EVIDENCE THAT Ca^{2+}-MODULATION OF THE STIFFNESS OF CUCUMARIA FRONDOSA DERMIS IS A RESULT OF Ca^{2+} -DEPENDENT CELLULAR PROCESSES

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The dermis of holothurians (sea cucumbers), as well as a number of other echinoderm tissues, has been shown to have neurally regulated mechanical properties (Motokawa, in "Echinoderm Biology", eds. Burke et al., Balkema, Rotterdam, pp. 39-54, 1988). Secretory cells within the tissues, possibly neurosecretory cells, are thought to be the effector cells, and the matrix between collagen fibrils is thought to be the site that is affected. Studies on freshly prepared specimens containing living cells have shown that the viscosity of holothurian dermis is directly correlated with the external calcium concentration. This "calcium effect" has been interpreted to be a result of reversible calcium-dependent crosslinks in the interfibrillar matrix, which are thought to be physiologically modulated by the cellular secretion and uptake of Ca^{2+} (Motokawa, ibid; Wilkie, J. Zool., London 228:5-26, 1992). However, no experiments have been reported previously that attempted to determine whether Ca^{2+} acts directly on the extracellular matrix or instead affects the matrix indirectly by playing an essential role in some cellular process(es). The experiments reported here support the latter hypothesis.

Standard specimens were prepared from the deep portion of the ventral interambulacral dermis of C. frondosa by slicing with razor blades. This portion of the dermis consists mainly of woven bundles of collagen fibrils the preferred orientation of which is parallel to the long axis of the animal. The specimens were 0.9 x 1.8 x 20 mm, and their long axes were aligned with that of the animal. Specimens were incubated and tested in solutions that were maintained at the temperature of the flowing sea water system of MDIBL (12-15°C). The viscoelastic properties of the specimens were determined using a creep test: the specimens were loaded with a constant tensile load, and their increase in length as a function of time was monitored by means of a linear variable differential transformer (LVDT), the output of which went to a chart recorder. To begin a test, a specimen was secured in specially constructed plexiglass clamps, using cyanoacrylate glue and stainless steel screws and nuts. Approximately 10 mm of dermis was suspended between the two clamps. The mounted specimen was then placed in a chamber containing the test solution. The creep test was begun by loading the specimen with a 200g weight, which produced a stress of 1.2×10^6 N m^{-2}. Each test lasted 30 min., or until the specimen broke. Because the creep rate is a function of the viscosity, this test measures the viscosity of the extracellular matrix under defined experimental conditions.

When tested in complete artificial sea water (ASW) containing 10mM $CaCl_2$, 10mM KCl, 50mM $MgCl_2$, 500mM NaCl, and 10mM MOPS buffer, pH 7.8-8.0, the specimens crept as shown in curve C, Fig. 1. For simplicity, only one curve is shown for each treatment, although each

treatment was repeated on at least three specimens. Specimens that were incubated for 20 min. or longer prior to testing in ASW in which the $CaCl_2$ was replaced by 7.2mM EGTA (E-ASW), and then tested in the same solution, crept more rapidly, and often broke before 30 min. (E, Fig. 1). Specimens that were incubated in E-ASW for 1 hr., then returned to ASW for 20 min. prior to testing (EC, Fig. 1), crept at the slower rate that was characteristic of the specimens that were constantly maintained in ASW. Figure 1 thus illustrates the "calcium effect" described above.

