Aragonite crystallization in primary cell cultures of multicellular isolates from a hard coral, *Pocillopora damicornis*

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Communicated by Steven M. Stanley, Johns Hopkins University, Baltimore, MD, August 20, 2001 (received for review May 10, 2001)

The foundation of marine coral reef ecosystems is calcium carbonate accumulated primarily by the action of hard corals (Coelenterata: Anthozoa: Scleractinia). Colonial hard coral polyps cover the surface of the reef and deposit calcium carbonate as the aragonite polymorph, stabilized into a continuous calcareous skeleton. Scleractinian coral skeleton composition and architecture are well documented; however, the cellular mechanisms of calcification are poorly understood. There is little information on the nature of the coral cell types involved or their cooperation in biocalcification. We report aragonite crystallization in primary cell cultures of a hard coral, *Pocillopora damicornis*. Cells of apical coral colony fragments were isolated by spontaneous *in vitro* dissociation. Single dissociated cell types were separated by density in a discontinuous Percoll gradient. Primary cell cultures displayed a transient increase in alkaline phosphatase (ALP) activity, to the level observed in intact corals. In adherent multicellular isolate cultures, enzyme activation was followed by precipitation of aragonite. Modification of the ionic formulation of the medium prolonged maintenance of isolates, delayed ALP activation, and delayed aragonite precipitation. These results demonstrate that *in vitro* crystallization of aragonite in coral cell cultures is possible, and provides an innovative approach to investigate reef-building coral calcification at the cellular level.

Hard corals are among the major calcium carbonate producers in marine coral reef ecosystems. Calcium carbonate is deposited as the aragonite polymorph, and is stabilized into a continuous calcareous skeleton by colonies of hexacoral polyps covering the surface of the reef (1). Studying hard coral calcification provides a better understanding of the biology of coral skeletogenesis, helps explain reef growth patterns, and may elucidate coral reef responses to environmental perturbations (2, 3).

The supportive role of reef-building hard coral skeletons is reflected in their stiff, biomineralized matrix composition. Skeleton composition and microarchitecture have been analyzed biochemically and ultrastructurally. The skeleton of *Pocillopora damicornis* is composed of the mineral aragonite (99.9% by weight), present as spheritic arrangements of submicroscopic crystals in an organic interstitial matrix (4, 5). This matrix has been shown to be composed of a network of chitin fibrils (5), embedded with proteins and Ca^{2+}-binding phospholipids (6).

As with any calcified tissue, skeleton formation in hard corals is a sequential process. Skeletogenic cells secrete and mature organic matrix components. This matrix concentrates Ca^{2+} ions and serves as a seeding site for calcium carbonate crystal nucleation in the aragonite polymorph (7, 8). Aragonite crystals grow and radiate into spheritic nodules, which are accreted into a continuous external skeleton (9). The actual cellular mechanisms of hard coral calcification, however, are still poorly understood, despite their fundamental interest in fields as diverse as biology, ecology, geology, and materials engineering.

As demonstrated by the progress achieved by using *in vitro* models of mammalian ossification, long-term primary cell cultures provide innovative tools to investigate skeleton formation at the cellular level (10, 11). In these models, the nature of the cells involved and the mechanisms of their cooperation in the regulation of calcification can be explored. Similar insights may be obtained from hard coral cell cultures.

Biomineralization of calcite spicules has been documented in cell cultures derived from a soft gorgonian coral (12). However, this coral differs from the majority of reef-building hard corals, including *P. damicornis,* in that gorgonian cells produce a fragmented skeleton. Coral soft tissues are composed of two superposed, ectodermal and gastroendodermal cell layers, separated by a gel-like matrix, the mesoglea. The gastroendodermal cells of hermatypic hard corals host symbiotic zooxanthellae (unicellular algae evolved from free-living dinoflagellates of the gymnodinioid form, *Symbiodinium* sp.). *In vivo* histochemical and ultrastructural observations of calcifying apical branches of hard coral (13, 14), and on radiochemical studies of $^{45}Ca^{2+}$ incorporation into the skeleton (15), have shown that calcification takes place in the calicoblastic epidermis. Autotrophic zooxanthellae symbionts provide part of the coral nutrients and their activity facilitates calcification (16).

