MECHANICAL PROPERTIES OF THE COLLAGENOUS MESOHYL OF *CHONDROSIA RENIFORMIS*: EVIDENCE FOR PHYSIOLOGICAL CONTROL

IAIN C. WILKIE*, FRANCESCO BONASORO**, GIORGIO BAVESTRELLO***, CARLO CERRANO**** & MARIA DANIELA CANDIA CARNEVALI**

*Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow G4 0BA, Scotland
**Dipartimento di Biologia, Via Celoria 26, I 20133 Milano, Italy
***Dipartimento di Scienze del Mare, Via Brecce Bianche, I 60131 Ancona, Italy
****Dip.Te.Ris., Corso Europa 26, I 16132 Genova, Italy
E-mail: i.wilkie@gcal.ac.uk

ABSTRACT

Incidental observations suggest that the collagenous mesohyl of *Chondrosia reniformis* can undergo reversible changes in stiffness. We investigated the possibility that the mechanical properties of the mesohyl are under direct physiological control by observing the effects of various treatments on the flexural stiffness of beam-shaped samples subjected to bending tests in which their deflection under gravity was recorded after a fixed time interval. The mesohyl is stiffened by elevated Ca^{2+} concentrations and by the inorganic calcium channel blockers Co^{2+} and Mn^{2+}, and it is destiffened by Ca^{2+}-free seawater. Treatments that cause membrane disruption stiffen the mesohyl irreversibly, and the mesohyl is also stiffened by a water-soluble factor released when mesohyl is minced. These results suggest that the passive stiffness of the mesohyl is modulated directly by calcium-dependent cellular activities that may include the secretion of a stiffening molecule that interacts directly with the extracellular matrix.

KEY WORDS

Chondrosia reniformis, connective tissue, mechanical properties, variable tensility.

INTRODUCTION

Sponges belonging to the genus *Chondrosia* completely lack a spicular skeleton. The bulk of their body consists of a collagenous mesohyl that is located between the external and internal epithelia (*exopinacoderm* and *endopinacoderm*, respectively) and is by far the main determinant of the passive mechanical properties of the whole animal (GARRONE *et al.,* 1975; BONASORO *et al.,* 2001).

When previously undisturbed specimens of *Chondrosia reniformis* Nardo are prodded repeatedly with a finger, they feel softer the first time they are touched than on second and subsequent stimulations. We have noticed this stiffening response in animals both in the sea and in laboratory aquaria. In the sea *C. reniformis* and related demosponges also show a form of opportunistic asexual reproduction in which a loosening of the substrate under part of an animal is followed by the slow elongation of that part under gravity and its eventual separation from the parent sponge, a

process that may involve destiffening or plasticisation of the sponge body (GAINO & PRONZATO, 1983; BONASORO *et al.,* 2001; ZANETTI, 2002). These stiffening and destiffening phenomena are indications that the mechanical properties of the mesohyl could be under direct physiological control and are reminiscent of the variable tensility demonstrated by the mutable collagenous tissue of echinoderms, which can undergo drastic, nervously mediated changes in its mechanical properties within a timescale of less than a second (WILKIE, 1996, 2002; TROTTER *et al.,* 2000).

The aim of this investigation was to test the hypothesis that the mechanical properties of the mesohyl in *C. reniformis* are under physiological control by determining if mesohyl stiffness is altered by agents that would be expected to affect the activities of cellular components.

MATERIALS AND METHODS

Specimens of *Chondrosia reniformis* were collected by scuba divers at Portofino on the Italian Ligurian coast. They were transported to the University of Milan and maintained in 50 l tanks of artificial seawater at 14 - 16° C.

