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ROLE OF Ca²⁺ IN EXCITATION–CONTRACTION COUPLING IN ECHINODERM MUSCLE: COMPARISON WITH ROLE IN OTHER TISSUES

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Summary

The longitudinal muscle of the body wall of *Isostichopus badionotus* may be considered a model for excitation–contraction coupling in echinoderm muscle. Other echinoderm muscles are reviewed by comparison with the model. Echinoderm muscle is also of interest as a model for 'mutable collagenous tissue'; however, in that tissue, Ca²⁺ has been proposed to function both in living control systems and in regulation of non-living interstitial substance.

Key words: echinoderm, physiology, muscle, ultrastructure, connective tissue, contraction, tissue lysis, tissue stiffening, tissue softening.

Introduction

Insofar as it deals with muscle, this review may be considered a successor to a review of echinoderm muscle physiology by K. Takahashi (Takahashi, 1966). Neuromuscular systems in echinderms were reviewed contemporaneously by Cobb and Laverack (Cobb and Laverack, 1967). At that time, amongst other matters, Takahashi reviewed quick stretch and release experiments with contracting echinoderm muscles carried out by Hill (Hill, 1926) and 'work/speed' curves determined by Levin and Wyman (Levin and Wyman, 1927). Cobb and Laverack discussed motor and inhibitory pathways. In 1987, J. L. S. Cobb reviewed echinoderm neurobiology (Cobb, 1987). His review covered neural innervation of echinoderm muscle extensively, as well as 'muscle tails', which probably function like innervation, and provided a thorough coverage of the innervation of connective tissue and other non-muscular tissues to that date. None of this definitive review will be recapitulated here.

In the current issue, Elphick and Melarange (Elphick and Melarange, 2001) review neural control of muscle relaxation in echinoderms, Landeira-Fernandez et al. (Landeira-Fernandez et al., 2001) review Ca²⁺ transport by subcellular vesicles from echinoderm muscle and Devlin et al. (Devlin et al., 2001) review membrane receptor types in echinoderm muscle. None of the subject matter of these other reviews will be covered in the present review, which will deal mainly with the role of Ca²⁺ in echinoderm muscle at the cellular and tissue levels. The discussion will update and expand on a section of a review published by the present author in 1993. Much of the work reported on the role of Ca²⁺ in excitation–contraction coupling deals with holothurian

longitudinal muscle of the body wall (LMBW), and much of that deals with LMBW of *Isostichopus badionotus* or closely related forms. The LMBW will therefore be the red thread that runs through this review, with observations on other muscles strung on where available. Elphick and Melarange (Elphick and Melarange, 2001) have suggested that Ca²⁺ may play a role in muscle relaxation through inhibition of muscle Ca²⁺ channels, but this hypothesis has yet to be investigated.

Ca²⁺ has also been thought to regulate 'mutable connective tissue'. This has been reviewed elsewhere (Wilkie, 1984; Wilkie, 1996) but may be discussed here since, in some ways, the behavior of mutable connective tissue resembles that of muscle. Ca²⁺ has been identified as a messenger for excitation–contraction coupling in both muscle and mutable collagenous tissue. Some aspects of control by Ca²⁺ have been reviewed, both for echinoderm muscle (Hill, 1993) and for mutable collagenous tissue (Wilkie, 1996). Work to be reported here concentrates mainly on the class Holothuroidea. One justification may be that holothuroids together constitute possibly 'one of the dominant large animals on earth' (Kerr and Kim, 1999). The LMBW of holothuroids is extremely extensible without physical damage. This muscle was used as a classical object both for the demonstration of active state and for the bioassay for acetylcholine (ACh). It has been demonstrated that contraction in the LMBW depends in the short term on the release of Ca²⁺ from subsarcolemmal vesicles and in the long term on the influx of Ca²⁺ across the sarcolemma. However, contractions induced by ACh are dependent on the presence of extracellular Ca²⁺ in the bathing medium and are blocked by Mn²⁺ or La³⁺. To abolish contractility, the LMBW must be soaked for a long period in

Ca^{2+}-free solution, suggesting that there may be extracellular stores of Ca^{2+} in the secluded extracellular space in the 'lumen' of each muscle bundle. After soaking, the LMBW quickly and reversibly regains contractility in artificial sea water and loses contractility in Ca^{2+}-free sea water. Stores from which caffeine releases Ca^{2+} for excitation–contraction coupling can be reloaded by depolarization or treatment with ionophores. The role of Ca^{2+} in the control of a number of other echinoderm muscles has also been reviewed (Hill, 1993).

Muscle

Ca^{2+} storage sites

Subsarcolemmal vesicles have been shown to be Ca^{2+} storage sites in fibers of the LMBW of *Isostichopus badionotus* (Suzuki, 1982). Flattened subsarcolemmal vesicles are found 7–10 nm from the plasma membranes of the bodies and extensions of LMBW cells. Pyroantimonate precipitate, which may indicate Ca^{2+} localization, aggregates at the subsarcolemmal vesicles and along the inner surface of the plasma membrane in cells fixed in the resting state, but in contracted cells the precipitate appears diffusely distributed in the myoplasm. X-ray microanalysis indicated that the precipitate does in fact contain Ca^{2+}. Sugi et al. (Sugi et al., 1982) looked for sources of activator Ca^{2+} for the LMBW of *Stichopus japonicus*. High-magnification electron microscopy revealed extended flattened subsarcolemmal vesicles parallel to the sarcolemma at a distance of 10 nm. In this species also, Suzuki and Sugi (Suzuki and Sugi, 1982) showed that, in resting fibers, Ca^{2+}-containing pyroantimonate precipitate was concentrated at the vesicles and on the inner surface of the plasmalemma, but was diffusely distributed in activated fibers. Flattened cellular extensions were also seen, as in other echinoderm muscles. On morphological grounds, Hill et al. (Hill et al., 1978) suggested that a great enlargement of potential Ca^{2+} storage sites is offered by the extended sarcolemmal area on the 'frills' of each muscle cell, extending into the 'lumen' of each muscle bundle in the LMBW of *I. badionotus*. Membranous sites are abundant adjacent to the 'lumen', which will be defined as the secluded extracellular space walled off down the length of each bundle of LMBW fibers by the extensive area of pentalaminar junctions between the muscle fibers and their frills. Adjacent membranous sites include the nuclei, mitochondria and Golgi apparatus. The periluminal membranous sites may explain how these fibers, with a relatively small provision of subsarcolemmal vesicles, function in many ways like cells with a sarcoplasmic reticulum (reviewed below).

There are several unusual features in the ultrastructure of LMBW cells of *I. badionotus* (Hill et al., 1978) (Fig. 1). No sarcoplasmic reticulum (SR) vesicles are found among the muscle filaments in the interior of the cells. The surface membranes of cells in the same muscle bundle are extensively tightly coupled, with continuous surface contacts ranging from 16% to 65% of the surface of any cell sectioned. The proportion tightly coupled where facing other cells in the bundle is higher than this, since a large proportion of the surface area of each cell faces adjacent bundles separated by collagen and a mucopolysaccharide matrix. Numerous cellular extensions are frequently seen in echinoderm muscle, but the unusual feature in holothurian LMBW is that several, or possibly all, of the cells in a bundle have frills that run into and wind up in the center of the bundle, forming the 'lumen' or secluded extracellular space. Most of each frill is very thin, but there are bulges, still in the 'lumen', containing nuclei, mitochondria and Golgi apparatus. The nature of the extensive tight coupling between cells in the same bundle is discussed by Hill et al. (Hill et al., 1978). In high-magnification electron micrographs, it can be seen that doublet leaflet membranes fuse to form pentalaminar junctions (Hill et al., 1982).

