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The Involvement of Calcium in Heat-induced Coral Bleaching

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Shiao-Ping Huang, Ku-Lin Lin and Lee-Shing Fang (1998) The involvement of calcium in heat-induced coral bleaching. *Zoological Studies* **37**(2): 89-94. Coral bleaching is inducible by elevated sea water temperature. At the subcellular level the mechanism of bleaching is unclear. We investigated the relationship between intracellular calcium concentration and bleaching induced by elevated temperature. Intracellular calcium concentration rose in proportion to the duration of heat treatment. Bleaching was inhibited after applying the calcium chelator EGTA and the cation ionophore A23187 to deplete extra- and intracellular calcium concentrations. Further, heat-induced coral bleaching was inhibited when the ambient calcium concentration in sea water was reduced or depleted. The extent of bleaching was correlated with the calcium concentration of the sea water. These findings indicate that calcium plays a pivotal role in the subcellular mechanism of coral bleaching.

Key words: Coral bleaching, Calcium concentration, Exocytosis.

Most reef corals host large populations of endosymbiotic dinoflagellate algae known as zooxanthellae. Zooxanthellae are located in vacuoles within coral cells (Gates and Muscatine 1992). This endosymbiotic relationship can be dramatically affected by temperature change (Cook et al. 1990, Gates 1990, Glynn and D'Croz 1990, Hayes and Buch 1990, Jokiel and Cole 1990), resulting in loss of zooxanthellae from coral or the decrease of pigment in the zooxanthellae, both of which cause bleaching of the coral (Glynn and D'Croz 1990, Hayes and Buch 1990, Jokiel and Coles 1990). Other stresses which can result in bleaching include high levels of UV radiation and salinity shock (Harriot 1985, Hoegh-Guldberg and Smith 1989, Gleason and Wellington 1993). Cellular mechanisms associated with coral bleaching have not been well studied, despite which, several potential mechanisms such as exocytosis, apoptosis, necrosis, pinching off, and host cell detachment have been suggested (see Gates et al. 1992 for review).

According to Steen and Muscatine (1987), exocytosis is involved in the release of symbiotic algae from sea anemones exposed to unusually low temperatures. A similar phenomenon was not observed in experiments with several scleractinian corals (Steen and Muscatine 1987). Muscatine et al. (1991) suggested that release of symbiotic algae from corals could involve thermotropic effects on host cell membranes after cold shock. Fang et al. (1997) found that the concentration of intracellular calcium in coral cells increased following exposure to elevated temperatures. It was demonstrated that the increase of intracellular calcium appeared to trigger the exocytosis of cortical granules from fertilized oocytes (for review, see Knight et al. 1989) and synaptic vesicles from synaptic terminals (Heidelberger et al. 1994). Therefore, the increasing calcium may act as a signal that plays an important role in the mechanism of coral bleaching, such as exocytosis of intracellular vacuoles which contain the zooxanthellae.

In experiments described here, we measured the change in concentration of intracellular calcium after cells were subjected to elevated temperatures. Heat-induced bleaching in normal samples was compared with bleaching in samples in which intracellular calcium had been depleted by treat-

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ment with EGTA and cation ionophore A23817. Finally, the extent of heat-induced coral bleaching was measured in artificial sea water having different ambient calcium concentrations.

MATERIALS AND METHODS

Specimens of the staghorn coral, *Acropora grandis*, were collected by scuba diving from about 6 m depth on reefs in Kenting National Park, Nanwan, Taiwan (21°56'N, 120°44'E). They were transported to the laboratory in aerated sea water within 3 h, and were kept in a filtered sea water aquarium at 25 °C with light for 16 h and at 23 °C without light for 8 h daily. Samples were used within 2 wk after collection.

Preparation of sea water with different calcium concentrations

Artificial sea water (ASW) was prepared from the following salts: 24.7 g NaCl, 0.7 g KCl, 6.3 g $MgSO_4 \cdot 7H_2O$, 4.6 g $MgCl_2 \cdot 6H_2O$, 1.0 g anhydrous $CaCl_2$, and 0.2 g $NaHCO_3$, dissolved in water and diluted to 1 l. Calcium-free sea water (CFSW) was prepared the same as ASW except $CaCl_2$ was excluded. Various calcium concentrations in ASW were obtained by adding different amounts of calcium to CFSW.

Experiments with isolated cells

A knife was used to scrape coral tissues from their skeletons. The coral cells were obtained by crushing coral tissues with a mortar, after which they were homogenized, then centrifuged, and resuspended in ASW. Zooxanthellae were pelleted by centrifugation in 22 × g for 15 min, then the coral cells in the upper layer were collected. The integrity of the cells was frequently examined by light microscope (Fang et al. 1997). FURA 2/AM (Calbiochem, CA, USA), a penta-acetoxymethyl ester of a calcium-sensitive fluorescent dye, was used to measure intracellular calcium concentrations in cytosolic fluid, following the method of Grynkiewicz et al. (1985). FURA 2/AM is a membrane-permeable ester derivative which can penetrate into cells. Cell suspensions from the above preparation (1.2×10^6 cells in 1.5 ml) were incubated with 30 μM FURA 2/AM in ASW for 2 h at 25 °C. The cells were then centrifuged (1000 × g for 10 min) and the pellet was resuspended in CFSW containing 0.1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma Chem., MO, USA), since EGTA could chelate possible calcium contamination from other compounds in the formula. To examine the variation of intracellular calcium under elevated temperature, coral cells were incubated at either 33 °C or 25 °C (control) for 24 h. Fluorescence of intracellular FURA 2, formed within the cells by hydrolysis of FURA 2/AM preloaded into the cells, was measured hourly using a fluorospectrophotometer (HITACHI F3000) and a 1×1 cm^2 quartz cuvette. Intracellular calcium concentration is proportional to the ratio of the fluorescence at 510 nm of excitation at 340 nm and 380 nm (5 nm bandwidth) wavelengths.