Compared with cell cultures from mammals, fish, and insects, the development of cell cultures from marine invertebrates has lagged. Specific *in vitro* growth and attachment factors are not available, and it has proven difficult to maintain axenic cultures from marine invertebrate species that are naturally associated with diverse microbial communities. Marine invertebrate cells are maintained in primary cultures with limited or no growth, surviving for periods ranging from a few hours to several months, and no immortal marine invertebrate cell lines have been established (17). Cultured coral cells are characterized based on their *in vitro* morphology and behavior, which are highly plastic, and can differ considerably from *in vivo* phenotypes (18). Functional cell viability is limited: single cells usually die within a day, survival is prolonged 4–7 days when cells are allowed to attach to a substrate, and 10–14 days when cells are maintained as tissue fragments (19). We have had previous success in establishing multicellular isolate cultures from the hard corals *Acropora microphthalma* and *P. damicornis*, in which the ciliary activity of gastroendodermal cells remained functional for 5 days (20). In this study, we extend the use of such primary cell cultures to obtain aragonite precipitates of calcium carbonate.

Materials and Methods

Small colonies of *P. damicornis* (Coelenterata: Anthozoa: Scleractinia), indigenous to the Indo-Pacific, were obtained from the Pittsburgh Zoo and maintained in artificial seawater (Reef

Abbreviations: ALP, alkaline phosphatase; TEM, transmission electron microscopy.

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Crystals, Aquarium Systems, Mentor, OH) at 35–36‰ salinity, pH 8.0–8.3, 23–25°C, in a 208-liter aquarium equipped with a biological filtration system, illuminated by using a full-spectrum halide bulb (400W, Spartan Laboratory Animals, Haslett, MI) on a 15 h/9 h dark/light photoperiod.

All reagents were purchased from Sigma unless otherwise noted. Calcium-free seawater (CaFSW) was prepared according to Frank *et al.* (18) (23 g NaCl/0.763 g KCl/1.89 g $MgSO_4 \cdot 7H_2O$/10.45 g $MgCl_2 \cdot 6H_2O$/3.0 g Na_2SO_4/0.25 g $NaHCO_3$ and 0.026 g $SrCl_2$ per liter of deionized H_2O). Artificial seawater A (ASWA) was prepared by adding 1.53 g $CaCl_2$ per liter of CaFSW. Artificial seawater B (ASWB) was prepared with 1 g $CaCl_2$/52.2 g NaCl/0.763 g KCl/1.89 g $MgSO_4 \cdot 7H_2O$/20.615 g $MgCl_2 \cdot 6H_2O$/3.0 g Na_2SO_4/0.25 g $NaHCO_3$ and 0.026 g $SrCl_2$ per liter of deionized H_2O. Powdered DMEM (GIBCO/BRL) was reconstituted in 1 liter of deionized H_2O, supplemented with 0.52 g KCl/1.64 g $CaCl_2$/6.07 g $MgSO_4 \cdot 7H_2O$/21.2 g Hepes/0.04 g taurine/3.7 g $NaHCO_3$, and the pH was adjusted to 8.25 with 1 M NaOH (supplemented DMEM).

Culture medium was prepared according to Kopecky and Ostrander (20) with (vol/vol) 50% supplemented DMEM, 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA), and 1% antibiotics–antimycotics (GIBCO/BRL), in ASWA or ASWB. Two ionic media formulations were tested, based on the type of artificial seawater used for dilution, ASWA for medium A and ASWB for medium B. Medium A was 800 millosmolar per liter (salinity 30‰), Mg/Ca = 2.9 and Na/K = 25, after Kopecky and Ostrander's optimization for *Acropora microphthalma* coral cell culture (20). Medium B was 1150 millosmolar per liter (salinity 36‰), Mg/Ca = 5 and Na/K = 44. Medium B more closely matched present-day surface seawater chemistry.

Culture dishes were 6- and 24-well flat-bottom Falcon Primaria tissue-culture plates (PGC Scientifics, Gaithersburg, MD). Primaria is a permanent, stable modification of the polystyrene surface, incorporating anionic and cationic functional groups, developed to grow primary cells that attach poorly to conventional negatively charged surfaces.

To initiate cell cultures, we modified our previously described protocol (20). Fast-growing apical fragments (0.5–0.7 cm long) were excised from parent coral colonies with side-cutting pliers, placed in a Petri dish in CaFSW with 3% (vol/vol) antibiotics–antimycotics, and incubated for 2–4 h in a shaking water bath (24°C, 30 rpm). Each fragment was then plated into explant primary culture in individual wells of 6-well plates, in 5 ml of medium. Cultures were incubated at 24°C in 5% CO_2/95% air, under 24-h illumination, and checked daily for spontaneous dissociation of coral soft tissues from the skeleton. Confirming our previous observations (20), this protocol yielded a mix of single cells and incompletely dissociated tissue fragments, called isolates, over the first 2–4 days. Isolates were active in that they displayed intense ciliary activity, detected by either their spinning or streaming media, depending on the isolate's size and consequent drag.

