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NOTE

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Introduction

Investigations of various aspects of calcification in corals have frequently made use of artificial seawaters from which calcium has been omitted or has been present in low concentrations compared to standard seawater (Chalker 1976; Krishnaveni et al. 1989; Ip and Krishnaveni 1991; Al-Moghrabi et al. 1996; Tambutte et al. 1996; Gattuso et al. 2000). The possibility of undesirable effects resulting from exposure to such media does not appear to have been considered. There appears to have been no attempt to investigate the non-physiological effects of calcium depletion in seawater even though it has been long known that calcium has profound effects on membrane permeabilities and numerous physiological processes (Hoar 1966; Manery 1966; Giese 1979).

The purpose of the present investigation was to ascertain whether calcium-free seawater, or seawater containing low calcium concentrations, had any obvious effects on the polyps of *Galaxea fascicularis*. A number of artificial seawater formulations have been used by different investigators. We report the effects of three low calcium or calcium-free formulations, used by previous investigators, and make comparisons with the corresponding standard artificial seawaters and natural standard seawater.

Materials and methods

Colonies of the scleractinian zooxanthellate coral *Galaxea fascicularis* were collected from the reef flat of Heron Reef, GBR. They were transferred in plastic buckets filled with seawater to outdoor aquaria at Heron Island Research Station and provided with a continuous flow of fresh seawater. The colonies were allowed to recover for a minimum of 3 d. The well-spaced polyps are joined by a cellular coenosteum that is easily broken. The polyps were readily separated, without damaging the tissues of the polyps, by teasing them apart with forceps. The individual, intact, polyps were kept in trays with constantly flowing seawater for a further 2 d before being used in experiments.

Calcium-free artificial seawater and standard artificial seawater was prepared according to Krishnaveni et al. (1989). Standard artificial seawater and low-Ca artificial seawater (2.8 mmol l^{-1}) was prepared according to Gattuso et al. (2000). The Ca concentration used in the low-Ca artificial seawater was that used in the experiments of Gattuso et al. (2000). Standard artificial seawater and artificial seawater containing a range of Ca concentrations (0–6 mmol l^{-1}) was prepared according to Benazet-Tambutte et al. (1996). The pH of all media fell well within the range of pH 8.0–8.2. Standard artificial seawater was also prepared according to Krishnaveni et al. (1989), but Ca was replaced by Sr.

Small polyps were incubated in the various seawater formulations for 15–30 min. Following incubation, the polyps were observed and photographed with a dissecting microscope while immersed in the incubation medium. Care was taken not to disturb the polyps or agitate the incubation media prior to, and during, microscopic observation. All experiments were replicated five times. The incubation temperature was 24°C. Polyps similarly treated were rapidly frozen in liquid propane cooled with liquid nitrogen to −190°C. Prior to freezing the external mucus layer was removed by rinsing in the incubation medium. This was done to facilitate rapid freezing. The frozen polyps were stored in liquid nitrogen until freeze-substituted and embedded in araldite according to a previously described protocol (Marshall 1980; Marshall and Wright 1991). Sections of embedded polyp tissue were prepared for both light microscopy and x-ray microanalysis (Marshall and

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Wright 1991; Marshall and Wright 1995). X-ray microanalysis was carried out on a JEOL 1200EX STEM fitted with an Oxford Instruments x-ray detector interfaced to a Macintosh Quadra700 computer via a 4Pi Scanning Interface. Quantitative elemental images were obtained using custom designed software (LeFurgey et al. 1992; Marshall and Clode 1998).

