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Mechanical adaptability of a sponge extracellular matrix: evidence for cellular control of mesohyl stiffness in *Chondrosia reniformis* Nardo

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Summary

The marine sponge *Chondrosia reniformis* Nardo consists largely of a collagenous tissue, the mesohyl, which confers a cartilaginous consistency on the whole animal. This investigation was prompted by the incidental observation that, despite a paucity of potentially contractile elements in the mesohyl, intact *C. reniformis* stiffen noticeably when touched. By measuring the deflection under gravity of beam-shaped tissue samples, it was demonstrated that the flexural stiffness of the mesohyl is altered by treatments that influence cellular activities, including [Ca^{2+}] manipulation, inorganic and organic calcium channel-blockers and cell membrane disrupters, and that it is also sensitive to extracts of *C. reniformis* tissue that have been repeatedly frozen then thawed. Since the membrane disrupters and tissue extracts cause marked stiffening of mesohyl samples, it is hypothesised that cells in the mesohyl store a stiffening factor and that the physiologically controlled release of this factor is responsible for the touch-induced stiffening of intact animals.

Key words: connective tissue, extracellular matrix, mechanical properties, mutable collagenous tissue, sponge.

Introduction

Chondrosia reniformis Nardo is a marine sponge that lacks calcite or siliceous spicules or the reinforcing spongin fibres present in many other members of the phylum Porifera (Garrone et al., 1975; Harrison and De Vos, 1991). The bulk of the body of *C. reniformis* consists of a collagenous material, the mesohyl, which is located between the external epithelium and the internal epithelia that line the inhalant and exhalant canals. Although, as reflected in the generic name, the mesohyl confers on the whole animal a cartilaginous consistency, it also demonstrates plasticity in terms of both shape and mechanical properties. For example, within 40–60 h after excision, fragments of *C. reniformis* undergo rounding off of cut surfaces and marked bending (Nickel and Brümmer, 2003). Whole sponges or tissue explants can lose rigidity under continuous compression (Garrone et al., 1975; Garrone, 1978), and in the sea, loosening of the substrate under part of a sponge is followed by the slow elongation of that part under gravity and its eventual separation from the still attached portion, a process that may be regarded as a form of opportunistic asexual reproduction (Sarà and Vacelet, 1973; Bonasoro et al., 2001; Zanetti, 2002).

The work described herein was prompted by the observation that, when previously undisturbed specimens of *C. reniformis* in the sea or laboratory aquaria are touched repeatedly, they feel softer the first time they are touched than on second and subsequent occasions. The fact that morphological studies of *C. reniformis* had not revealed the presence of potentially contractile cells in a quantity or disposition sufficient to account for this phenomenon (Bonasoro et al., 2001) (F. Bonasoro, unpublished observations), raised the possibility that the mechanical properties of the mesohyl itself are under physiological control. A precedent for this is provided by the 'mutable' collagenous tissue of echinoderms, the variable tensility of which is neurally modulated and which is involved throughout the phylum in the energy-sparing maintenance of posture and in the rapid detachment of anatomical structures at autotomy (Trotter et al., 2000; Wilkie, 2001; Wilkie, 2005).

The main aim of this investigation was to test the hypothesis that the passive mechanical properties of the mesohyl of *C. reniformis* are under direct cellular control by determining the effect on the flexural stiffness of the mesohyl of a range of agents that influence cellular activities in other animals. A preliminary account of some of the results was presented at the 6th International Sponge Conference, Rapallo, Italy 2002 (Wilkie et al., 2004).

Materials and methods

Specimens of *C. reniformis* were collected by scuba divers at Portofino and Bergeggi on the Italian Ligurian coast. They were transported to the University of Milan and maintained in 50 l tanks of artificial seawater (ASW: 'Instant Ocean' Aquarium Systems, Sarrebaug, France) at 14–16°C.

For convenience of description we regard an individual *C. reniformis* as consisting of an outer epithelium (exopinacoderm) and 'internal tissue', the latter term encompassing both the collagenous mesohyl and the internal epithelia (endopinacoderm and choanoderm). The internal tissue comprises two macroscopically distinguishable regions: an outer cortex, or ectosome, which is penetrated by a relatively small number of aquiferous canals and is densely collagenous, and a medulla, or choanosome, which contains choanocyte chambers and many more canals and is less densely collagenous (Fig. 1A–C). Beam-shaped samples 2.5 mm×2.5 mm×15 mm in size were cut from both the ectosome and choanosome regions, as shown in Fig. 1A, using two parallel-mounted razor blades. 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 exopinacoderm (to eliminate any contribution it might make to the mechanical responsiveness of the samples) and variable, but always small, amounts of choanosome. Samples traversed by large canals were discarded. Each sample was fixed to a 20 mm×20 mm 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. 1D). This orientation was chosen to minimise variability caused by the slow bending of ectosome samples, which occurs only in a plane orthogonal to the external surface (personal observation). To minimise variability due to handling, 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, each sample was lifted gently from the solution whilst the coverslip was kept horizontal, a stop-clock being started as the lifting began, and the coverslip was then clamped horizontally with the sample projecting in front of a 0.5 mm grid (Fig. 1E). Care was taken to avoid jolting the sample during its transfer from the test solution to the clamp. The sample usually bent under gravity and at a predetermined time (45 s unless stated otherwise) after the stop-clock was started the amount of deflection was recorded to the nearest 0.5 mm by viewing the grid through a horizontally mounted binocular microscope. Deflection in a fixed time period is inversely proportional to flexural stiffness, and therefore provides an indication of the relative stiffness of the mesohyl. This procedure is a modified version of that developed by Trotter and Koob (Trotter and Koob, 1995) to investigate the variable tensility of holothurian dermis. Trotter and Koob explained the theoretical background to this technique and discussed its limitations with respect to the quantification of absolute mechanical properties (Trotter and Koob, 1995).