Once soft coral tissues had dissociated from the skeleton, 2–4 days after initiation, the skeleton and skeletal debris were removed, and cells and isolates were transferred into primary cultures in individual wells of 24-well plates filled with 2 ml of fresh medium. Isolate cultures were plated by gravity sampling, and transfer of only intact active isolates (6 to 8 isolates per well) took place. Individual cell cultures were plated by mechanically dispersing dissociated cells and isolates into a single cell suspension in CaFSW. This suspension was centrifuged at $230 \times g$ onto a cushion of 60% Percoll in CaFSW (density = 1.09 g/ml) to remove original skeletal debris. Cells at the interface were recovered, washed once in CaFSW, and plated in fresh medium at 1.5 to 8×10^6 cells per well.

Primary cultures were fed once every 2 weeks by adding 1 ml of freshly prepared medium. Morphology was observed daily with a Nikon (type 104) phase-contrast/bright-field inverted microscope equipped with a camera. Cell types were classified according to Frank *et al.* (18), based on *in vitro* morphology. Isolate size was measured over time with a calibrated micrometer at 100×, by averaging the diameter (mm) of 15–20 spherical isolates (nonspherical isolates had their surface estimated and adjusted to a sphere whose diameter was calculated). Differences in isolate size between treatments were tested by using a Student's *t* test. To estimate cell density, single cell suspensions were prepared (tissue or isolates mechanically dispersed), and cell numbers were counted with a Levy–Neubauer hemocytometer (two or three countings averaged per suspension).

Endogenous alkaline phosphatase (ALP) activity was measured in the primary cultures by using a Sigma colorimetric assay, according to the method of Sud *et al.* (21). As a control, intact tissue was assayed and compared to both individual cell cultures and isolate cultures, at plating and over time. Three replicate cultures were assayed for each time point. Cells were suspended in CaFSW, cell densities were estimated, and suspensions were sonicated for 5 min at 4°C. Cell extracts were kept on ice until ALP determination. Each cell extract was assayed in triplicate: 50 μl of *p*-nitrophenyl phosphate substrate (Sigma 104, 40 mg in 10 ml of deionized H_2O) was diluted with 50 μl of alkaline buffer solution (Sigma 221) and mixed with 10 μl of cell extract. The reaction was incubated at 37°C for 30 min and stopped by adding 1 ml of 0.05 M NaOH. The yellow *p*-nitrophenol color liberated by ALP was quantitated colorimetrically by reading a first absorbance (A_1) at 405 nm. The reaction mix was decolorized by adding 200 μl of 1 M HCl and any residual color was read (A_2) at 405 nm, and subtracted to obtain corrected ALP activity ($A_2 - A_1$). ALP Sigma units corresponding to this activity were determined from a calibration curve of *p*-nitrophenol standard solution (Sigma 104–4) diluted with 0.02 M NaOH (slope was 0.080). Triplicate measures were taken for each cell extract and averaged to give the ALP activity of the cell extract. Intra-assay variability was 5–10% as calculated by the ratio of standard deviation to the mean of triplicate measures. Our preliminary experiments established that ALP activity was proportional to coral cell density. Thus, for comparison purposes between treatments, ALP activities were adjusted to densities of 5×10^6 cells per ml and each treatment was repeated three times. Interculture variability was 15–30%, as calculated by the ratio of standard deviation to mean ALP activity of three replicate culture extracts.

Single dissociated cells were transferred from a 3-day explant culture and separated according to their densities over a discontinuous Percoll gradient, adapting a protocol developed for marine sponge cells (22). Percoll dilutions [60%, 45%, 30%, and 15% (vol/vol)] were prepared in CaFSW (respective densities were 1.09, 1.07, 1.055, and 1.04 g/ml), and layered in a 15-ml centrifuge tube. A 1-ml single dissociated cell suspension ($\approx 10^7$ cells, density 1.02 g/ml) was loaded on top of the gradient, and was centrifuged for 10 min at $230 \times g$ at 24°C. Cells were collected with a fine pipette at the 0–15% (S0), 15–30% (S1), and 30–45% (S2) density interfaces. They were rinsed once in CaFSW, and plated in 2 ml of fresh medium in 24-well plates. The separation experiment was repeated four times. To evaluate the efficiency of the separation, cell type proportions (percent of total cells in each population) were compared, and differences between distributions were tested for significance by using a one-way ANOVA (with arcsine data transformation). To identify which cell type displayed ALP activity, each Percoll-separated cell population and the control pre-Percoll mixed cell population were assayed for ALP activity. This experiment was repeated four times and cell type proportions were correlated with ALP activities (Spearman's coefficient, $P < 0.05$).