Structures that resemble hemi-desmosomes face each other across the gap between bundles, and may form a mechanical link between adjacent bundles in LMBW of *Sclerodactyla briareus*, since they appear on the plasma membrane where it makes direct contact with external lamina (Chen, 1983). The structural link between adjacent bundles is 80–100 nm wide where fused external lamina link two facing hemi-desmosomes.

The subsarcolemmal vesicles can be made more evident by osmotic manipulation while fixing (Hill et al., 1982) and then appear as long shallow vesicles underlying the sarcolemma. Chen (Chen, 1983) has suggested that they may sequentially release Ca^{2+} as a fiber contracts. In *Sclerodactyla briareus*, these 'SR sacs' may reach half the length of a thick filament and contact the plasma membrane with material resembling SR feet. Taken together, the plasma membrane, the SR sac and the material between resemble a triad from which excitation of the cell may release Ca^{2+} over a significant section of a muscle fiber (Chen, 1983).

Experiments with Ca^{2+}-deprivation

LMBW of Isostichopus badionotus

Dependence on activator Ca^{2+} has been assessed in contractions induced by K^+ or ACh. K^+ and ACh have been shown to depolarize muscle fibers of *I. badionotus* (Hill et al., 1978) and *Holothuria cinerascens* (Hill, 1987). Spontaneous contractions of the isolated LMBW of *Holothuria cinerascens* occur locally with spontaneous localized spiking. However, bath application of ACh or KCl induces dose-dependent overall depolarization and contraction (Hill, 1987; Hill, 1993). On anatomical grounds, it has been supposed that LMBW muscle bundles of *I. badionotus* may have secluded extracellular Ca^{2+} storage spaces (Hill et al., 1978). In fact, consideration of cell structure led to the supposition that the small cells, with SR represented only by superficial vesicles (adjacent to the sarcolemma), might be directly dependent on Ca^{2+} in the extracellular space for excitation–contraction coupling. However, contraction was not abolished by a 30 min superfusion of an isolated muscle with Ca^{2+}-free solution containing EGTA. In *I. badionotus*, all contractile response to 50 mmol l^{-1} KCl was lost after 12 h in the Ca^{2+}-free solution of chelating agent but, when transferred to 9 mmol l^{-1} Ca^{2+} (in
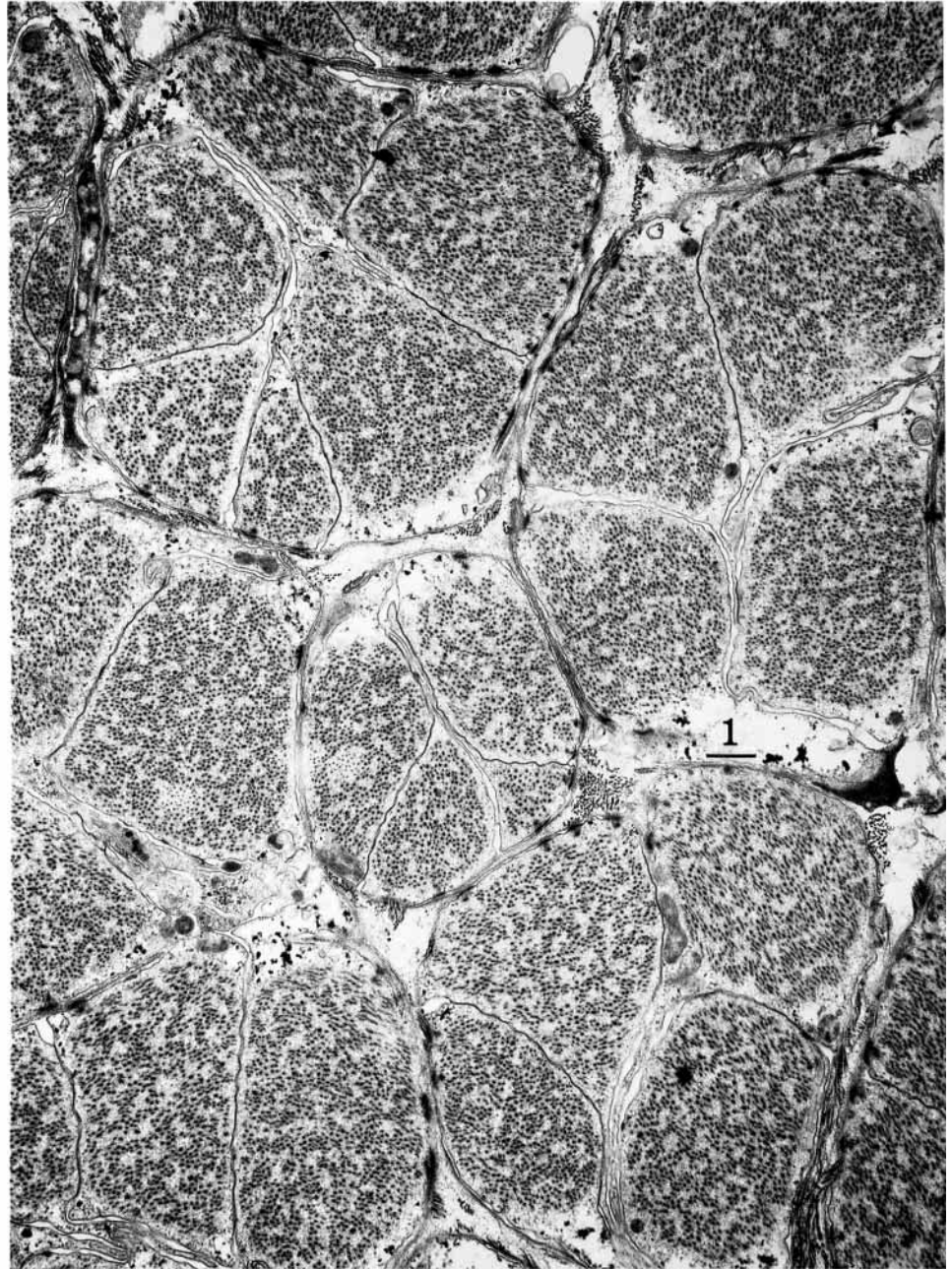
Fig. 1. A cross section of several bundles of the longitudinal muscle of the body wall of *Isostichopus badionotus* showing tight surface contacts between cells in each bundle and structures like hemidesmosomes between facing cells in adjacent bundles. Extensions of each cell into the 'lumen' of each bundle provide an increase in sarcolemmic area. The sarcoplasmic reticulum is represented by small vesicles associated with the cell surface. These features are also apparent in an enlarged view of the luminal area reproduced elsewhere (Landeira Fernandez, 2001). Scale bar, 1 µm. This figure is slightly modified from Fig. 1 (Hill et al., 1978).

artificial sea water without EGTA), the LMBW regained contractility (Hill et al., 1978). This may be taken to mean that storage sites became reloaded with Ca^{2+}. The reloaded muscle then reliably, reproducibly and reversibly lost contractility in a graded fashion, in Ca^{2+}-free solution, with a 70 % loss after 1 h.

Contractions induced by 10^{-3} mol l^{-1} ACh were abolished by pre-soaking for 30 min in a Ca^{2+}-free solution containing 5 mmol l^{-1} EGTA. The greater susceptibility to Ca^{2+} deprivation of ACh responses that is observed may indicate that K^+ depolarization uniformly affects all excitable membrane of the fibers, whereas depolarization by ACh may act on specific receptor sites. Thus, it may be hypothesized that ACh-induced depolarization is linked to specific intracellular storage sites, whereas depolarization induced by an elevated $[K^+]$ may be more refractory to Ca^{2+} if overall membrane depolarization affects widespread Ca^{2+} stores. In a medium lacking Ca^{2+} and Mg^{2+}, the isolated LMBW contracts slowly. This may be attributed to the release of Ca^{2+} from the inner surface of the plasma membrane (Suzuki, 1982).