Three groups of experiments were conducted.

(1) Mechanical behaviour of samples before treatment with chemical agents

The recovery of samples from the stiffening effect of the excision procedure was observed as follows. Immediately after each sample was attached to a coverslip, its stiffness was measured, as described above, and it was then immersed in ASW. Some samples were left at room temperature (24.5–26°C) and some were left in a cold-room at 14°C. All were tested again after 1 h, 3 h, 5 h, 7 h and 9 h.

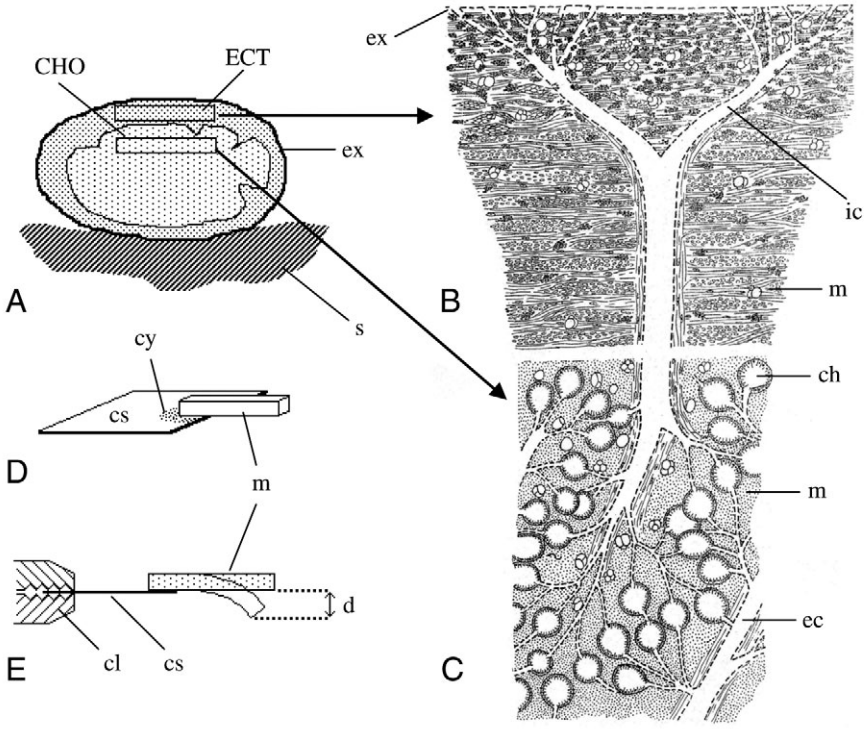

Fig. 1. Samples and experimental setup. (A) Diagrammatic vertical section through a whole specimen of *C. reniformis*, showing location and orientation of choanosome (CHO) and ectosome (ECT) samples. ex, exopinacoderm; s, substrate. (B,C) Drawings of vertical sections through the ectosome and choanosome. ch, choanocyte chamber; ec, exhalant canal; ic, inhalant canal; m, collagenous mesohyl. (D) Drawing showing a sample attached to a glass coverslip (cs) with cyanoacrylate cement (cy). (E) Diagrammatic lateral view of experimental set-up in which the coverslip is held horizontally in a clamp (cl) and the deflection (d) of the sample under gravity is measured after a predetermined time period (45 s unless stated otherwise). [B and C are from Grassé (Grassé, 1973)].

(2) Effects of membrane disrupters and ionic manipulation

The effects of these agents were determined by measuring the deflection of samples on a single occasion after immersion in the relevant media for usually 1–2 h. The results are expressed as mm deflection.

(3) Effects of tissue extracts

In these experiments, the time course of potential effects was monitored by measuring the deflection of each sample immediately before immersion in the relevant control or test medium, and at hourly intervals thereafter. The results are expressed as the difference in mm between the initial deflection and deflection at later times. Tissue extracts were used to investigate the possible presence of an intracellularly stored stiffening factor. These were prepared by slicing up a large sponge finely with razor blades. Half of the resulting material was stirred in 5 volumes of seawater for 3 h, subjected to 5 cycles of freezing at –80°C for 2 h and thawing for 2 h, then centrifuged at 25 420 ***g*** for 30 min and the supernatant retained. This will be called 'extract 1'. The other half of the sliced material was stirred in seawater and centrifuged without freeze–thawing. The supernatant was retained ('extract 2') and the residue was then stirred in seawater, subjected to five freeze–thaw cycles, centrifuged and the supernatant retained ('extract 3'). All extracts were stored frozen at –80°C.