The sponge mesohyl consists of an outer cortex, or *ectosome*, which contains fine inhalant canals and is densely collagenous, and a medulla, or *choanosome*, which contains choanocyte chambers and larger exhalant canals and is less densely collagenous. Beam-shaped samples 2.5 x 2.5 x 15 mm in size were cut from both the ectosome and choanosome regions, using parallel-mounted razor blades. As illustrated in Fig. 1A, two opposite long sides of the samples were roughly parallel to the external surface of the animal (and so the other two, anatomically 'lateral', long sides were perpendicular to the external surface). Ectosome samples included no, or very little, exopinacoderm. Each sample was fixed to a glass coverslip using cyanoacrylate cement, with a 'lateral' surface in contact with the coverslip and with exactly 10 mm projecting from the edge of the coverslip (Fig. 1B). The samples were transferred to and from test solutions by gripping the coverslip with forceps, never by gripping the tissue itself. After immersion in the test solution for 2 - 4 h at room temperature (21 - 26° C), each sample was lifted gently from the solution while a stop-clock was started, and the coverslip was clamped horizontally with the sample in front of a 0.5 mm grid (Fig. 1C). The sample usually bent under gravity and exactly 45 s after the stop-clock was started the amount of deflection was recorded to the nearest 0.5 mm. This procedure was done in a standardised way by the same researcher for all the experiments. Since deflection in a fixed time period is inversely proportional to flexural stiffness, this method provided an indication of the relative stiffness of the mesohyl.

The possibility that cells in *C. reniformis* contain a factor that influences mesohyl stiffness was investigated using tissue extracts. A large sponge was chopped up finely. Half of the mince was stirred in 5 volumes of seawater for 3 h, subjected to two cycles of freezing at –20° C for 2 h and thawing at room temperature for 2 h, then centrifuged at 37,000 rpm for 30 min and the supernatant retained. The other half of the mince was stirred in seawater and centrifuged (without freeze-thawing). The supernatant was retained and the residue was then stirred in seawater, subjected to two freeze-thaw cycles, centrifuged and the supernatant retained.