Nodules, which were defined as two or more gray, extracellular granules, were detected with phase-contrast light microscopy at

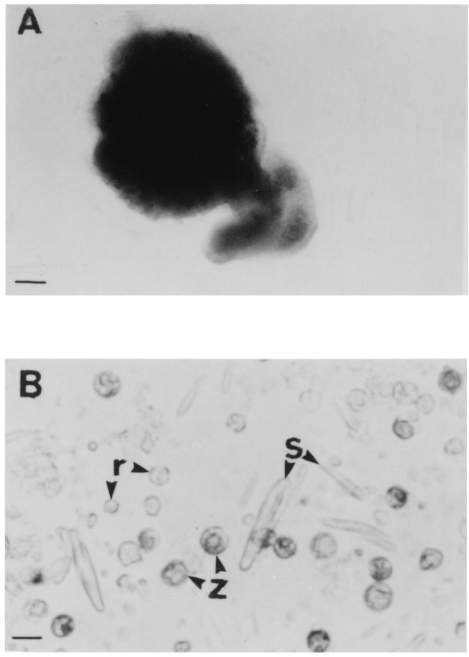

Fig. 1. Phase-contrast light micrographs of *P. damicornis* primary cell cultures at plating. (*A*) Multicellular isolate in medium B. (Scale bar, 100 μm.) Two tentacles are visible in the fragment of polyp tissue. (*B*) Individual cell culture in medium A. (Scale bar, 10 μm.) z, Zooxanthellae; s, spindle cells; r, round cells.

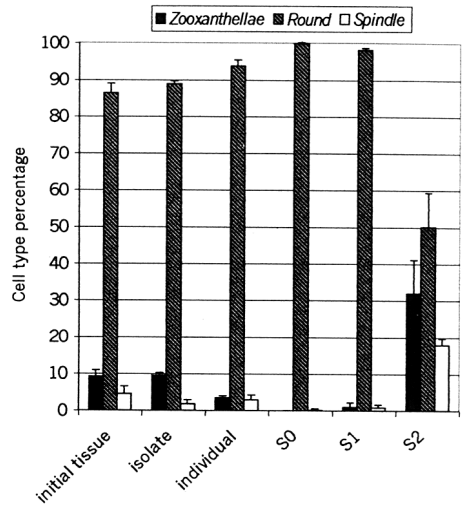

Fig. 2. Morphological cell type distribution in control initial tissue versus multicellular isolate cultures, individual cell cultures, and the three Percoll gradient fractions (S0, S1, and S2) at plating. The vertical lines indicate 1 standard deviation.

200× and 400× in each adherent isolate grown in medium A or medium B. For each medium the number of nodules per isolate was counted in 15–20 isolates from three replicate cultures.

Nodules were extracted from 19-day isolate cultures grown in medium B, for chemical and structural characterization in the transmission electron microscope. Nodule-containing isolates were sampled, mechanically dispersed, and sonicated for 5 min at 4°C. The resulting cell extract was frozen at −20°C, thawed, rinsed twice in deionized H_2O, and placed on holey carbon-coated support grids. Transmission electron microscopy (TEM) was conducted with a Philips CM-300 FEG instrument, operating at 300-kV accelerating potential. Selected-area electron diffraction, diffraction-contrast imaging, and analytical electron microscopy (AEM) were used to identify and characterize the nodules. The AEM analysis comprised energy-dispersive x-ray spectroscopy using an Oxford detector and EMiSPEC control system to determine chemical composition. Camera lengths for diffraction analysis were calibrated by using sputtered gold standards. Electron diffraction analysis included pattern indexing of unknowns as well as comparison of standard aragonite collected from a control sample of *P. damicornis* skeletons grown *in vivo*.

Results

Single cells and tissue fragments were isolated from the hard coral *P. damicornis* through spontaneous dissociation of soft tissues from the skeleton. Total yield for a 0.3- to 0.5-mm-long coral branch was 0.5 to 1 $\times$ 10^6 cells over the first 3 h, and 5 to 10 $\times$ 10^6 cells over the first 3 days in culture.

Tissue fragments, single dissociated cells, and skeletal debris were separated by density, establishing two types of primary cultures free of original skeletal pieces. Multicellular isolate cultures consisted of incompletely dissociated tissue fragments, transferred intact (Fig. 1*A*). Their multicellular structure was confirmed by TEM. Individual cell cultures consisted of mechanically dispersed single cells (Fig. 1*B*). Both primary cultures were heterogeneous, containing only coral cell types able to survive *in vitro*. Three morphologically different coral cell types were distinguished according to the terminology of Frank *et al.* (18) and were comaintained in primary cultures.