Other echinoderm muscles

Contractions are Ca^{2+}-dependent in other holothurian muscles (Prosser and Mackie, 1980), in muscle tissue of a sea urchin (Tsuchiya and Amemiya, 1977) and in muscle tissue of a starfish (O'Neill, 1994).
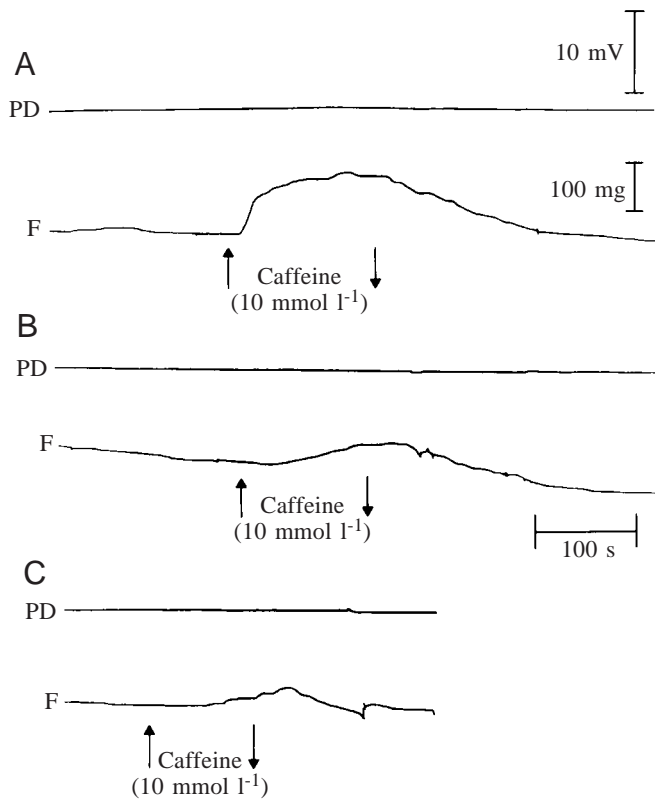
Fig. 2. 20 min after a conditioning depolarization of 3 min in 50 mmol l^{-1} KCl, caffeine induces force without depolarization in isolated longitudinal muscle of the body wall of *Holothuria cinerascens* mounted in a single sucrose gap. In a sequential series of exposures to 10 mmol l^{-1} caffeine, the first contraction (A) is relatively large, the second contraction (B) is small and slow, and the small third contraction (C) has a long latency. F, force; PD, membrane potential.

Fig. 3. (A) No depolarization and very little force is evoked in the first exposure to 10 mmol l^{-1} caffeine of the longitudinal muscle of the body wall of *Holothuria cinerascens* on the left side of a sucrose gap. (B) In comparison, depolarization and force are evoked in the first exposure to 100 mmol l^{-1} KCl of tissue on the right side of the gap. This figure appeared as Fig. 2 in Hill (1987). LF, left force; RF, right force; PD, membrane potential.

Experiments with caffeine

LMBW of *Isostichopus badionotus*

Caffeine induces contractions in Ca^{2+}-free solutions, even after 24 h of soaking in Ca^{2+}-free solution with EGTA (Hill et al., 1978). Caffeine (5 mmol l^{-1}), in Ca^{2+}-free solution, induces a slow contraction that reaches roughly the same amplitude as a contraction induced by 500 mmol l^{-1} KCl in normal extracellular [Ca^{2+}]. That is to say, a relatively low dose of caffeine induces a slow contraction, equivalent to the contraction induced by a supermaximal dose of KCl, even in the absence of extracellular Ca^{2+}. This suggests release of Ca^{2+} from one of the intracellular sites discussed above. When the isolated LMBW is treated sequentially with repeated doses of 10 mmol l^{-1} caffeine, alternating with washes, the muscle undergoes a series of contractures that diminish progressively, from 29 % of resting length to 2.5 % of resting length (Hill, 1980). These contractures are not accompanied by any depolarization in this muscle (Hill et al., 1978) or in the LMBW of *Holothuria cinerascens* (Hill, 1987) (Fig. 2). Caffeine-induced contractures occurred even in muscles that did not contract in response to depolarization (Hill et al.,
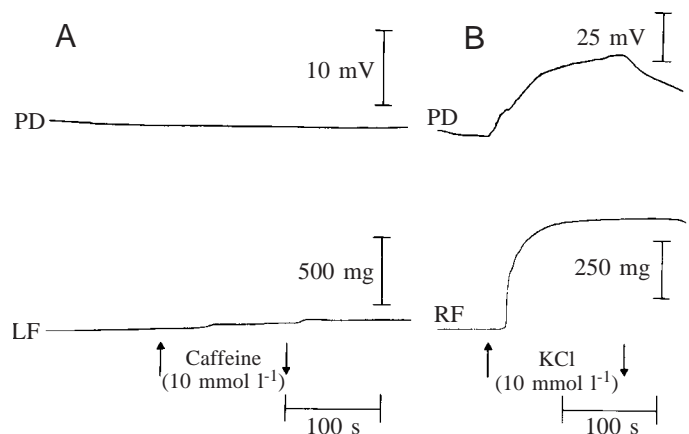
1978). Thus, the progressively diminishing caffeine-induced contractures may be due to release of Ca^{2+} into the cytoplasm from relatively quickly loading and unloading intracellular storage sites; e.g. at the level of the subsarcolemmal vesicles. Once reduced, in a series of responses, amplitude was not restored by 30 min of incubation in sea water (9 mmol l^{-1} Ca^{2+}), in 27 mmol l^{-1} Ca^{2+} solution, or in a solution containing 27 mmol l^{-1} Ca^{2+} and 2 mmol l^{-1} caffeine. However, the amplitude of the responses was restored to approximately 50 % of the initial value by 60 min of incubation in 100 mmol l^{-1} Ca^{2+} solution and to 100 % of the initial value by a 3 min depolarization induced by 10^{-7} mol l^{-1} ACh or by 50 mmol l^{-1} KCl (Hill, 1980) (Figs 3, 4). Thus, depolarization quickly reloads the Ca^{2+} stores depleted by caffeine treatment. It seems that extracellular Ca^{2+} is made available more effectively by membrane depolarization than by an enhanced transmembrane Ca^{2+} gradient.

LMBW of other species

The mechanical responses of the LMBW of *Sclerodactyla briareus* to 50 mmol l^{-1} KCl or to 10^{-7} mol l^{-1} ACh are also Ca^{2+}-dependent (Chen, 1986). Successive caffeine-induced contractures dwindle similarly, but contractility is again restored after a contraction induced by KCl or ACh. The overall pattern of excitation–contraction coupling therefore appears identical to that observed in *Isostichopus badionotus*. Contractions of the LMBW of *Stichopus japonicus* are again Ca^{2+}-dependent, but the intracellular Ca^{2+} storage sites may only be capable of activating approximately 30 % of a maximal contraction (Sugi et al., 1982). These sites appear to be localized along the inner surface of the plasma membrane and at the flattened subsarcolemmal vesicles (Suzuki and Sugi, 1982).
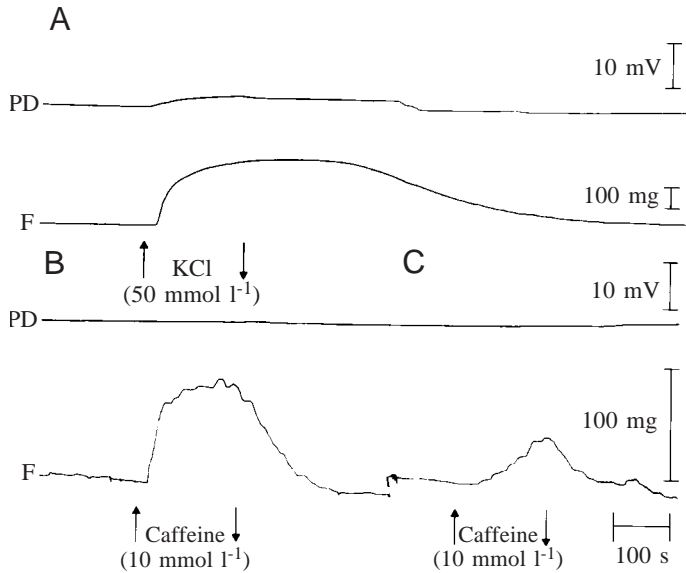
Fig. 4. (A) 50 mmol l^{-1} KCl induces depolarization and contraction in tissue that contracted feebly, without depolarization, in 10 mmol l^{-1} caffeine (see Fig. 3A). (B) 20 min after washout of KCl, 10 mmol l^{-1} caffeine induced a larger contraction (without depolarization). (C) A second response to 10 mmol l^{-1} caffeine was slow and small. This figure appeared as Fig. 3 (Hill, 1987). F, force; PD, membrane potential.