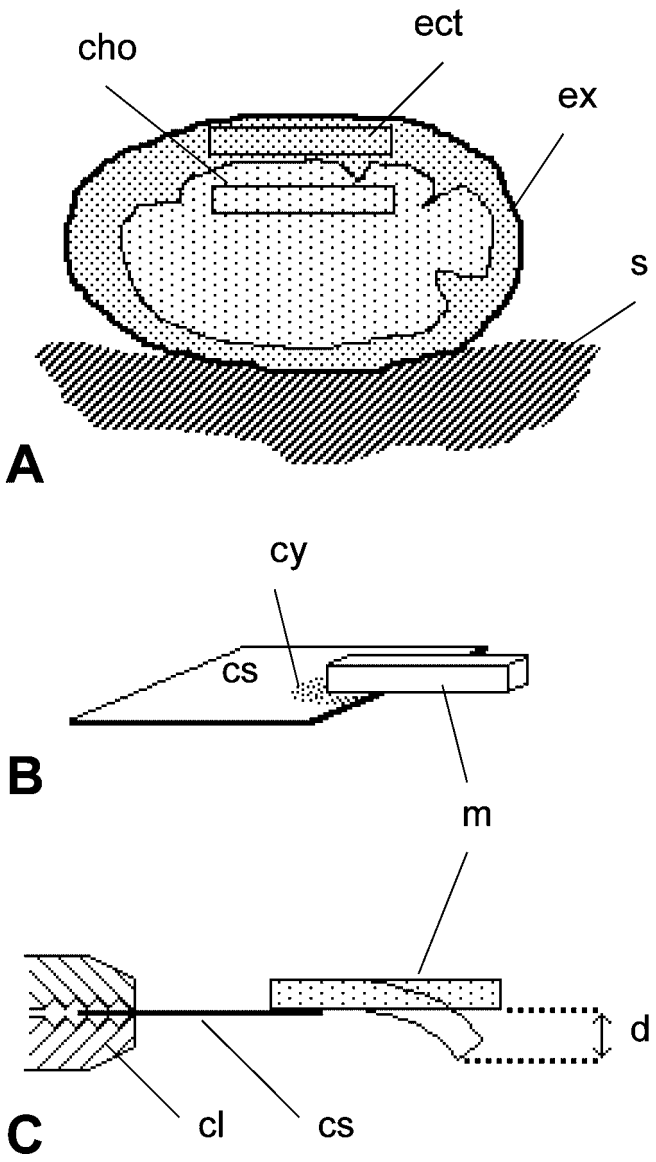

Fig. 1. Preparation and testing of mesohyl samples. **A,** Diagrammatic section through whole sponge showing orientation and location of ectosome (ect) and choanosome (cho) samples. ex, exopinacoderm; s, substrate. **B,** Mesohyl sample (m) attached to glass coverslip (cs) with cyanoacrylate cement (cy). **C,** Diagrammatic lateral view of test apparatus. cl, clamp; d, deflection in 45 s measured using 0.5 mm grid positioned vertically behind sample (not shown).