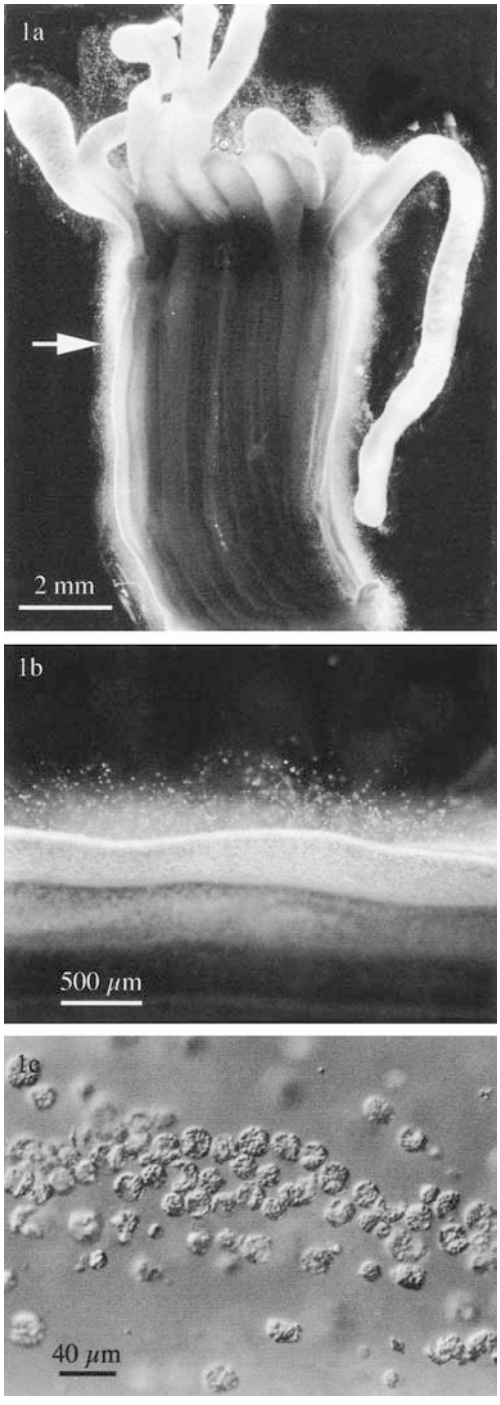


Fig. 1 (**a**) *Galaxea fascicularis* polyp incubated in Ca-free artificial seawater for 15 min. A layer of extruded mucocytes and mucus surrounds the polyp (*arrow*) and this material is also being extruded from the mouth. (**b**) Higher magnification showing strands of extruded mucocytes on the surface of the oral ectoderm. (**c**) Extruded mucocytes photographed with Nomarski interference contrast

Results and discussion

Exposure of *Galaxea* polyps to either Ca–free or to low-Ca (2.8 mmol l^{-1}) seawater resulted in a rapid extrusion of mucocytes (Fig.1 a,b,c). This occurred within 10 min of the start of incubation. Within 15 min, the extended tentacles of the polyps no longer responded to tactile stimuli. The polyps were anaesthetized and extruded enormous numbers of mucocytes. The mucus granules were slowly released from the cells to form a viscoelastic gel of considerable proportions around the polyp. After 2 h of exposure, polyps returned to standard seawater appeared to be fully recovered after a period of 12–24 h, despite the loss of vast numbers of mucocytes. Polyps incubated in the corresponding standard artificial seawaters showed no evidence of mucocyte extrusion and responded rapidly to tactile stimuli. They appeared to be normal in all respects. Polyps incubated in artificial seawater with a range of Ca concentrations for 20 min, showed a response that varied with Ca concentration (Table 1). No effect was evident at Ca concentrations higher than 5 mmol l^{-1}, although the polyps responded only slowly to tactile stimuli at 6 mmol l^{-1}. No effects were observed in polyps incubated in the corresponding standard artificial seawater. Similarly, no effects were observed in polyps incubated in natural standard seawater. The substitution of Sr for Ca did not prevent the massive extrusion of mucocytes seen in Ca-free seawater.