All group 2 and group 3 experiments were carried out at room temperature and were repeated at least once to confirm the reproducibility of results. The sample number was usually 5 (range 4–7). Statistical significance was evaluated by means of Student's *t*-tests or, for multiple comparisons, ANOVA and Bonferroni *post-hoc* tests.

Results

Destiffening of untreated samples

All groups of samples destiffened progressively over 7–9 h (Fig. 2). Only the ectosome samples at room temperature appeared to reach a condition of maximal compliance after 7 h. At either temperature there was no significant difference between the behaviour of the ectosome and choanosome, except after 3 h at room temperature when choanosome samples were significantly stiffer. The rate of destiffening was slower at 14°C than at room temperature: after 3 h, 5 h and 7 h both ectosome and choanosome samples were significantly stiffer at 14°C than at room temperature, and this applied to choanosome, but not ectosome, samples after 9 h.

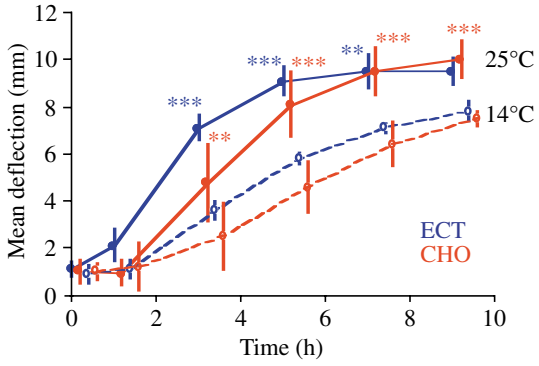

Fig. 2. Effect of temperature on mesohyl destiffening. In this and all subsequent figures ectosome (ECT) results are coloured blue and choanosome (CHO) red, vertical bars represent standard deviations and asterisks indicate statistically significant differences between means: **P*<0.05, ***P*<0.01, ****P*<0.001. Although all deflections were measured at 0, 1, 3, 5, 7 and 9 h, in this figure the means for the different groups have been staggered to avoid overlap.

In subsequent experiments, chemical agents were tested on both 'maximally stiffened' (MS) samples, which were placed in experimental media immediately after attachment to a coverslip, and 'partially destiffened' (PD) samples, which, after attachment, were left undisturbed in ASW for 3–4 h, before being tested mechanically, then immersed in experimental media.

Effects of membrane disrupters and ionic manipulation

A range of treatments that cause cell membrane disruption or permeabilisation by various mechanisms all strongly inhibited the destiffening of MS samples. This effect was produced by deionised water, which causes osmotic lysis, the detergents Triton X-100 (1% in ASW) and *Quillaja* saponin (0.1% in ASW), which dissolve membrane lipids, freezing at –24°C for 18 h to 3 days followed by thawing, which disrupts cellular structure through ice crystal formation, and the pore-forming antibiotic nystatin (0.05% in ASW; Fig. 3A,C). The effect of the first four of these treatments was irreversible: even after prolonged (up to 20 h) immersion in ASW, samples showed almost no deflection under gravity (Fig. 3B). Nystatin had a less extreme effect and retarded destiffening, rather than blocking it altogether (Fig. 3C,D). All of these treatments, except freeze–thawing, were shown to restiffen PD samples, with deionised water having the most pronounced such action (Fig. 3E). The effect of freeze–thawing on PD samples was not determined because of difficulties encountered in maintaining adhesion between the samples and coverslips.

Media with an elevated [Ca^{2+}] inhibited the destiffening of MS samples (Fig. 4A). A solution of 0.38 mol l^{-1} $CaCl_2$ alone (which is isosmotic with seawater) had a more pronounced effect than ASW containing 100 mmol l^{-1} Ca^{2+}. The effect of the latter, but not the former, was reversed by immersion in ASW alone (Fig. 4A). Although ASW containing 100 mmol l^{-1} Ca^{2+} had no effect on PD samples, 0.38 mol l^{-1} $CaCl_2$ partially reversed the destiffening of PD ectosome samples (i.e. partially restiffened them) and stopped further destiffening of choanosome samples (Fig. 4B).

Calcium-free ASW (CaFASW) accelerated the destiffening of MS samples (Fig. 4C). Paradoxically, CaFASW containing EGTA, a divalent cation chelator, inhibited destiffening. This effect was produced by 1–5 mmol l^{-1} EGTA but not by 100 μmol l^{-1} EGTA (Fig. 4C,D). EGTA also reversed the destiffening of PD samples in a dose-dependent manner