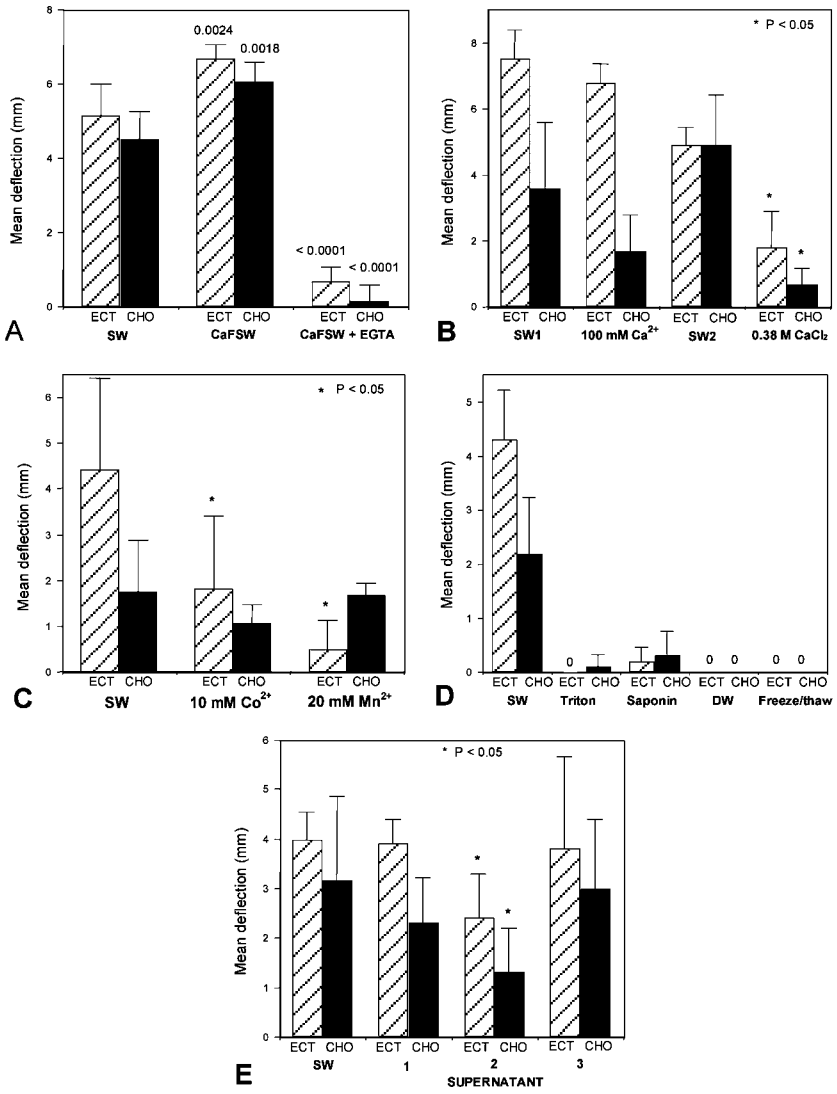

Fig. 2. Effects of experimental treatments on flexural stiffness of mesohyl samples. Bar charts show mean deflections after 45 s; vertical bars represent one standard deviation; in all cases n = 5 or 6. CHO, choanosome; ECT, ectosome; SW, artificial seawater. **A,** Effect of Ca^{2+}-free SW alone and with 5 mM EGTA; values are two-tailed probabilities generated by Student's t-tests comparing test and control means. **B,** Effect of SW containing 100 mM Ca^{2+} and 0.38 M $CaCl_2$; these were compared with separate control groups (SW1 and SW2 respectively). **C,** Effect of SW containing 10 mM Co^{2+} or 20 mM Mn^{2+}. **D,** Effect of 1 % Triton X-100, 0.1 % saponin, distilled water and freeze-thawing. **E,** Effect of supernatants from frozen minced sponge (1), unfrozen minced sponge (2) and residue from frozen minced sponge (3).

RESULTS

Effect of changes in external Ca^{2+} concentration

Nominally Ca^{2+}-free seawater (Ca^{2+} substituted with Na^{+}) caused a significant increase in deflection, *i.e.* reduction in stiffness, of both the ectosome and choanosome (Fig. 2A), which was reversible (not illustrated). However, Ca^{2+}-free seawater containing 5 mM EGTA, a Ca^{2+}-specific chelator, caused a drastic reduction in deflection, *i.e.* increase in stiffness (Fig. 2A), which was irreversible (not illustrated). Elevating the Ca^{2+} concentration from 10 mM to 100 mM caused a small, statistically insignificant increase in stiffness, whereas 0.38 M $CaCl_2$ alone, which is isotonic with seawater, caused a significant increase in stiffness (Fig. 2B).

Effect of Co^{2+} and Mn^{2+}

Seawater containing 10 mM Co^{2+} or 20 mM Mn^{2+} ions significantly stiffened ectosome samples. Although they appeared to have no significant effect on the choanosome, the control choanosome samples were themselves very stiff in this experiment, which could have masked any action of Co^{2+} or Mn^{2+} (Fig. 2C).

Effect of membrane disrupters

Mesohyl samples were subjected to a variety of treatments that cause membrane disruption and cell lysis. These were: 1 % Triton X-100 in seawater, 0.1 % saponin in seawater, distilled water, and freezing at –20° C for up to 18 h followed by thawing. All of these treatments stiffened the mesohyl dramatically: the samples bent very little or not at all (Fig. 2D). For all treatments this effect was irreversible (not illustrated).

Effect of tissue extracts

Neither the supernatant from the frozen mince nor that from the frozen residue had a significant effect. The supernatant from the unfrozen mince increased significantly the stiffness of both the ectosome and choanosome (Fig. 2E).

DISCUSSION AND CONCLUSIONS

Ca^{2+}-free seawater destiffened and elevated Ca^{2+} concentrations stiffened the mesohyl. These results could be due to effects of Ca^{2+} on the extracellular matrix (ECM) and/or on cells that influence the tensile properties of the ECM. Ca^{2+} contributes directly to intermolecular cohesion in the ECM of both mammals and echinoderms (STEVEN, 1967; DIXON *et al.,* 1972; EYLERS, 1982; EYLERS & GREENBERG, 1989). However, the sensitivity of echinoderm mutable collagenous tissue (MCT) to $[Ca^{2+}]_o$ manipulation is due mainly to the disturbance of cellular activities, which could include secretory mechanisms and impulse conduction (TROTTER & KOOB, 1995; SZULGIT & SHADWICK, 2000). That mesohyl tensility can be affected by a Ca^{2+}-dependent cellular mechanism was indicated by the stiffening action of 10 mM Co^{2+} and 20 mM Mn^{2+}, which are inorganic Ca^{2+}-channel blockers (LEYS *et al.,* 1999).

The extreme stiffening induced by different agents that cause membrane disruption and cell lysis provides strong evidence for cellular involvement and indicates that the destiffened condition is dependent on the presence of intact cells.