Zooxanthellae were unicellular spherical microalgae of the genus *Symbiodinium* sp., 8 μm in diameter, identified by their orange-brown pigmentation in phase-contrast light microscopy and by their chloroplasts in TEM. They were observed either as symbiont form, within 10- to 12-μm diameter endoderm coral host cells, or free in the medium. With routine light microscopy we could not conclusively distinguish between free-living and symbiont forms of zooxanthellae. They mostly separated in the 30% Percoll fraction (1.055 g/ml).

Spindle cells were spindle-shaped cells, 3–8 μm in diameter by 15–30 μm long, with an intracellular capsule containing a thread-like filament coiled within or discharged into the culture medium. Based on their ultrastructure, these capsules are nematocysts and spindle cells are cnidoblasts (cnidarian stinging cells adapted to catching prey). The smaller spindle cells may be immature cells, in the process of differentiating into full-size cnidoblasts. All spindle cells coseparated with the zooxanthellae in the 30% Percoll fraction (1.055 g/ml).

The remaining coral cell types were round, including 10- to 12-μm diameter granular cells, 10-μm diameter flagellate cells, and 5- to 10-μm smooth cells. Round cells mostly separated above (1.02 g/ml) and in the 15% Percoll fraction (1.04 g/ml) with some in the 30% Percoll fraction (1.055 g/ml).

Cell-type distributions of multicellular isolates and individual cell cultures were compared with control intact tissue, at plating and after density separation in a Percoll discontinuous gradient. The results showed significant differences in the proportions of all 3 cell types (Fig. 2). As compared with control tissue, multicellular isolates contained the same proportion of zooxanthellae, whereas individual cell cultures contained a 50% lower proportion of zooxanthellae ($P < 0.001$). Cell-type separation resulted in selective 4- to 8-fold enrichment of the cell population in zooxanthellae and spindle cells ($P < 0.001$).

Both multicellular isolate cultures and individual cell cultures were maintained over 4 weeks *in vitro*, with medium addition once every 2 weeks. Although individual cells remained in suspension, multicellular isolates adhered to the Primaria dish substratum on the first day of transfer and their cells migrated outward in a circular outgrowth, spreading the isolate mass. In

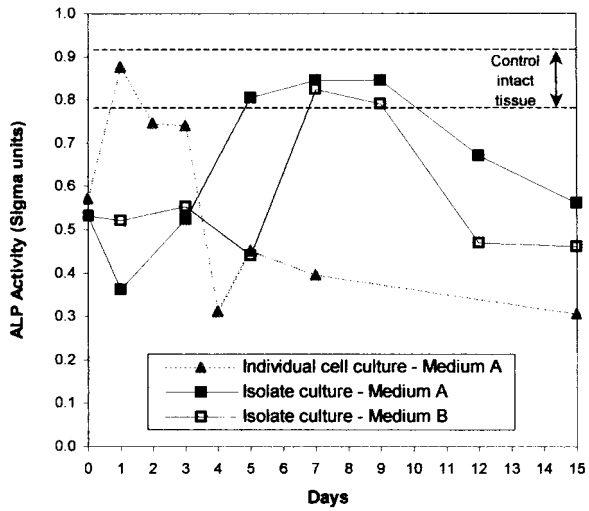

Fig. 3. Mean ALP activity of primary cell cultures. The mean activity ± 1 SD of the initial intact tissue is shown as a comparative control (dotted line box extended across the figure). Error bars are omitted for clarity. Interculture variability, calculated from the standard deviation-to-mean ratio of triplicate cultures, was 15–30%.

both types of primary cultures, cell yield (i.e., total number of cells in culture) decreased over time. Spindle cells survived the longest, resulting in a gradual enrichment of the primary cultures in these cell types. Despite the presence of wide-spectrum antibiotics–antimycotics in the medium, 15–25% of the primary coral cell cultures had to be discarded because of contamination.

Modification of the culture medium's ionic formulation affected isolate maintenance. Medium B had higher salinity and twice the Mg/Ca and Na/K ionic ratios of medium A. Once transferred, all isolates adhered and spread onto the Primaria substratum. In medium B, however, isolates remained significantly smaller (Student's t test, $P < 0.05$), with a well-defined core. The difference was greatest on day 1. In medium A, isolates spread more quickly, doubling their size within the first day. Over time, cells in both media migrated out and away from the isolates, individually in medium B, and as loose cell sheets still connected to the isolate mass in medium A. Compared with medium A, the use of medium B delayed the dissociation of multicellular coral isolates into single cells and prolonged their maintenance in primary culture.