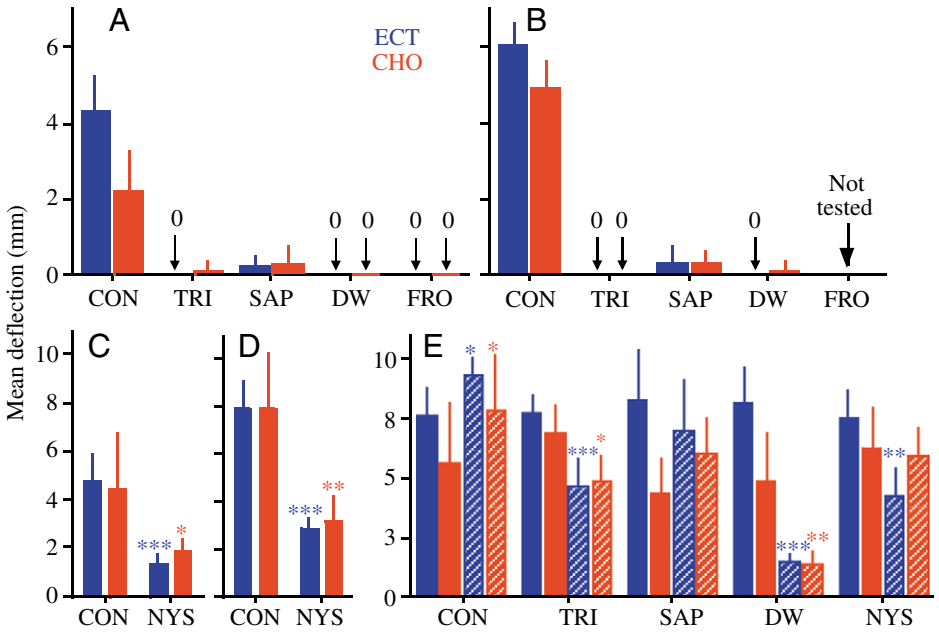

Fig. 3. (A–D) Effect of cell membrane disrupters on maximally stiffened samples (i.e. protocol 1). (A) Effect of immersion for 2.5–3 h in ASW (control; CON), 1% Triton X-100 (TRI), 0.1% *Quillaja* saponin (SAP), deionised water (DW) and freezing at –24°C for 3 days followed by thawing (FRO). In all cases where statistics could be applied (i.e. where the standard deviation was not zero), $P<0.01$. (B) Reversibility of effects of membrane disrupters. After treatment and testing, all samples used for the experiments in A were left in ASW for 3.5 h then retested. In all cases where statistics could be applied, $P<0.01$. (C) Effect of immersion for 2 h in 0.05% nystatin (NYS). (D) Reversibility of effect of nystatin. After treatment and testing, the samples used in the experiments in C were left in ASW for 2 h then retested. (E) Effect of membrane disrupters on partially destiffened samples (i.e. protocol 2). After excision, samples were left for 5–6 h in ASW then tested (solid bars). They were then left for 2 h in the stated media (CON=ASW) and retested (cross-hatched bars). In A–D, statistical comparison is between mean deflections of ASW control and treated samples; in E, statistical comparison is between mean deflections of each group of samples before (solid bars) and after (cross-hatched bars) treatment. Vertical bars represent standard deviations and asterisks indicate statistically significant differences between means: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

(Fig. 4F) and 5 mmol l^{-1} EGTA caused irreversible stiffening (Fig. 4C). Whereas EGTA does not penetrate cell membranes, its acetoxymethyl ester (EGTA-AM) does, and thereby acts as an intracellular Ca^{2+} chelator. EGTA-AM (100 μmol l^{-1}) retarded the destiffening of MS ectosome and choanosome samples, and 50 μmol l^{-1} EGTA-AM had a significant effect on only ectosome samples (Fig. 4E).

Magnesium-free ASW had no consistent effect. In four experiments it respectively inhibited significantly the destiffening of ectosome and choanosome samples, accelerated the destiffening of both significantly, destiffened choanosome samples but had no effect on ectosome samples, and affected neither (data not shown).

Cobalt and manganese ions, which can block calcium-specific membrane channels, inhibited destiffening of MS samples. The effect of 20 mmol l^{-1} Mn^{2+} was consistently greater than that of 20 mmol l^{-1} Co^{2+}; the latter effect, but not the former, was partially reversible (Fig. 4G). PD samples were restiffened by 20 mmol l^{-1} Mn^{2+} but not by 20 mmol l^{-1} Co^{2+} (Fig. 4H).

The organic calcium channel antagonists verapamil (100 μmol l^{-1}) and nimodipine (100 μmol l^{-1}) both retarded destiffening of MS samples (Fig. 4I).

Effects of tissue extracts

All three tissue extracts inhibited destiffening, though to varying extents and with varying degrees of consistency. The extract of frozen tissue inhibited destiffening strongly in two separate experiments. The extract of unfrozen tissue had the weakest inhibitory effect in both experiments. Although in the illustrated example (Fig. 5A,B) this effect was statistically significant for the ectosome at all times, in the other experiment there were no statistically significant differences between the test and control groups. In the illustrated experiment the extract prepared after freezing the residue belonging to extract 2 inhibited destiffening as strongly as extract 1, but in the other experiment its effect was almost as weak as that of extract 2. In both experiments the inhibitory effect of all three extracts was more pronounced on ectosome samples than on choanosome samples.