Other echinoderm muscles

Contractions in response to electrical stimulation, ACh and KCl are Ca^{2+}-dependent and Mn^{2+}-sensitive in the radial muscles of the soft-bodied sea urchin *Asthenosoma ijimai*, but caffeine-induced contractions, which only reach a fraction of the response to KCl or ACh, are not Ca^{2+}-sensitive (Tsuchiya and Amemiya, 1977). Thus, excitation–contraction coupling may be primarily dependent on entry of extracellular Ca^{2+}. Prosser and Mackie (Prosser and Mackie, 1980) studied contractions elicited by quick stretch in the five longitudinal

retractors (=LMBW) of *Stichopus parvipennis*, *Parastichopus californicus*, *Eupentacta pseudoquinquisemita*, *Cucumaria minata* and *Leptosynapta clarki* as well as the pharyngeal retractors of *C. minata*. Treatment with Ca^{2+}-free artificial sea water, Mn^{2+}, Co^{2+} or verapamil was used to assess the Ca^{2+}-dependence of contraction. These agents abolished both the neurally mediated responses to stretch stimulation and responses to direct electrical stimulation and spontaneous rhythmic contractions. Prosser and Mackie (Prosser and Mackie, 1980) suggested that these results indicate both the presence of Ca^{2+} action potentials and a role of Ca^{2+} in excitation–contraction coupling.

Experiments with a Ca^{2+} ionophore

LMBW of Isostichopus badionotus

The Ca^{2+} ionophore X-537A was used to test for the hypothesized caffeine-depleted storage sites (Hill, 1980). It was predicted that, once the ionophore had partitioned into the cell membrane, the submembrane storage sites should reload more readily. In fact, in the presence of X-537A, a few minutes of incubation in sea water was sufficient to restore contractility lost in a series of caffeine-induced contractures (Fig. 5A-E). Subsequently, after 60 min of treatment with X-537A, caffeine contractures remained reproducible 'permanently' (after the ionophore had been washed out of the bath) (Fig. 5F). These results suggested that molecules of X-537A had partitioned into the cell membrane, where they acted as Ca^{2+} carriers and enhanced reloading of the stores from which caffeine releases Ca^{2+}. Experiments conducted after ionophore treatment provided evidence that intracellular sites may be reloaded from extracellular Ca^{2+} (Hill, 1983a). After caffeine responses in sea water had been made reproducible by treatment with X-537A, caffeine responses in Ca^{2+}-free solution still diminished sequentially. However, contractility lost in a caffeine-induced series of contractures was now restored by brief treatment with 50 mmol l^{-1} KCl. Restoration of contractility was time-dependent, but was

Fig. 5. (A,B) A fresh longitudinal muscle of the body wall of *Isostichopus badionotus* responds to sequential exposures to 10 mmol l^{-1} caffeine with isotonic contractions of diminishing amplitude. (C) After a 30 min treatment with a Ca^{2+} ionophore (10^{-5} mol l^{-1} X-537A), 10 mmol l^{-1} caffeine induces a large contraction but, after washing out the ionophore, the next caffeine response is back in the sequence of diminishing amplitudes. (D) After a further 30 min treatment with the Ca^{2+} ionophore, there is again a large response to 10 mmol l^{-1} caffeine. (E) Repeated doses of 10 mmol l^{-1} caffeine, each time in the presence of ionophore, are reproducible. (F) After a 60 min treatment with ionophore, 10 mmol l^{-1} caffeine continues to elicit reproducible isotonic responses, even though the ionophore has been washed off. (The maximum contraction shown is to 29 % of resting length.)
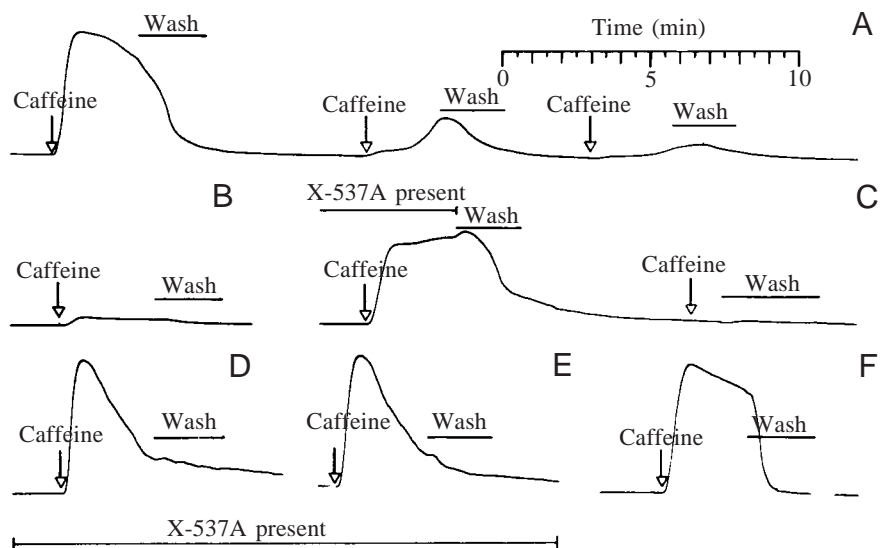
completed in 3 min. The extent of a similar restoration of contractility by ACh, after ionophore treatment, was time-dependent, and concentration-dependent between 10^{-8} and 10^{-6} mol l^{-1} ACh. ACh depolarization and ionophore treatment have strikingly different effects on the restoration of contractility. After the Ca^{2+} ionophore has partitioned into the membrane, the stores (subsarcolemmal vesicles?) recharge quickly in 9 mmol l^{-1} external [Ca^{2+}] and the isolated muscle responds reproducibly to repeated challenges with caffeine. However, one depolarization only recharges the muscle once, and repeated challenges with caffeine then result in a renewed rundown of contractility. What is the location of the slowly loading and unloading Ca^{2+} stores? In other words, is the Ca^{2+} in the secluded extracellular space handled differently from that in the abundant extracellular space outside the bundles?

Ca^{2+} antagonists

Studies with Ca^{2+} antagonists may aid in differentiating between Ca^{2+} stores used in reproducible contractions and Ca^{2+} stores used in rapidly extinguishing contractions (Hill, 1983b). In typical reproducible contractions, responses to tetanizing stimuli or to ACh can be evoked in an isolated muscle for hours, in a bath medium consisting of filtered sea water or artificial sea water with a Ca^{2+} content equivalent to that of sea water. Responses to applications of caffeine in the same bath medium decline rapidly, unless the isolated muscle has been pretreated with a Ca^{2+} ionophore (Hill, 1980; Hill, 1983a; Hill, 1983b). Agents that block membrane Ca^{2+} channels may cause reproducible responses to resemble rapidly extinguishing responses.