All *P. damicornis* primary cell cultures displayed transient high levels of ALP enzyme activity. This assay had a 15–30% interculture variability, reflecting the variability typical of primary cultures, but a common trend was recognizable (Fig. 3). There was an initial decrease in ALP activity of 3-day individual cells and 2-day isolates as compared with control intact tissue. However, after plating in primary culture free of skeletal debris, an increase in ALP activity was observed in all types of culture, reaching the intact tissue level and then gradually declining. In individual cell cultures, ALP activity peaked one day after plating, with no significant differences between medium A and medium B (data not shown). In isolate cultures, a similar peak in ALP activity was observed, but it was delayed to 5 days and 7 days after plating in medium A and medium B, respectively. The ALP activity was significantly lower at 5 days in medium B than in medium A (one-way ANOVA, $P < 0.001$). Timing of the increase in ALP activity depended on the culture conditions. The peak was delayed in isolate as compared with individual cell cultures, and this delay was extended in isolate cultures by the use of medium B as compared with medium A.

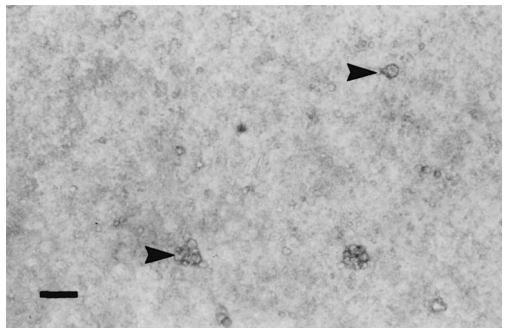

Fig. 4. Phase-contrast light micrograph of a 23-day multicellular isolate culture in medium B. Arrows point to typical calcium carbonate nodules. (Scale bar, 25 μm.)

ALP activities were compared between each Percoll-separated cell fraction from a primary individual cell culture, at plating. Activity was greatest in those fractions where round cells were most abundant. Although this correlation was not significant (Spearman's coefficient, $P > 0.05$), there was a significant ($P < 0.05$) negative correlation between ALP activity and the percentage of spindle cells, and a nonsignificant ($P > 0.05$), but negative, correlation between ALP activity and the percentage of zooxanthellae. Spindle cells and zooxanthellae did not appear to directly express ALP activity.

Precipitates were detected with an inverted light microscope in adherent multicellular isolate cultures but not in control medium without cells. They were spherical (5- to 20-μm diameter) to spindle-shaped (20 μm long) nodules (Fig. 4). They were preferentially localized in the areas of lesser cell density, around the isolate's edges, where the tissue was in the process of dissociating into single cells. The number of precipitates per isolate increased with time, with a delay between cultures in medium A and medium B: 7-day cultures contained 30 ± 5 precipitates per isolate in medium A, versus 18 ± 7 only in medium B; at 15 days, precipitates were 45 ± 11 in medium A versus 25 ± 7 in medium B.

Nodules were extracted from 19-day multicellular isolate cultures grown in medium B, for chemical and structural characterization by TEM. Fig. 5 is a bright-field image of these nodules, showing rod-shaped grains, varying in size from ≈50 nm to more than 1 μm long. In our samples, the grains were typically clustered into aggregates.

Chemical analysis was accomplished by energy-dispersive x-ray spectrometry (EDS) and demonstrated that each grain was, indeed, calcium carbonate. Fig. 6 is a typical x-ray spectrum on a portion of a grain and shows prominent calcium, carbon, and oxygen peaks as well as a minor magnesium peak. These spectra show that the grains are nearly pure $CaCO_3$, with $MgCO_3$ solid

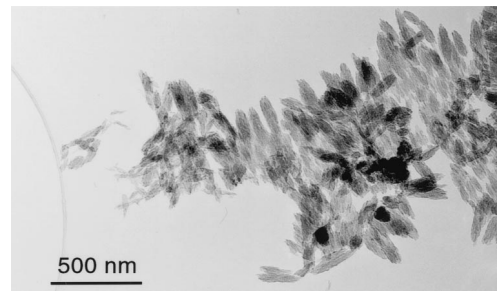

Fig. 5. Bright-field transmission electron micrographs of aragonite rods extracted from multicellular isolate cultures grown in medium B for 19 days. Individual rods are polycrystalline and typically clustered in aggregates.

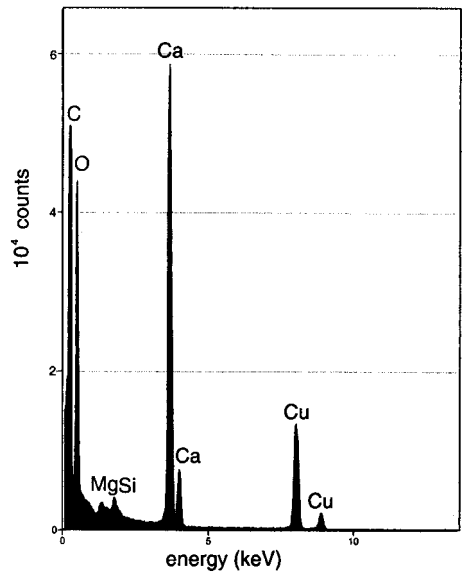

Fig. 6. Energy-dispersive, x-ray spectrum of aragonite rod showing calcium carbonate composition. Cu and Si peaks are from support grid and film.

solution <2%. Meaningful quantification of the carbon and oxygen contents of the samples is not possible because of sorption of contamination from the atmosphere during sample preparation, traces of vacuum pump oils in the microscope, and the carbon-coated support grid used to hold the sample. In addition, the silicon and copper peaks are always present in the EDS analyses because the support film contains silicon and the grid itself is copper metal.