Discussion

Mechanical behaviour of samples before treatment with chemical agents

Although contractile phenomena in sponges have been investigated extensively (see Simpson, 1984; Harrison and De Vos, 1991; Nickel, 2004), less attention has been paid to the passive mechanical properties of sponge tissues. Chanas and Pawlik (Chanas and Pawlik, 1995) measured the tensile strength of frozen then thawed samples of unspecified orientation and anatomical location from 71 sponge species, and Garrone et al. (Garrone et al., 1975) measured the stiffness and ultimate properties of ectosome samples from *C. reniformis* that were in an unspecified physiological state. It had been noted previously that the mesohyl of *C. reniformis* 'flows' under prolonged compression or tension (Sarà and Vacelet, 1973; Garrone et al., 1975; Bonasoro et al., 2001;

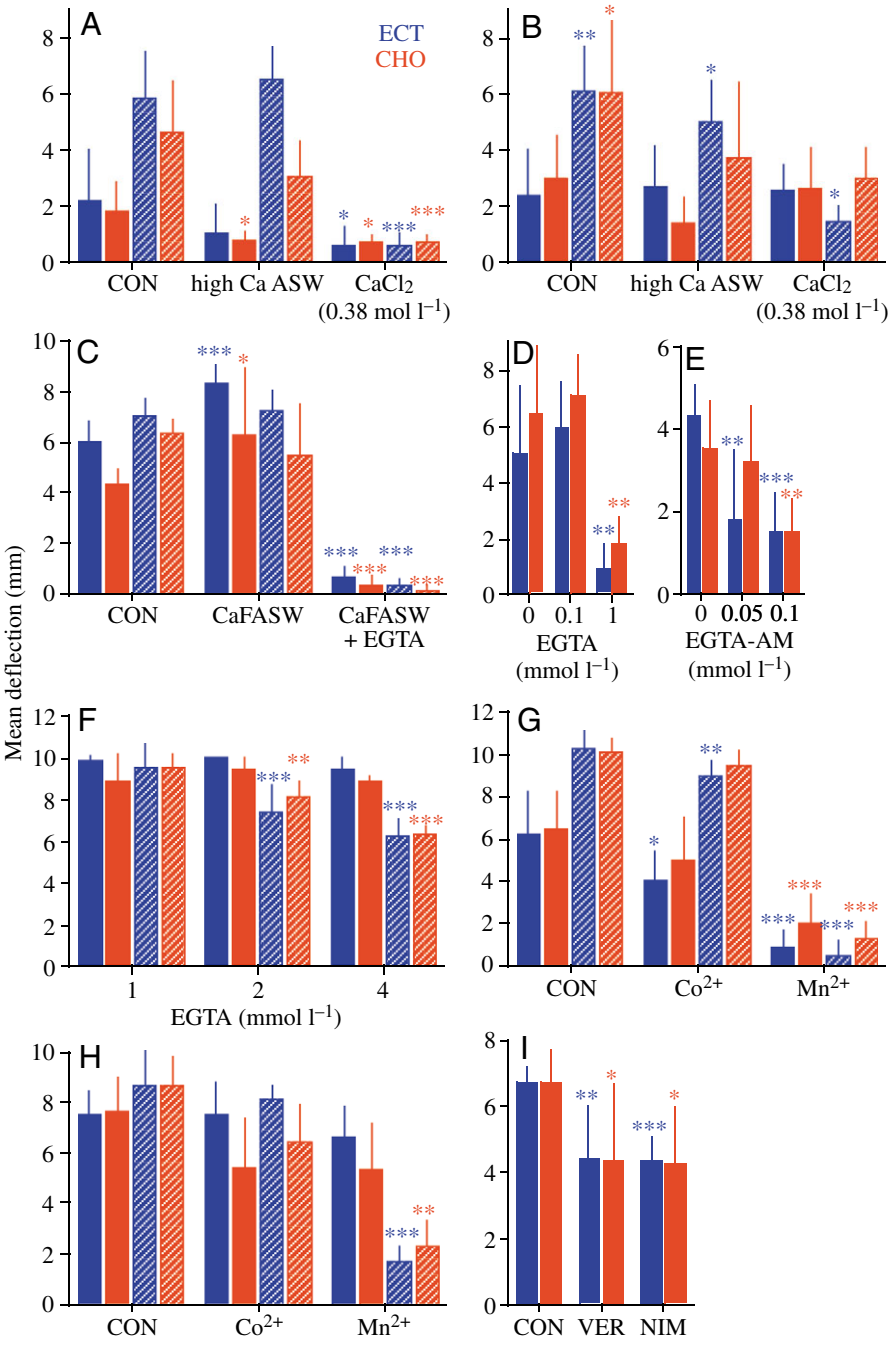

Fig. 4. Effect of ion manipulation. (A) Elevated [Ca^{2+}]. Deflection of maximally stiffened samples immersed for 2 h in normal ASW (CON), ASW containing 100 mmol l^{-1} Ca^{2+} or 0.38 mol l^{-1} $CaCl_2$ (solid bars), and of the same samples after a subsequent wash for 2 h in ASW (cross-hatched bars). (B) Effect of the same agents on partially destiffened samples. After excision, samples were left for 3–4 h in ASW then tested (solid bars). They were then left for 2 h in the stated media and retested (cross-hatched bars). (C) Ca^{2+} depletion. Deflection of maximally stiffened samples (solid bars) immersed for 2 h in normal ASW, calcium-free ASW alone (CaFASW) or CaFASW containing 5 mmol l^{-1} EGTA, and of the same samples after a further wash for 4 h in ASW (cross-hatched bars). (D) Effect on maximally stiffened samples of 2 h exposure to low concentrations of EGTA (dissolved in CaFASW). (E) Effect on maximally stiffened samples of 2 h exposure to EGTA-AM (dissolved in CaFASW containing 1% DMSO). (F) Effect of EGTA on partially destiffened samples. After excision, samples were left for 3.5 h in CaFASW then tested (solid bars). They were then left for 2 h in CaFASW containing 1, 2 or 4 mmol l^{-1} EGTA and retested (cross-hatched bars). (G) Inorganic Ca^{2+}-channel blockers. Deflection of maximally stiffened samples (solid bars) immersed for 1–2 h in normal ASW (CON), ASW containing 20 mmol l^{-1} Co^{2+} or ASW containing 20 mmol l^{-1} Mn^{2+}, and of the same samples after a further wash for 2 h in ASW (cross-hatched bars). (H) Effect of inorganic