LMBW of Isostichopus badionotus

Mn^{2+}, La^{3+} and dantrolene were used as Ca^{2+} antagonists in a series of trials intended to differentiate the sources of Ca^{2+} used in reproducible and rapidly extinguishing contractions. Responses to tetanizing shocks at 5 s intervals were recorded with a free-loaded isotonic lever, as were responses to 10 mmol l^{-1} caffeine or 10^{-7} or 10^{-8} mol l^{-1} ACh. No anticholinesterases were employed. The untreated LMBW of *I. badionotus* rapidly loses responsiveness to caffeine, so isolated muscle strips were treated with X-537A to establish a steady state in which Ca^{2+} enters quickly to recharge the stores from which caffeine discharges Ca^{2+}. The order of effectiveness of Ca^{2+} antagonists in blocking caffeine contractures (in ionophore-treated muscle) was then $La^{3+}>Mn^{2+}>$dantrolene. The order of reversibility was $Mn^{2+}>$dantrolene$>La^{3+}$. The effects of Ca^{2+} antagonists indicate simple reduction of Ca^{2+} entry in the ionophore-treated preparation although, in preparations not treated with ionophore, Ca^{2+} antagonists do not all act alike. La^{3+} and Mn^{2+} block tetanic free-loaded contractions or contractions induced by 10^{-8} mol l^{-1} ACh, but dantrolene enhances both types of contraction. It could be hypothesized that dantrolene blocks Ca^{2+} loss from subsarcolemmal vesicles to the extracellular medium, without blocking the release of Ca^{2+} to the cytoplasm involved in excitation–contraction coupling (Hill, 1983b).

Procaine reduces the response by a fraction, which may correspond to the proportion of excitation–contraction coupling related to intracellular Ca^{2+} release (Suzuki, 1982).

Diltiazem and verapamil block auxotonic contractions in response to 10^{-6} mol l^{-1} ACh (Devlin, 1993). Diltiazem is more effective than verapamil which, however, also reduced responses to 10 mmol l^{-1} caffeine. The mechanism of action in echinoderm muscle of these mammalian Ca^{2+} blockers could repay further electrophysiological investigation.

LMBW of Stichopus japonicus

The LMBW is directly dependent on external Ca^{2+} in contractions induced by ACh or KCl, which are abolished after a few minutes in Ca^{2+}-free solution containing EGTA. ACh- and K^+-induced contractures are reduced but not abolished by 10 mmol l^{-1} Mn^{2+}. ACh-induced force is more sensitive to procaine, as is the response to caffeine. The isolated LMBW contracts slowly when deprived of Ca^{2+} and Mg^{2+} in the bathing medium (Sugi et al., 1982) as in *I. badionotus* (Suzuki, 1982).

LMBW of Sclerodactyla briareus

Chen (1986) has found that the LMBW of *Sclerodactyla briareus* reacts somewhat differently to Ca^{2+} antagonists. Mn^{2+} decreases responses to KCl or ACh, but has no effect on responses to caffeine. Procaine decreases responses to ACh and to caffeine, but has no effect on responses to KCl. Chen (Chen, 1986) drew a distinction between (i) Ca^{2+}-induced release of Ca^{2+}, (ii) Ca^{2+} release by membrane depolarization, (iii) direct Ca^{2+} influx and (iv) a combined mechanism. She concluded that depolarization by KCl evokes force by mechanism (iv), ACh evokes force by mechanisms (iii) and (i), while caffeine evokes force by mechanism (i). Devlin and Smith (Devlin and Smith, 1996) have observed that ACh induces a Ca^{2+} efflux from the LMBW of *S. briareus*, which is inhibited by Co^{2+}, La^{3+}, diltiazem and verapamil. They propose that this efflux stems from an early ACh-induced Ca^{2+} influx, which is too rapid to be resolved by their techniques.

Connective tissue

Ca^{2+} may be involved in two kinds of responses in echinoderm connective tissues: in changes of state or in contraction and relaxation. Clear cases of contraction and relaxation have been reported only infrequently. A prime example may be the downward power stroke of the swimming arms of comatulid crinoids (Wilkie, 1996). Changes of state may be irreversible or reversible. Contractions of the longitudinal and circular muscles of the body wall of holothurians drive extensive changes in configuration that are hard to understand in species with thick firm body walls, except by invoking change of state of the connective tissue of the body wall (Motokawa, 1982a). Along similar lines, the body wall and skeleton of a starfish can be distorted to allow the ingestion of extraordinarily large prey. Wilkie (Wilkie, 1984; Wilkie, 1996) has reviewed the extensive literature describing the

mutable connective tissue invoked to explain such phenomena. Variable tensility in echinoderm collagenous tissues has been of fundamental interest for some time (Wilkie, 1984). There are two cases: switching reversibly between stiffening and softening and irreversible disintegration (Wilkie, 1996). Reversible structures are said to have 'mutable collagenous tissue', and the switching is attributed to changes in the extracellular matrix.

Excitable connective tissue

Actual contraction must be a different phenomenon from changes in stiffness. In most cases, 'mutable connective tissue' (MCT) displays variable tensility, but for a long time instances have been reported in which MCT appears to contract like muscular tissue (Wilkie, 1996). Wilkie points out that connective tissue with variable tensility may take the place of muscle in such functions as 'detachment of body parts and fixation of posture'. There are reports of active contraction in holothurian dermis, but Motokawa (Motokawa, 1982b) determined, using electron microscopy, that there are no muscle fibers in the dermis of *Stichopus chloronotus* except in the walls of water vascular canals. Thus, any response to stimulation must be responses of connective tissue.

Birenheide and Motokawa (Birenheide and Motokawa, 1996) report that arm ligaments of crinoids lack muscle cells but contract actively on stimulation. This provides an aboral power stroke that is antagonized by muscles that bend the arms orally. Upon depolarization with 100 mmol l^{-1} KCl, the arm ligaments contract actively to less than 50 % of their resting length; this contraction is blocked by anesthetization with menthol. The active contraction is rapid, but the ligaments go into a state of 'catch' when the depolarizing solution is washed off. Donovan (Donovan, 1989) reviews reports of contraction in stems of extant crinoids, which lack muscle fibers. A thorough discussion and review of modern and extinct crinoids leads to the conclusion that bending of crinoid columns is always controlled by MCT. This may, then, be under Ca^{2+} control of extracellular matrix (reviewed below), but it is not yet clear whether excitable connective tissue is under Ca^{2+} control.

Muscle versus connective tissue

Some MCTs have been reported to contain a proportion of muscle cells. For instance, compass depressors of *Stylocidaris affinis* contain dispersed myocytes (Wilkie et al., 1998). Catch ligaments of the primary spines of *Eucidaris tribuloides* contain a small proportion of muscle cells, which insert directly on collagen columns (del Castillo et al., 1995). (The function of the latter muscle cells gives rise to a lively controversy, reported in other reviews in this issue.) However, there have been relatively few studies comparing the activation of echinoderm muscle to activation of echinoderm collagenous connective tissue.

Applied vibration reduces the force of contraction of isolated LMBW of *Stichopus japonicus* that has been activated with ACh or K$^+$ (Kobayashi et al., 1994). The reduction in mean active force may reflect an adverse effect on actin/myosin links. Force recovers at the end of the period of vibration, which would not be the case in a catch state. However, pretreatment with ACh or with 100 mmol l^{-1} K$^+$ solution does not affect vibration-induced stiffness changes in isolated dermis strips from *Stichopus japonicus* (Shibayama et al., 1994). Vibration induces passive force in the strips. Stretch force then provides a measure of stiffness. The outcome was the observation that vibration itself induced an increase in stiffness, followed by a transient increase in stiffness during a pause in vibration. Ca^{2+}-dependence was not tested in the muscle study, but in the dermis study (from the same laboratory) neither Ca^{2+}-free nor 100 mmol l^{-1} Ca^{2+} solutions affected vibration-induced stiffness changes, which appear to affect catch rather than activation.