To assess crystallinity and structure of the calcium carbonate in the grains, selected-area electron diffraction (SAED) patterns were collected from 14 grains. Fig. 7*A* is a dark-field electron micrograph of an intermediate-sized carbonate rod. In this image, regions of the sample with different contrast are in different crystal orientation. The image shows, therefore, that the individual rod actually comprises numerous crystalline domains of different orientation, with scale on the order of one to tens of nanometers across; the rods are nanocrystalline. Fig. 7*B* is a SAED pattern of one of these nanocrystalline domains. Indexing of this diffraction pattern, and all others collected from our samples, identifies the calcium carbonate in these grains as the aragonite polymorph. This analysis thus clearly establishes that the calcium carbonate precipitated in our coral cell cultures is aragonite.

Discussion

Extending work by Kopecky and Ostrander (20), *P. damicornis* cell cultures were initiated by spontaneous dissociation of hard coral colony fragments, yielding a mix of single cells and intact, ciliary-active, tissue fragments, called multicellular coral isolates. Within each multicellular isolate, mesoglea, the native coral extracellular matrix separating ectodermal from gastroendodermal cell layers, was likely to be preserved. We suggest that adherence of each isolate was indirect, the result of partial adsorption of this mesoglea onto the Primaria dish substratum. Mesoglea may then support attachment, spreading, and outward migration of individual cells. Species-specific extracellular matrices play a crucial role in the *in vitro* functional maintenance of marine invertebrate cells, including coral cells (19). Scyphozoan jellyfish mesoglea has recently been identified as an optimal substrate to maintain cells isolated from sea anemones, scleractinian, and alcyonacean corals (23). Establishing multicellular

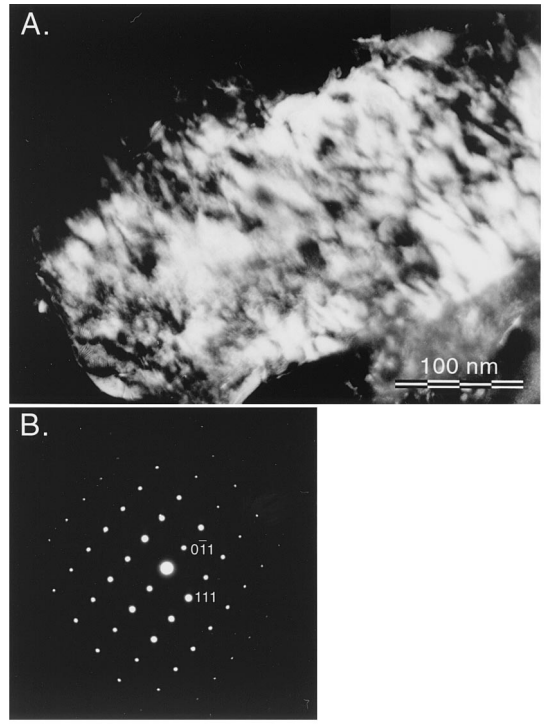

Fig. 7. (*A*) Dark-field transmission electron micrograph of aragonite rod showing polycrystalline nature. Regions of different contrast are in different crystallographic orientation. (*B*) Selected-area electron diffraction pattern of the [$\bar{2}$11]-zone of aragonite taken from a single crystalline domain in the rod.

isolate cultures may be an innovative solution to provide scleractinian (hard coral) cells their species-specific extracellular matrix components and maintain their functionality. This may explain why calcium carbonate nodules were detected only within adherent multicellular isolates.