Ca^{2+}-channel blockers on partially destiffened samples. After excision, samples were left for 3–4 h in ASW then tested (solid bars). They were then left for 2 h in the stated media and retested (cross-hatched bars). (I) Effect of verapamil (VER; 100 μmol l^{-1}) and nimodipine (NIM; 100 μmol l^{-1}) on partially destiffened samples. Control: 1% methanol in ASW. In D–F and I deflections were measure after 35 s. In A,C–F,I statistical comparison is between mean deflections of ASW control and treated samples; in B,G, statistical comparison is between mean deflections of each group of samples before (solid bars) and after (cross-hatched bars) treatment. Vertical bars represent standard deviations and asterisks indicate statistically significant differences between means: *P<0.05, **P<0.01, ***P<0.001.

Zanetti, 2002). However, as far as we are aware, ours is the first investigation to have demonstrated that the stiffness of sponge mesohyl can be changed by an intrinsic mechanism.

Whole sponges stiffen in response to brief mechanical stimulation. Since the handling of sponges that is necessary to prepare isolated tissue samples constitutes intense and prolonged mechanical stimulation, it is not surprising that, immediately after excision, such samples were in a stiffened condition, as indicated by the fact that they bent very little under gravity. Both ectosome and choanosome samples then destiffened over a period of hours, although after 9 h, when the experiment was terminated, only the ectosome samples at 25°C had reached a plateau of maximal compliance, which may represent the ‘resting’ condition of the internal tissue in unstimulated sponges. Destiffening occurred more rapidly at room temperature (24.5–26°C) than at 14°C, 26°C and 14°C being the upper and lower limits of the sea temperature range to which *C. reniformis* is exposed at the collection sites (G.

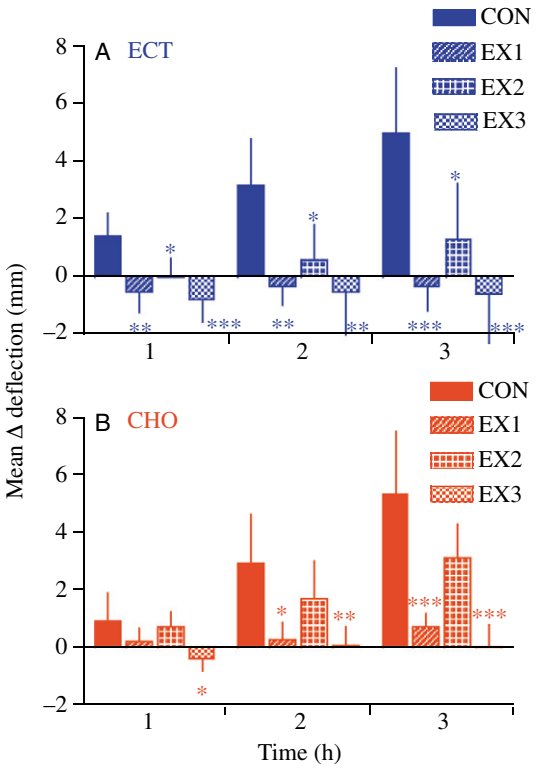

Fig. 5. Effect on maximally stiffened samples of treatment for 1, 2 and 3 h with an extract of frozen minced tissue (EX1), unfrozen minced tissue (EX2) and the frozen residue from EX2 (EX3). Control: ASW. Deflections were measured after 35 s.

Bavestrello, personal observation). However, the overall shape of the deflection-time curves was similar at the two temperatures, suggesting that the physiology of the internal tissue was qualitatively similar and that it was therefore valid to conduct all subsequent experiments at room temperature.