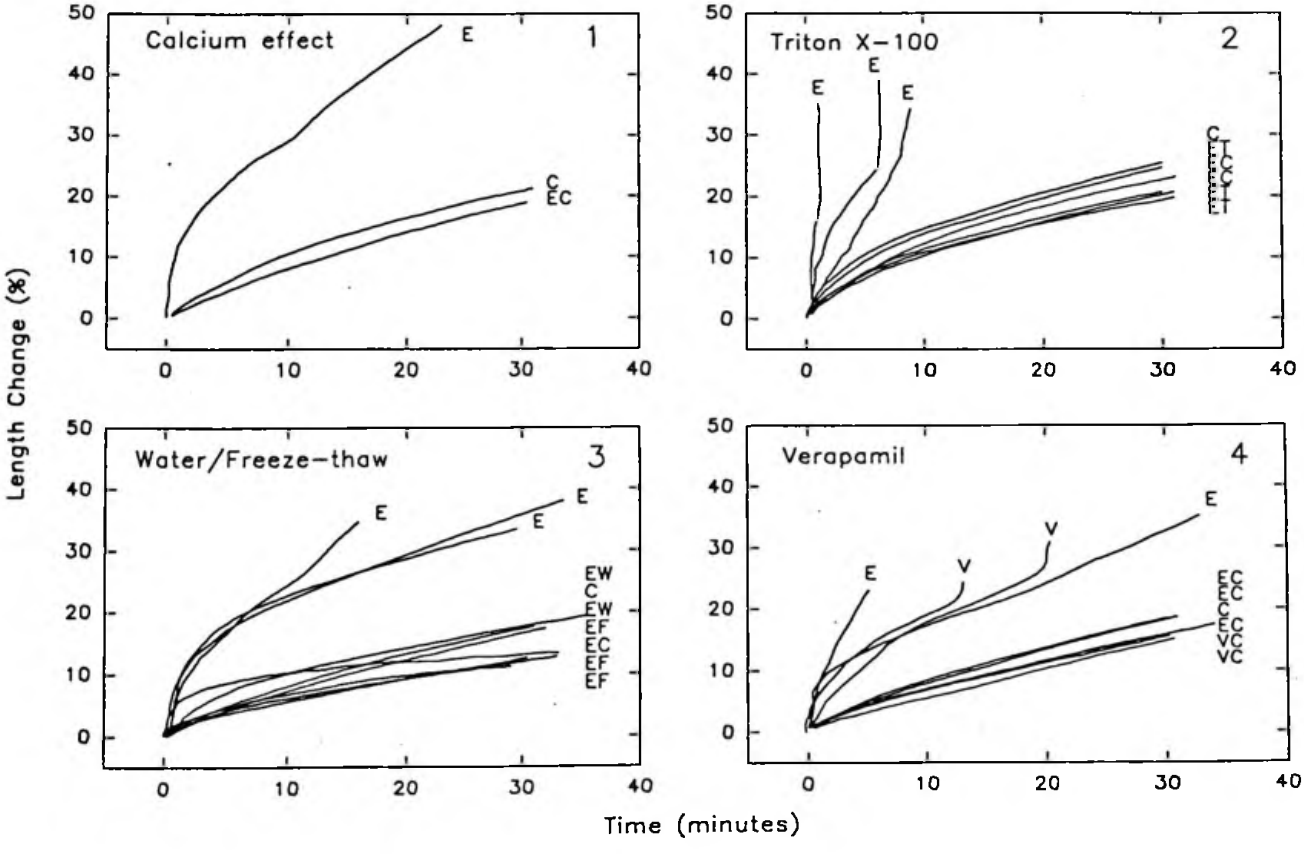

Fig. 1-4. Creep tests. See text for explanation. The variable effects of EGTA seen in Figs. 1-4 are due to animal to animal variability.

Specimens that were incubated sequentially in E-ASW, E-ASW containing 1% Triton X-100 (a nonionic detergent), and E-ASW (1 hr. ea.) prior to being tested in E-ASW crept at the rate characteristic of specimens in ASW (ET, Fig. 2). The addition of the detergent thus caused the specimens to become stiff even in the presence of a calcium chelator. The most likely mechanism of action of Triton X-100 was cell lysis. To determine whether cell lysis per se was sufficient to stiffen the matrix, other specimens were incubated in E-ASW for 1 hr., then were either exposed for 1 hr. to deionized water, or were frozen (-70°C, 30 min.) and thawed (15°C) five times before being tested in E-ASW (EW & EF, Fig. 3). Both treatments had the same effect as Triton X-100. Samples from each of these tests were analyzed by transmission electron microscopy, which confirmed that the cells were indeed lysed by all three treatments. Since the three treatments lyse cells by entirely different mechanisms, it appears that cell lysis per se, even in the presence of a

calcium chelator, is sufficient to increase the viscosity of the matrix to the same extent that calcium does. The most likely explanation of this phenomenon is that cell lysis causes the release from cells of the same stiffening agent that is released when specimens are placed in ASW after being incubated in E-ASW. This agent is clearly not Ca^{2+}.

To quantify the effect of EGTA and the other treatments on the calcium content of the tissue, 5 specimens from each of 5 different animals were treated as above before being processed as previously described (Trotter & Koob, Bull. MDIBL 31:16-17, 1992) to determine their calcium concentrations by atomic absorption spectroscopy. The results showed that the Ca contents in mmol/kg wet weight (+/- SD) of the specimens were : ASW - 8.9 (1.2); E-ASW - 0.58 (0.22); EW - 0.22 (0.18); ET - 0.14 (0.04); EF - 0.08 (0.10). The total calcium content was thus lower in the specimens that were stiffened by cell lysis in the presence of EGTA than in the unlysed specimens.

The above results indicate that the "calcium effect" is a cellular rather than a matrix effect. To probe the locus of the cellular calcium effect specimens were incubated sequentially in E-ASW and ASW, as described for Fig. 1, except that the ASW contained the calcium channel blocker verapamil in concentrations from 0.1 to 1mM. In concentrations greater than 0.2mM verapamil prevented the stiffening effect of calcium (V, Fig. 4 shows the results of 0.5mM verapamil). Although verapamil's site of action is likely to be the cell membrane, these results need to be interpreted cautiously because the effective concentrations are high compared to those needed to block voltage-dependent calcium channels in other animals. A result similar to that seen with verapamil was obtained with a 1mM concentration of the calcium "antagonist" 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (results not shown). The effects of both drugs were completely reversible (VC, Fig. 4).

These results demonstrate that the dermis of C. frondosa not only becomes stiff in the presence of a calcium chelator (when the cells are lysed), but also fails to become stiff in the presence of normal amounts of Ca^{2+} (when cellular calcium antagonists are also present). The findings are significant because they strongly suggest that organic molecules, rather than inorganic ions, are what cells secrete to plasticize and stiffen the dermis, and raise the possibility that the secretions could be purified from experimentally softened and stiffened dermis.

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