Irreversible change of state

'Die Haut der meisten Holothurien und in Speciellen der Aspidochiroten ist in manche Hinsicht von der Haut der Wirbelthiere und andere Wirbellosen nicht nur anatomisch, sondern auch physiologisch verschieden.'

(Lindemann, 1900).

Isostichopus badionotus

Early in the twentieth century, the cloaca and anal sphincter of *I. badionotus* were used for a wide range of studies in basic general cellular physiology. Later, cloacal rhythmicity was the endpoint for studies of respiratory physiology (e.g. Crozier, 1920; Lutz, 1930) and the object of pharmacological studies intended to identify neurohumoral transmitter substances (for a review, see Hill, 1970). When the cloacal end of *Isostichopus badionotus* is amputated, a progressive local degeneration sets in immediately at the cut end. Crozier (Crozier, 1916) described this as a swelling and mucoid degeneration. The nature of the mucoid degeneration has been of interest as a model for massive tissue lysis, but it may well be related to irreversible softening in MCT. For instance, the puckering and division of the body wall during fission (Crozier, 1917) resembles local degeneration. Progressive degeneration (Crozier, 1916) may be an example of irreversible disintegration or an early step in wound healing (Cowden, 1968; Szulgit and Shadwick, 1998).

In a dramatic progressive degeneration, the major part of the initially firm body wall flows out in a sticky mass during, for instance, dissection of the cloaca and body wall muscles (Hill, 1966) (Fig. 6). In focal local degeneration, white softened spots appear patterned by finger pressure, if one swims along grasping a freshly collected specimen firmly (R. B. Hill, personal observation). Junqueira et al. (Junqueira et al., 1980) studied induced quick tissue degradation as a step in autotomy. After painting and washing off 10 % formalin or Bouin's fluid, applied to the posterior third of a specimen of *I. badionotus*, white spots began to appear all over the animal. In a few minutes, the spots coalesced and the whole body flowed away as a soft viscid mass. This effect has been called 'local degeneration' in a number of publications and it is blocked
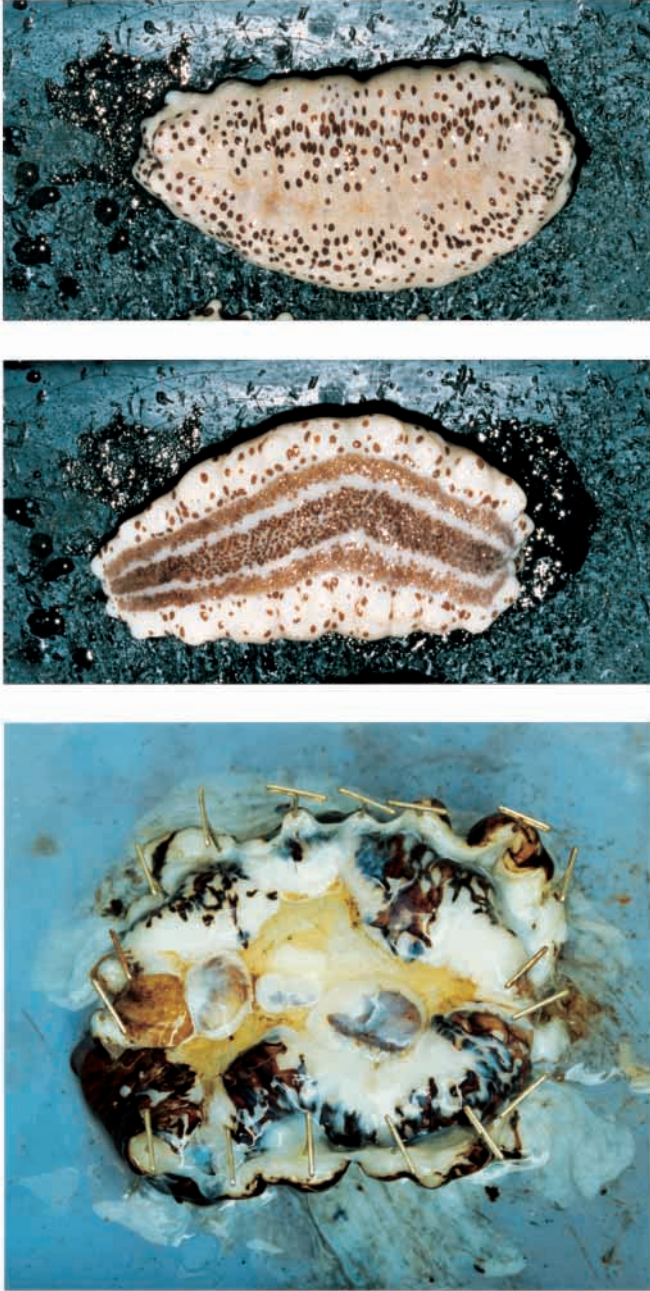
Fig. 6. After handling during dissection, progressive local degeneration of the body wall of *Isostichopus badionotus* proceeds to a point at which the form of the animal is hardly recognizable in the sticky frothy ooze produced. The form of these 'sea puddings' is highly labile, but the upper (dorsal) and middle (ventral) views are representative of a 'resting' state, as seen in animals handled gently, which have not been treated with a relaxing agent. Tube feet and tentacles are retracted in an animal approximately 20 cm long at rest. After 30 min of vigorous handling during dissection of the longitudinal muscle of the body wall and the cloaca, the remainder of the animal achieves a state beyond softening, as seen in the lower view.

when an extended dissection is carried out under sea water saturated with propylene phenoxetol, after the specimen has been relaxed with the same substance. Propylene phenoxetol

has anti-oxidant and local anesthetic properties and has long been used to relax molluscan tissues (Owen, 1955). It relaxes *Isostichopus badionotus* very effectively, but it was a surprise to find that it also blocks local degeneration (Hill, 1966). The propylene phenoxetol, long used as a relaxant, is 1-phenoxypropan-2-ol (obtained from NIPA Laboratories).

Junqueira et al. (Junqueira et al., 1980) found that the body wall of *I. badionotus* contains collagen fibers in a matrix rich in proteoglycans. They suggest that a protease of the body wall frees proteoglycans from interaction with collagen. (This could be a Ca^{2+}-activated collagenase.) A diffusible substance released from softened body wall induces softening in normal segments of body wall. This supports the concept of release of a triggering substance from secretory cells embedded in the living body wall.

Local degeneration was assessed in tests in which manual massage of blocks of living body wall, mimicking handling during dissection, produced local degeneration, which was assessed *versus* treatment with a range of antioxidants and local anesthetics. The four most effective antioxidants were ethyl gallate, propylene phenoxetol, butylated hydroxyanisole (BHA) and propyl gallate (Hill and Reinschmidt, 1976).

In further tests, with an Instron apparatus, the results supported the reported action of propylene phenoxetol. Compressive blows were applied to plugs of intact living body wall with epithelium, circular muscle layer and longitudinal muscle. Increased 'give', as compressions were repeated, was significantly diminished by treatment with propylene phenoxetol (T. Fort, personal communication).

Collagen breakdown products have been detected in LMBW of *I. badionotus* undergoing local degeneration (W. R. Ellington, unpublished observations). The amino acid composition of the ooze released by local degeneration suggests the presence of proteoglycans (F. Rahemtulla, personal communication). Hypothetically, local degeneration may involve a sequence of events beginning with pressure-activated cellular influx of Ca^{2+} and leading to release of proteoglycans by Ca^{2+}-activated collagenase as tissue integrity is lost. Uptake of water by the proteoglycans may produce the characteristic mucinous ooze. Antioxidants may retard local degeneration by blocking the release of active radicals and reactive oxygen species.

