-lysis causes the release of one or more soluble factors that act to increase the stiffness of the extracellular matrix. This suggestion was tested by incubating freshly excised specimens in EGTA-ASW in order to plasticize them, followed by incubation in either EGTA-ASW, ASW, an extract of minced tissue made by freezing and thawing it in EGTA-ASW, or a similar extract made without the freezing and thawing cycles. The specimens were made nearly as stiff by the freeze-thaw extract as they were by ASW. The stiffening activity was destroyed by boiling, indicating that it was not a neurotransmitter. Elimination of the freeze-thaw cycles produced an extract without stiffening effect. Also without much stiffening effect was a freeze-thaw extract of body-wall muscle, which shows that the stiffening activity may be specific to connective tissue cells, and is not related to constitutive cellular contents.

The results obtained in these studies are consistent with the "cellular calcium regulation" hypothesis previously suggested by Diab and Gilly (J. Exp. Biol.111:155-170, 1984) and by Szulgit and Shadwick (Amer. Zool. 32:129a, 1992), and further suggest that the stiffening response is effected by the Ca^{2+}-dependent secretion of one or more stiffening factors, which can also be obtained by lysing the cells. EGTA, verapamil, and TMB-8 plasticize the dermis by inhibiting the Ca^{2+}-dependent secretion of stiffener. The characteristics and mode of action of the stiffener(s) are currently under investigation.

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