Examination of sections of tissue from freeze-substituted polyps confirmed that entire mucocytes appeared to be extruded when polyps were exposed to Ca-free or low-Ca seawater. Mucocytes were abundant and occupied a large volume of the oral and aboral epithelia in polyps exposed to standard artificial seawater or natural standard seawater (Fig. 2a) but were sparse in the epithelia of polyps exposed to Ca-free or low-Ca artificial seawater (Fig. 2b). In the latter polyps, it can also be seen that the oral ectoderm contains spaces that were previously occupied by mucocytes (Fig. 2b) and that the coelenteron is filled with organic material (Fig. 2b) that is absent in the

Table 1 Effect of low Ca artificial seawater (Benazet-Tambutte et al. 1996) on the release of mucocytes from *Galaxea fascicularis*. The number of +'s indicates the degree of the response as judged visually, where five +'s represents the maximum response. *NE* no visible effect

Ca concentration (mmol l^{-1})	Release of mucocytes
0	+ + + + +
1	+ + + + +
2	+ + + +
3	+ + +
4	+ +
5	+
6	NE

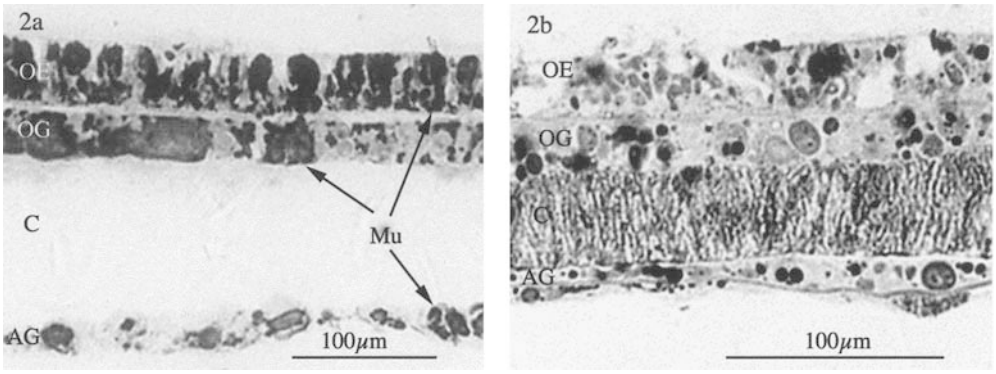
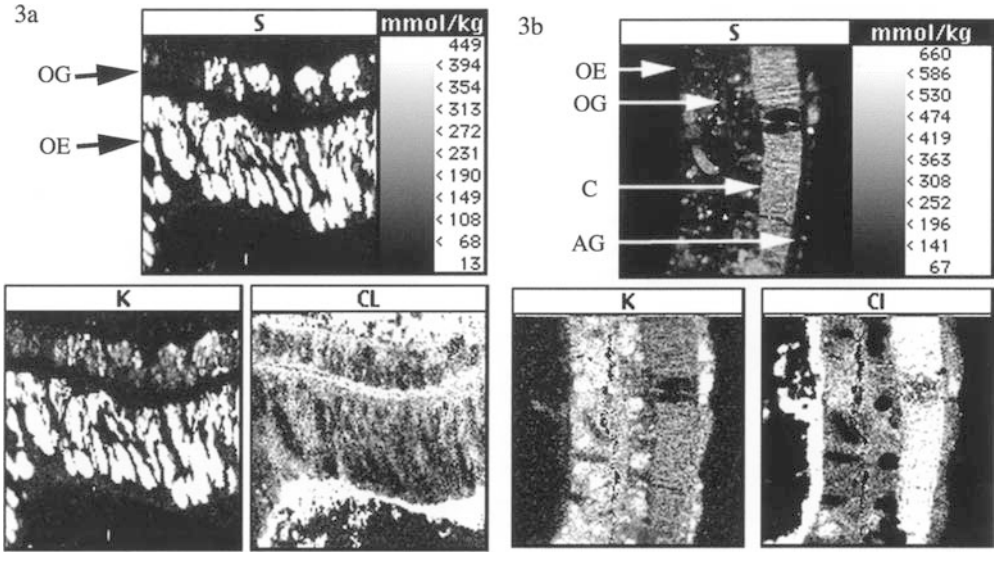