ALP is a type I phosphomonoesterase that hydrolyzes short-chain esters. It is a nonspecific enzyme, which has been related to the process of *in vitro* crystalline biomineralization in mammalian ossification models of bone-forming osteoblasts (10) and dentin-forming odontoblasts (11). In those models, ALP activity peaks before nucleation and growth of calcium phosphate crystals (apatite). Thus, it is used as a marker of early biomineralization activity. Increased ALP activity is interpreted as a prerequisite initial step, possibly related to cell-mediated secretion of structural organic matrix, which then mineralizes in a second step into the final calcified tissue product. The type of protein deposited in marine invertebrate matrix controls the type of mineral subsequently grown (7, 8). In *Madreporaria* corals, extranuclear ALP has been correlated with the distribution of a neutral mucopolysaccharide-like material (4). Sud *et al.* (21) have recently reported a peak in ALP activity in cell cultures from the shell-making mantle of the gastropod *Haliotis tuberculata*. A parallel study using the same gastropod cell system has demonstrated *in vitro* production of structural matrix components (24), but it was not followed by mineralization.

Here, we report that primary cultures of cells from the reef-building coral *P. damicornis* displayed a transient increase in ALP activity. This increase was followed, in multicellular isolate cultures, by crystallization of the aragonite polymorph of calcium carbonate.

ALP expression was delayed by 4 to 6 days in multicellular isolates as compared with individual cell cultures. This delay was extended in medium B as compared with medium A, with a corresponding delay in the detection of calcium carbonate

nodules. The ionic composition of medium B extended the maintenance of cell-to-cell contacts within the isolates. The Mg/Ca and Na/K ratios of mineral B matched the ratios of Stanley and Hardie's model of ocean carbonate productivity to favor the precipitation of aragonite over calcite (2). If ALP activity marks the differentiation of coral cells into the biomineralizing calicoblast phenotype, these results suggest that differentiation and senescence was delayed in compact multicellular isolates. Dissociating coral tissue into single cells may result in immediate cell differentiation, followed by autodigestion and death. Maintenance of cell-to-cell contacts within multicellular isolates may preserve the viability and functionality of differentiated coral cells, and delay cellular aging and death, thus allowing *in vitro* calcification. This hypothesis is supported by observations that marine sponge cells have a higher proliferative capacity (measured by telomerase activity) when maintained within multicellular aggregates (primmorphs) than as single cells (25). The link between ALP activity and mineralization is not clear. Investigation of bone nodules formed *in vitro* has suggested that ALP may be necessary only for initiating mineralization (26), either by increasing the local concentration of inorganic phosphate or by hydrolyzing inhibitors of mineralization. In coral, ALP might hydrolyze phosphate inhibitors of calcification, such as bisphosphonate chelating agents, which have been shown *in vivo* to inhibit calcification of hard coral *Stylophora pistillata* (27).

In our hard coral cell system, at ambient atmospheric pressure and temperature (24°C), aragonite crystals of calcium carbonate were detected in adherent multicellular isolate cultures, but not in control medium A or B without cells. Original skeletal debris was removed from the primary cultures at the time of their establishment. Thus, these aragonite nodules are *de novo* material. In inorganic systems, the aragonite polymorph of calcium carbonate is thermodynamically unstable at near-surface conditions (28). Natural occurrences and laboratory experiments have clearly established, however, that the elevated concentration of Mg^{2+} in modern seawater causes metastable precipitation of aragonite rather than calcite (29–31). Morse *et al.* (32) have shown that between 6°C and 35°C, the Mg/Ca ratio of the solution is the principal control on the type of carbonate polymorph precipitated. In our work, however, the absence of carbonate precipitation in control experiments and the location of precipitates within the cultured tissue itself indicates that this aragonite is biogenic and not inorganic precipitation. Micromolar concentrations of phosphate have also been shown to favor metastable nucleation of aragonite (31). It is possible, therefore, that the observed ALP activity plays a primary role in the specification of carbonate polymorph.

When examined by TEM without fixation or staining for organic matrix, the aragonite nodules were clusters of nanocrystalline grains. It is not clear, however, if these aggregates are representative of the nodule structure as grown *in vitro*, or if they were modified during the extraction and deposition onto TEM grids. Further work is necessary to determine patterns of crystal accretion and evaluate this aggregate structure in undisturbed nodules, fixed within cultured coral isolates.

Our research demonstrates that it is possible to obtain aragonite crystallization in primary cell cultures established from a hard coral, *P. damicornis*. This provides an innovative, *in vitro*, model to study the biological control of calcification in reef-building corals at the cellular level. It is a tool to investigate the cooperation of coral cell types in the calcification process. It is also a tool to investigate the nucleation and growth requirements of calcium carbonate crystals and their stabilization by organic matrices.

We thank Ken Billin, Jim Prappas, Michael McCaffery, David Veblen, Steven Stanley, Lawrence Hardie, and Joe Geraci for their helpful comments, discussion, and encouragement. Support was provided by the National Aquarium in Baltimore (to G.K.O.), The Eppley Foundation (to G.K.O.), the Department of Energy/Basic Energy Sciences (to D.C.E.), and the Howard Hughes Medical Institute (to P.S.C.).

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