The anomalous stiffening effect of 5 mM EGTA may also be due to cell lysis, since high concentrations of EGTA damage mammalian cells and lead to necrosis (WARING & SJAARDA, 1989). The treatments used in this investigation (with the exception of EGTA) also stiffen certain examples of echinoderm MCT, an effect that has been shown to result from the release from damaged cells of an organic stiffening factor that interacts directly with ECM molecules. The echinoderm factor can be extracted from minced tissue subjected to freeze-thaw cycles (TROTTER & KOOB, 1995; SZULGIT & SHADWICK, 2000). When we applied a similar protocol to sponge mesohyl, significant stiffening activity was detectable only in the extract from unfrozen tissue and not in that from frozen tissue. This provides some evidence for the presence of an intracellularly sequestered stiffening factor in the mesohyl. Our results suggest that mincing of the mesohyl without freeze-thawing causes enough cell damage to release a stiffening factor, though in lower amounts than when 'intact' tissue samples are subjected to a single freeze-thaw cycle. The reduction in activity after the two freeze-thaw cycles involved in producing the supernatant may be caused by the time- or temperature-dependent degradation of the factor.

This investigation has shown that treatments that would be expected to disrupt cells or alter cellular activities change the properties of the mesohyl within a short timescale (2 - 4 h), indicating that cells in the mesohyl have the capacity to regulate its mechanical properties. Cells could achieve this either through contractile activity, in the way that myocytes influence the wall stiffness of mammalian blood vessels, or through their ability to alter the mechanical properties of the ECM, as occurs in echinoderm MCT (WILKIE, 1996, 2002; TROTTER *et al.,* 2000). Our results indicate that the former is highly unlikely, because, since active cellular contraction would stiffen the mesohyl, a necessary corollary is that cell-damaging treatments should destiffen it, whereas we found that cell damage resulted in an extremely stiffened condition. Moreover, although there are cells in the mesohyl with a contractile phenotype, their number appears to be too low for them to have a significant influence on the passive mechanical properties of the mesohyl (BONASORO *et al.,* 2001), especially with regard to the highly collagenous ectosome whose tensile strength and stiffness can be close to those of bovine nasal cartilage (GARRONE *et al.,* 1975).

It must be concluded, therefore, that cells can bring about changes in the passive mechanical properties of the mesohyl by a mechanism that modifies directly extracellular macromolecules or the interactions between them. Whilst our results indicate that mesohyl cells contain a stiffening factor, it remains to be demonstrated that this is a component of a regulatory system, comparable with that of echinoderm MCT, rather than an experimental artefact. There are interesting similarities between MCT and the mesohyl of *Chondrosia reniformis*: the mechanical properties of both are sensitive to $[Ca^{2+}]_o$ and are affected dramatically by treatments that cause cell lysis; cells in both contain a water-soluble stiffening factor; both consist mainly of cross-striated collagen fibrils organised into parallel bundles (*i.e.* fibres) and interconnected by proteoglycan-like molecules (GARRONE *et al.,* 1975; WILKIE, 1996); and there is ultrastructural evidence that, as in MCT, changes in the tensile properties of the mesohyl depend on adjustments in interfibrillar cohesion, not in the collagen fibrils themselves (BONASORO *et al.,* 2001). Furthermore, the 'global contractions' observed in sponge mesohyl, the cellular basis of which has not been established (SIMPSON,

1984; HARRISON & DE VOS, 1991), recall the force-developing capacity of some echinoderm ligaments (BIRENHEIDE & MOTOKAWA, 1996).

This investigation has provided preliminary evidence that sponge mesohyl, one of the first fibrous connective tissues to have evolved, shows short term mechanical adaptability that is under physiological control. This phenomenon has been demonstrated in only one other phylum - the Echinodermata, in all five classes of which it is of crucial importance for energy-sparing posture maintenance and for the detachment mechanisms associated with autotomy (WILKIE, 2001, 2002). Further research is needed to determine if the similarities between mesohyl and MCT are due to convergence or homology. However, even if it emerges that they are underpinned by different molecular mechanisms and/or cellular processes, the mystery will remain as to why such an apparently advantageous feature as connective tissue mutability is not expressed more widely throughout the animal phyla.

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