Fig. 2 (**a**) Section of the extrathecal epithelia of a freeze-substituted polyp of *Galaxea fascicularis* incubated in standard seawater (*stained with toluidine blue*). Numerous dark stained mucocytes (*Mu*) are visible in the oral ectoderm (*OE*), oral gastrodermis (*OG*), and aboral gastrodermis (*AG*). The coelenteron contains only faint ice crystal segregation zones from the frozen seawater. (**b**) Section of the extrathecal epithelia of a freeze-substituted polyp of *Galaxea fascicularis* incubated in Ca-free artificial seawater for 30 min (*stained with toluidine blue*). Only the dark stained remnants of mucocytes remain in all the cell layers and the coelenteron (*C*) is filled with stained organic material believed to be extruded mucus coelenteron of polyps exposed to standard artificial seawater or standard seawater (Fig. 2a).

The dissociation of cells in Ca-free and low-Ca seawater is consistent with the observations of Hobmayer et al. (2001) on cell aggregation in *Hydra*. These authors have shown that homotypic cell aggregation (i.e., the association of ectodermal or endodermal cells with other ectodermal or endodermal cells) after mechanical dissociation of *Hydra* will not proceed in the absence of Ca and is greatly reduced in low-Ca water (Hobmayer et al. 2001). It is well established that depletion of Ca ions has profound effects on cell junctions and cell adhesion (Manery 1966).

The elemental composition of *Galaxea* mucus has been previously described (Marshall and Wright 1995). The mucus granules within mucocytes are characterized by a very high S content and substantial concentrations of K, Sr, and Ca (Fig. 3a). In polyps exposed to Ca-free or low-Ca seawater, it can be seen in elemental images that the coelenteron contains very high concentrations of S and that the concentration of K is elevated above normal seawater levels (Fig. 3b). A mucus layer is not apparent on the external surface of the oral ectoderm because it was necessary to remove the thick mucus layer enveloping the polyp by washing with the incubation medium prior to freezing the polyp. Failure to remove

Fig. 3 (**a**) Quantitative element distribution maps from a dry cut, unstained section of the oral ectoderm (*OE*) and oral gastrodermis (*OG*) of a freeze-substituted *Galaxea fascicularis* polyp incubated in standard seawater—same polyp as in Fig. 2a. The mucocytes are characterized by high concentrations of sulfur (*S*) and potassium (*K*). The presence of seawater on the external surface of the oral ectoderm and in the coelenteron is indicated in the chlorine (*Cl*) image. (**b**) Quantitative elemental distribution maps from a dry cut, unstained section of a freeze-substituted polyp of *Galaxea fascicularis* incubated in Ca-free artificial seawater for 30 min; same polyp as in Fig. 2b. The oral ectoderm (*OE*) and oral gastrodermis (*OG*) show little indication of sulfur-containing mucocytes in the sulfur image (*S*), but a high concentration of sulfur is present in the coelenteron (*C*) as also is potassium (*K*). The distribution of seawater on the external surface of the oral ectoderm and in the coelenteron is indicated in the chlorine (*Cl*) image



the mucus layer would have resulted in a low freezing rate and poor cryofixation.

In *Galaxea*, mucus granules are composed of S-rich polyanionic compounds with which are associated extremely high concentrations of K, Sr, and Ca. The consequences of the mucocyte release, induced by seawater with low calcium concentration, must include physical changes to the epithelia due to the shedding of cells, the formation of extensive Donnan matrices surrounding the polyp externally and internally within the coelenteron, and the release from the mucus of K, Ca, and Sr ions. This inevitably must affect the transport of Ca^{2+} across the epithelia and compromise any experiments in which calcium transport is a factor. We do not know at present whether this is a general effect seen in all scleractinian corals. It would be prudent, however, to exercise caution particularly since the effect may not be so visually evident in coral colonies with very small polyps. That the phenomenon is attributable to a lack of Ca ions is demonstrated by the apparently healthy state of polyps after incubation in standard artificial seawater, i.e., artificial seawater containing the same calcium concentration as standard seawater, and the similarity of calcium incorporation (measured as Ca^{45} uptake) in polyps incubated in standard seawater and in standard artificial seawater (Marshall and Clode 2002).

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