Reversible change of state

Mutable collagenous tissues change state rapidly and reversibly under neural control and serve a wide variety of functions in echinoderm life (for a review, see Wilkie, 1996). Here, the main concern will be to review the role of Ca^{2+} in coupling response to excitation in MCT. MCTs have been reported to show abrupt changes in stiffness or viscosity in response to electrical stimulation, K^+ or ACh (Wilkie, 1996). These changes have been interpreted as evidence for neural control. Structures containing MCT have been studied *in vitro* in responses to agents such as K^+ or ACh, employing bending tests, deformation stress/strain tests, forced vibration tests and toughness in response to punch force. Ca^{2+} may be involved

in the regulation of MCT at the level of the extracellular matrix, or as an extracellular or intracellular messenger acting on or in living cells.

Definitions of the Ca^{2+} regulation hypotheses

It may be useful here to define the 'Ca^{2+} regulation hypotheses', restated from Wilkie et al., 1999, who cite Trotter and Koob, 1995. Numerous publications related to the hypotheses are cited by Trotter and Koob, 1995, Wilkie, 1996 and Wilkie et al., 1999. The definitions as restated here are as follows.

(i) Neurosecretory-like cells modulate MCT tensility by adjusting the Ca^{2+} concentration of extracellular fluid. Usually, increased external [Ca^{2+}] ([Ca^{2+}]$_o$) stiffens MCT, while decreased [Ca^{2+}]$_o$ softens MCT by acting on Ca^{2+}-dependent crosslinks in the extracellular matrix. This is the 'extracellular Ca^{2+} regulation hypothesis' (Trotter and Koob, 1995).

(ii) Experiments in which [Ca^{2+}]$_o$ is manipulated affect secretion, by regulatory cells, of factors that affect the tensility of MCT. This is an admittedly simplified restatement of the 'cellular Ca^{2+} regulation hypothesis' (Trotter and Koob, 1995).

Does $[Ca^{2+}]_o$ control MCT?

Hidaka (Hidaka, 1983) found that the collagenous connective tissue of the catch apparatus of *Anthocidaris crassispina* showed a decrease in viscosity in medium free of Ca^{2+}. Recently, Trotter and Chino (Trotter and Chino, 1997) have shown that the viscosity of the deep dermis of *Acinopyga agassizi* can be reduced by Ca^{2+} chelation or by the action of Ca^{2+} antagonists. A series of related experiments intervened and, by the end of 1983, Wilkie (Wilkie, 1984) could write about support for 'the hypothesis that variable tensility involves the active control of extracellular pH or Ca-ion availability'. He reviewed publications providing evidence for a direct action of Ca^{2+} on the extracellular compartment, in the sense that certain echinoderm MCTs are sensitive to change in [Ca^{2+}]$_o$, even after freezing in distilled water or narcotization with propylene phenoxetol (PP). By 1995, investigators generally agreed that control of MCTs is effected through the control of cohesion between collagen fibrils, with less ambiguity in studies with cell-dead preparations (Wilkie, 1996). Possible mechanisms have been discussed and modeled by Wilkie (Wilkie, 1996) who points out that, theoretically, changes in the mechanical properties of MCT could be due to changes in the tensility of collagen fibrils or to biochemical changes in the extracellular matrix, which may involve interaction between matrix molecules or with collagen.

Eylers and Greenberg (Eylers and Greenberg, 1989) studied swelling in the body wall of *Thyonella gemmata* . [Ca^{2+}] proved to affect the elastic modulus of the tissue. This was of interest because of the background of reports of neural modulation of the stiffness of echinoderm connective tissue. Ca^{2+} in the bath generally had been reported to increase the stiffness of these MCTs while decreasing viscous flow, creep compliance or stress relaxation. The authors evidently considered stiffness to be a function of non-living extracellular

matrix since they complained about the analysis being complicated by the presence of living cells in previous studies. They therefore soaked body wall connective tissue of *Thyonella gemmata* and *Pentacta pygmaea* in distilled water before conducting swelling experiments. The outcome was that swelling increased maximally with time in pure NaCl solution, but there was no swelling in pure CaCl$_2$ solution.

Swelling is interpreted as Na$^+$ disrupting crosslinks in the network of the extracellular matrix. Shrinking in Ca^{2+} solution (Eylers and Greenberg, 1988) suggested activation of Ca^{2+}-dependent reversible crosslinks. From this study of the biochemical and physical properties of non-living connective tissue, Greenberg and Eylers (Greenberg and Eylers, 1989) concluded that they were 'looking at the effects of cations on the matrix itself, unmasked by the activities of cells'. One way to look at the results in the literature has been to suggest that controlled plasticization in living MCTs and plasticization in non-living MCT models may both be phenomena related to the action of Ca^{2+} on cohesion between collagen fibrils. Certainly, a contribution from the action of Ca^{2+} on muscle fibers, where present, must be limited to living MCTs. Presumably MCTs contain living components that control the extracellular matrix or the interaction between collagen fibrils. They may also contain innervated muscle fibers that participate in mechanical adjustment of the tissue to demands of the echindoderm life style.

Do neurosecretory cells control $[Ca^{2+}]_o$?

In 1967, Doyle reported that there had been very few reports of neurosecretory activity in echinoderms. Doyle (Doyle, 1967) found nerve cells packed with vesicles in nerve strands of the hemal rete of *Cucumaria frondosa*. This may be the first clear indication of neurosecretory cells in echinoderms. However, Wilkie (Wilkie, 1984) reviewed a variety of granulated cells that could be effector cells for variable tensility as well as reports of processes, permeating MCTs, containing granules which may be synthesized in 'neurosecretory-like perikarya'. Wilkie (Wilkie, 1996) reviewed the widespread occurrence of neuron-like juxtaligamental cells, some of which contain granules that accumulate Ca^{2+}. However, in investigations reviewed by Wilkie (Wilkie, 1996), the evidence was that changes in tensile state are accompanied by a redistribution of Ca^{2+} in the postulated effector cells, not that the effector cells release Ca^{2+} that act on MCT. The literature to that date was carefully and critically reviewed by Wilkie (Wilkie, 1996). It may not be thought that neurosecretory cells control plasticity directly by regulating [Ca^{2+}]$_o$.

Plasticization

There may be a way to reconcile the mechanisms of irreversible rapid total lysis (progressive local degeneration) and plasticization. Trotter and Koob (Trotter and Koob, 1995) show that there are cells in holothurian dermis releasing an organic stiffening factor that turns on a Ca^{2+}-dependent decrease in plasticity. Other experimental results (Junqueira et

al., 1980) indicate that a diffusible substance can trigger softening, which may proceed to total lysis. Are there both Ca^{2+}-released stiffening factors and lysis-initiating factors?