Cellular basis of variable tensility

Anatomical structures that have a significant collagenous component, and that show rapid changes in passive mechanical properties, occur in other phyla. The variable tensility of these structures depends on either active force development by contractile cells, as in the walls of vertebrate blood vessels (Bank et al., 1996), or the direct, cell-mediated modulation of the tensile properties of the extracellular matrix, as in the mutable collagenous tissue of echinoderms (Wilkie, 2005).

Contractile cells may be present in the internal tissue of *C. reniformis*. The endopinacocytes lining the inhalant and exhalant canals of sponges are thought to be contractile, primarily on the grounds that they contain actin microfilaments (Simpson, 1984; Harrison and De Vos, 1991), and the mesohyl of *C. reniformis* includes a sparse and loose network of cell processes (possibly belonging to endopinacocytes) also furnished with microfilaments (Bonasoro et al., 2001). However, the very small volume ratio occupied by potentially contractile cells, particularly in the ectosome, which is penetrated by relatively few canals (Bavestrello et al., 1988), and the paucity of potentially tension-resistant cell–cell and cell–extracellular matrix (ECM) junctions (Bonasoro et al., 2001), suggest that these cells could not influence significantly the passive mechanical properties of the internal tissue. This morphologically derived inference was supported by the present investigation. The drastic disruption of cellular structure caused by deionised water and freeze–thawing prevented destiffening and restiffened partly destiffened samples, actions that could not have been due to cell-dependent contractile activity. Furthermore, because actin–myosin-based contractile systems, including those of sponges (Lorenz et al., 1996), have a universal dependence on Ca^{2+}, any agent that reduces the cytosolic [Ca^{2+}] inhibits such systems, a relevant example being the relaxing effect of calcium channel blockers on vertebrate smooth muscle. However, the destiffening (i.e. relaxation) of sponge tissue was inhibited by inorganic and organic calcium channel blockers, the extracellular chelator EGTA and the intracellular chelator EGTA-AM, all of which would be expected to depress $[Ca^{2+}]_i$. Our experimental results, therefore, preclude the possibility that touch-induced stiffening of the internal tissue of *C. reniformis* is due to cellular contraction and lead to the conclusion that the stiffness of the mesohyl ECM is itself under direct cellular control.

Theoretically, both stiffening and destiffening of the mesohyl, or only one of these, is an active, cell-mediated process. Suppressing cellular function by membrane disruption blocked irreversibly the destiffening of MS samples and restiffened PD samples. The irreversibility of the former effect is evidence that destiffening requires functioning cells. However, there are two possible explanations for the restiffening caused by these treatments. The first, and less likely of the two, is that stiffening is passive and occurs whenever there is cessation of cellular activity that causes and maintains destiffening. The second explanation is that cell lysis releases a chemical factor that stiffens the ECM directly. The presence of an intracellularly stored stiffening factor would explain why less disruptive membrane-permeabilising treatments – detergents and nystatin – had a generally weaker effect than deionised water and freeze–thawing, since membrane permeabilisation without cell lysis would be expected to cause less stiffening factor to be released from cells. The existence of such a factor was confirmed by the experiments using tissue extracts.

All three extracts inhibited destiffening, with those from frozen tissue (extracts 1 and 3) exerting the greater effect. The fact that the extract of unfrozen tissue (extract 2) also had some activity indicates that the more limited cell damage caused by mechanical disruption alone was sufficient to release a significant amount of the stiffening factor. The present investigation provides little insight into the nature of this factor, which is not necessarily a single chemical substance, although the ability of nystatin to inhibit and reverse destiffening suggests it is not composed of large molecules. Nystatin is a polyene antibiotic which introduces into cell membranes pores that permit the leakage of small ions and molecules (Gale et al., 1981). Work is currently under way to isolate and

characterise the stiffening factor and to determine its physiological significance: its action on the mesohyl may be incidental and its *in vivo* functions unrelated to variable tensility. This caveat notwithstanding, we hypothesise that touch-induced stiffening of the mesohyl of *C. reniformis* depends on the secretion from cells of a stiffening factor that interacts directly with the extracellular matrix.

The flexural stiffness of the mesohyl was changed by other agents that would be expected to modify cellular activities, rather than stop them altogether as in the case of membrane disrupters. The inhibition of destiffening caused by both inorganic and organic calcium channel blockers and by the intracellular chelator EGTA-AM suggests that the mechanical condition of the mesohyl is influenced by cellular processes involving transmembrane Ca^{2+} fluxes and changes in $[Ca^{2+}]_i$.