The evidence for the 'cellular Ca^{2+} regulation hypothesis' has been drawn from MCTs that are complex, in that they display both stiffening and plasticization in normal physiological activities (Wilkie et al., 1999). In contrast, Wilkie et al. (Wilkie et al., 1999) consider a case of irreversible plasticization that may be categorized as an example of simple MCT. The authors applied cell lysing agents to crinoid ligaments, which contain MCT that normally functions by disintegrating, allowing autotomy. The ligaments to be disrupted were anesthetized in 0.1 % PP, and the test solutions contained the same concentration. Anesthetized ligaments treated with agents that disrupt cell membranes became destabilized. That is, the joints fractured as in autotomy. In high $[Ca^{2+}]$ solutions containing PP, the same agents still destabilized, but exposure to $100 \, mmol \, l^{-1}$ Ca^{2+}, without lysing agents, did not destabilize. This may support the 'cellular' hypothesis, which states that effector cells store plasticizer that is released by cell-lysing agents when cell membranes are disrupted. This plasticizer could not be a Ca^{2+}-sequestering agent, since cell lysis still destabilizes in the presence of $100 \, mmol \, l^{-1} \, Ca^{2+}$. Wilkie et al. (Wilkie et al., 1999) conclude that, in the case of a simple softening MCT, the 'cellular Ca^{2+} regulation hypothesis' (cited from Trotter and Koob, 1995) is confirmed in that cells (possibly the granule-containing juxtaligamental cells) lyse to release a diffusible substance disrupting cohesive forces in connective tissue. One may ask whether irreversible softening of this MCT resembles progressive local degeneration. However, in irreversible change-of-state cell lysis, a disintegrating agent (or family of agents) is apparently released, whose action is blocked by antioxidants or local anesthetics (evidence reviewed above).

How does $[Ca^{2+}]_o$ affect holothurian dermis?

A review of previous results led Trotter and Chino (Trotter and Chino, 1997) to investigate the possibility that stiffening by cell lysis may indicate a cell signaling pathway leading to secretion of soluble stiffening and plasticizing factors. Repeated freezing and thawing of specimens caused lysis of resident granulated cells and an increase in stiffness of the specimens, which was independent of the presence or absence of Ca^{2+}. They made tissue extracts that should contain material from the granulated secretory cells seen in electron micrographs of dermis. These extracts stiffened specimens even in the absence of $[Ca^{2+}]_o$, suggesting that stiffening of living MCT may result from the release of a substance from the resident granulated cells.

Koob et al. (Koob et al., 1999) concluded that Ca^{2+} control of holothurian dermis is secondary to an action of Ca^{2+} on resident secretory cells, rather than a direct effect on connective tissue. They isolated an inner-dermis stiffening factor and an outer-dermis plasticizing factor. Two questions arise: (i) how does Ca^{2+} control release of the factors, and (ii) how do the factors control viscosity of MCT?

Koob et al. (Koob et al., 1999) review previous studies indicating that $[Ca^{2+}]_o$ affects the extracellular matrix of MCT, so that tissue is stiff in normal $[Ca^{2+}]_o$ and compliant in reduced $[Ca^{2+}]_o$. This view gave rise to the hypothesis that resident secretory cells regulated MCT by governing $[Ca^{2+}]_o$. However, in *Cucumaria frondosa* (Koob et al., 1999), as in *Anthocidaris crassispina* (Trotter and Chino, 1997), the stiffening response to Ca^{2+} is thought to be due to a Ca^{2+} stimulation of the secretion of a stiffening agent synthesized in granular cells. Living inner dermis specimens were plastic in chelated artificial sea water. Extracts of inner dermis specimens that had been subjected to freeze–thaw cycles stiffened live specimens in chelated sea water. These tissues were then as stiff as controls in artificial sea water. Living inner dermis specimens were stiff in normal artificial sea water. Extracts of outer dermis specimens that had been subjected to freeze–thaw cycles plasticized live specimens in normal artificial sea water. This could be an action on extracellular matrix or chemical properties of collagen, since killed specimens are also plasticized.

Granular cells in both the outer and inner dermis could be a source, directly or indirectly, of the release of stiffening and plasticizing factors when the tissues are subjected to freeze–thaw cycles. This is compatible with the cellular Ca^{2+} regulating hypothesis, defined above, implicating protein factors rather than Ca^{2+} as effectors for the modulation of connective tissue state (see references cited by Koob et al., 1999).

Szulgit and Shadwick (Szulgit and Shadwick, 2000) have addressed the necessity of distinguishing experimentally between the $[Ca^{2+}]_o$ model of direct Ca^{2+} control of MCT and the secreted substance model, in which Ca^{2+} would act on a neurosecretory or granulated cell, providing indirect control of MCT. These models, with references to the literature, have been reviewed (Szulgit and Shadwick, 2000). The models might work through one of two hypothetical mechanisms for viscoelastic stiffening of holothurian dermis: increased viscosity of the interfibrillar solution, or increased linking of the fibrils. The first mechanism (increased viscosity) has been proposed by a number of authors (for a review, see Szulgit and Shadwick, 2000). In the second mechanism (proposed by Szulgit and Shadwick, 2000), links between collagen fibrils would produce elastic elements spanning the tissue.

Szulgit and Shadwick tested their proposed mechanism using dynamic oscillatory shear tests (Szulgit and Shadwick, 2000). Oscillatory longitudinal displacement was applied to standard tissue specimens from hardened unanesthetized individuals of *Cucumaria frondosa*. These included living (non-frozen) specimens and 'freeze–thaw' specimens subjected to 'cell lysis' in a freeze–thaw cycle. Stiffness was determined by recording dynamic shear stress and displacement values. Inner dermis extracts (IDE) and outer dermis extracts (ODE) were prepared from the remaining tissue of each individual. Minced lysed freeze–thawed tissue was dissolved in artificial sea water (normal $[Ca^{2+}]$) or Ca^{2+}-chelated artificial sea water.

Living specimens of dermis of *C. frondosa* incubated in

artificial sea water retained their initial stiffness, while specimens incubated in Ca^{2+}-chelated artificial sea water became compliant. This is much as had been reported by a number of earlier authors (cited by Szulgit and Shadwick, 2000). However, the high initial stiffness is attributed to 'mechanical stimulation prior to cutting and as a result of the dissection itself'. This is then diametrically opposed to progressive local degeneration (reviewed above), in which mechanical stimulation leads to progressive massive softening. After a further incubation of 24 h, non-frozen tissues in artificial sea water lost stiffness, but such tissues in Ca^{2+}-chelated artificial sea water remained compliant. Both groups of non-frozen tissues became or remained relatively stiff when exposed to IDE but became compliant in ODE. Thus, neither IDE stiffener nor ODE softener showed Ca^{2+}-dependence.

Szulgit and Shadwick (Szulgit and Shadwick, 2000) conducted tests with freeze–thawed tissues to distinguish between the model of direct action of extracts on extracellular matrix and the model of indirect action of extract, on living secretory cells in the MCT. Freeze–thawing stiffened tissues, such as those in Ca^{2+}-chelated artificial sea water, which would otherwise have remained compliant. IDE in artificial sea water further stiffened freeze–thawed tissues, while IDE in Ca^{2+}-chelated artificial sea water left stiffness as great as in controls. This indicated that the stiffening agent is not free Ca^{2+}. ODE increased the compliance of freeze–thawed tissues, whether in artificial sea water or in Ca^{2+}-chelated artificial sea water solution, thus being effective either in the presence or in the absence of Ca^{2+}. These results are seen to support the hypothesis that *in vivo* stiffening results from the release into the extracellular matrix of factors that do not act through regulating $[Ca^{2+}]_o$. Viscoelastic models for holothurian dermis were discussed (Szulgit and Shadwick, 2000) and that tissue stiffening may result from the formation of links between collagen fibrils was proposed.

Thurmond and Trotter (Thurmond and Trotter, 1996) describe the morphology of the dermis of *C. frondosa*, in which the major components are collagen fibrils surrounded by a microfibrillar network. Other components include proteoglycans, glycoproteins, nerve fibers, cells that appear to be neurosecretory and water vascular channels (Motokawa, 1982b). Change in state of sea cucumber dermis is thus not necessarily to be modeled on Ca^{2+}-dependent change of state in sea cucumber muscle cells. Recent studies reviewed here do not support the hypothesis of Ca^{2+} regulation of extracellular matrix. Possibly Ca^{2+} regulation could be involved in progressive local degeneration. Yet to be explored are the differences between species in which mechanical stimulation induces stiffening and species in which mechanical pressure induces the onset of progressive local degeneration.

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