The effects of manipulation of the extracellular $[Ca^{2+}]$ are, however, problematical. Media with an elevated $[Ca^{2+}]$ inhibited destiffening and CaFASW by itself accelerated destiffening. CaFASW containing 1 mmol l^{-1} EGTA, in contrast, inhibited destiffening and CaFASW with 2 mmol l^{-1} EGTA restiffened PD samples. The contradictory effects of CaFASW with and without EGTA must result from interference with different processes. The effects of CaFASW alone and of elevated $[Ca^{2+}]$ are hard to reconcile with those of certain other agents. CaFASW would be expected, like EGTA-AM and calcium channel blockers, to depress $[Ca^{2+}]_i$, yet it accelerated rather than retarded destiffening. The fact that collagen fibrils can be isolated from the mesohyl of *C. reniformis* by a medium containing EDTA (Garrone et al., 1975) indicates that, as in mammalian and echinoderm collagenous tissue (Dixon et al., 1972; Steven, 1967; Wilkie, 2005), divalent cations contribute directly to the cohesion of the mesohyl ECM. Therefore, it is possible that the effects of elevated $[Ca^{2+}]$ and CaFASW alone are due to their direct influence on extracellular components of the mesohyl ECM and are not cell-mediated. CaFASW with EGTA restiffened PD samples and 5 mmol l^{-1} EGTA caused irreversible mesohyl stiffening. Therefore, like deionised water, freeze–thawing etc., EGTA may cause cell membrane permeabilisation and leakage of the stiffening factor. This makes it possible that EGTA removes extracellularly bound Ca^{2+} ions that are unaffected by CaFASW alone and that maintain cell membrane integrity in intact tissue.

We have provided evidence that the mechanical properties of the mesohyl ECM are under cellular control. At present we have no information on the identity of the effector cells. In addition to the endopinacocytes and choanocytes of the aquiferous system, the internal tissue of *C. reniformis* contains a variety of cell types, the functions of which are largely unknown (Bonasoro et al., 2001). These include cells with membrane-bounded cytoplasmic vesicles that in other sponges release their contents into the extracellular compartment. Amongst the materials thus released are lectins (Bretting and Königsmann, 1979), a class of small molecules that may have a structural role in mammalian collagenous structures (Tidball, 1994; Ozeki et al., 1995). Lectins are stored in the vesicles of spherulous cells (Bretting and Königsmann, 1979; Bretting et al., 1983), which in *C. reniformis* are particularly abundant in the ectosome (Bonasoro et al., 2001). In this investigation it was found that experimental treatments tended to have more pronounced effects on ectosome samples than choanosome samples. Since this usually took the form of a stronger stiffening response, it raises the possibility that the spherulous cells are the source of the stiffening factor in *C. reniformis* and that the stiffening factor is a lectin.

Molecular mechanism of variable tensility and evolutionary significance

The mesohyl ECM of *C. reniformis* consists of bundles of collagen fibrils interconnected by molecules that include complex carbohydrates (Garrone et al., 1975). Collagen fibrils have a high tensile strength and low extensibility (Sasaki and Odajima, 1996; Redaelli et al., 2003). However, the mesohyl of *C. reniformis* 'flows' under prolonged compression (Garrone et al., 1975; Garrone, 1978) or tension (Sarà and Vacelet, 1973; Zanetti, 2002). Since the latter occurs without rupture or lengthening of the collagen fibrils (Bonasoro et al., 2001), it is apparent that (1) the fibrils are discontinuous (i.e. individual fibrils do not completely encircle the body) and (2) the molecular interactions holding adjacent fibrils together are unstable enough to allow fibrils, or bundles of fibrils (i.e. fibres), to slide past each other. The latter is also demonstrated by the ease with which fibrils can be isolated by mild, non-denaturing methods (Garrone et al., 1975), in contrast to the inextractability of the collagen fibrils of adult mammalian connective tissue (deVente et al., 1997). Given the discontinuous nature of the collagen fibrils and the lability of interfibrillar cohesion in the unstimulated mesohyl, the only possible way in which stiffening can be effected is by the strengthening of the cohesive forces, thereby preventing interfibrillar or interfibre slippage. This must be the mode of action of the stiffening factor whose existence we infer from our experimental results.

There are similarities between the mesohyl of *C. reniformis* and the mutable collagenous tissue (MCT) of echinoderms, the mechanical properties of which can be altered rapidly under nervous control. The collagen fibrils of MCT form discontinuous arrays and are easily extracted, in the case of holothurian dermis, by prolonged immersion in seawater alone (Trotter et al., 1996). Like the mesohyl, holothurian dermis, the most intensively investigated mutable collagenous structure, stiffens rapidly in response to mechanical stimulation, then destiffens over a period of hours (Szulgit and Shadwick, 2000; Motokawa and Tsuchi, 2003). Holothurian dermis is also stiffened by cell membrane permeabilising treatments, which have been shown to release an intracellularly sequestered stiffening factor that strengthens interfibrillar cohesion (Tipper et al., 2003; Tamori et al., 2006). The phylogenetic significance of these similarities will not become clear until the molecular mechanisms and cellular processes underpinning the variable tensility of MCT and the mesohyl have been elucidated fully. This will clarify whether the short-term physiological control

of ECM mechanics is a primitive attribute of collagenous connective tissue, its absence from most phyla representing secondary loss, or whether it is a derived feature that has evolved independently in different groups. We suspect that the latter is the case, but that it depends on what may be a primitive feature of collagenous tissue, namely the lability of intermolecular linkages holding together adjacent collagen fibrils. That this is a primitive feature is suggested by the ease with which collagen fibrils can be extracted from the extracellular matrix of members of the first two multicellular animal phyla to appear in the fossil record: sponges (Garrone et al., 1975) and cnidarians (Deutzmann et al., 2000).

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