The same experiment was replicated several times to observe the reproducibility of the intracellular calcium change. The data could not be pooled because the penetration ratio of FURA 2/AM into cells can not be standardized.

Intact coral experiments

Bleaching assay

Branches of *A. grandis* about 15 mm long and 10 mm in diameter at their base were incubated in polypropylene tubes with 10 ml ASW. Bleaching was induced by raising the temperature to 33 °C for 24 h. Released zooxanthellae were pelleted by centrifuging at 500 × g for 10 min at 25 °C. The pellet was resuspended in 5 ml 95% ethanol and the algae were sonicated to extract pigments (Fang et al. 1995). The pigments have an absorption maximum at 440 nm and, consequently, for maximum sensitivity, absorption at 440 nm was used as an index of the amounts of zooxanthellae released into ASW. There was little interference from coral tissue because only zooxanthellae were collected for pigment extraction. At the end of each experiment, zooxanthellae remaining in the coral samples were collected by the procedure described above. The process was repeated several times until no zooxanthellae were found after pelleting. Amounts of zooxanthellae retained were then estimated from their absorption at 440 nm. Bleaching was calculated from the average ratio of absorption of pigments from released algae to total pigments. For each experimental condition, assays were performed in triplicate and results were averaged.

Depletion of intracellular calcium

A calcium chelator, EGTA, and the cation ionophore A23187 (Sigma Chem., MO, USA), were used to deplete intracellular calcium stores

(Coukell et al. 1992). A23187 is a lipid-soluble antibiotic that permits divalent cations to pass through cell membranes (Reed and Lardy 1972). Corals were incubated in ASW with 10 or 50 mM EGTA at 33 °C, or they were treated with EGTA and 10 μM A23187 (Brody and Mathews 1989) for different experiments.

Experiments using different treatments were applied on the bleaching assay described previously. For each treatment, 3 coral branches were used, and usually 5 replicates were performed. The treatment with A23187 and EGTA was designed to deplete intracellular calcium stores. The treatment with EGTA alone was to distinguish between the effects of A23187 and EGTA. Two doses, 10 mM and 50 mM, of EGTA were used to ensure the efficiency of calcium chelating. The heat-treated corals represented the normal extent of bleaching. The controls were samples treated at room temperature. The degree of bleaching was calculated 22 h later.

Effects of external calcium concentration

To investigate the possible participation of external calcium in the process of coral bleaching, we varied the amount of calcium in ASW as 9 mM, 0.9 mM, and depleted (normal, 1/10 normal, and calcium-free, respectively). Coral branches were incubated at 33 °C or room temperature and bleaching was measured after 24 h. Replicates were the same as in the above experiments.

RESULTS

Intracellular concentration of calcium in *A. grandis* cells rose proportionally with time during incubation at 33 °C. It also rose in the control, but the rate of increase was significantly lower (Fig. 1; ANCOVA test, $p < 0.05$). This experiment was performed in CFSW. Consequently, the increase in calcium concentration in the cytosolic fluid must have been due to release of calcium from intracellular stores, such as from the cell's endoplastic reticulum or mitochondria.

Figure 2 shows the extent of coral bleaching after either extra- or intracellular calcium was depleted. The 33 °C heat-treated (HT) coral samples released about 63% of their pigment within 24 h and released the same percentage of HT with 10 mM EGTA or HT with 10 mM EGTA and A23187 (Student-Newman-Keuls test after Kruskal-Wallis test, $p > 0.05$).

In the presence of 50 mM EGTA, the extent of pigment loss was reduced to 42% and was significantly lower than that of the HT, but significantly higher than both the room temperature control (RT) and HT with 50 mM EGTA and A23187 (Student-Newman-Keuls test after Kruskal-Wallis test, $p < 0.05$). The pigment loss in the presence of both 50 mM EGTA and A23187 was further inhibited (reduced to only 28%), and was the same as that of the RT (Student-Newman-Keuls test, after Kruskal-Wallis test, $p > 0.05$).

Incubation of corals in ASW with different calcium concentrations at 33 °C showed that the amount of pigment loss was inversely proportional to the ambient calcium concentration (Fig. 3). With the normal calcium concentration of sea water (9 mM), the percentage of pigments released into sea water from coral cells with heat treatment was significantly larger than with the room temperature treatment (Mann-Whitney test, $p < 0.05$). However, the percentages of pigments released into sea water in room temperature groups of all calcium concentrations were not significantly different (Kruskal-Wallis test, $p > 0.05$). Moreover, the heat-treated coral sample released much more pigment in normal sea water (1 x Ca) than did those of the

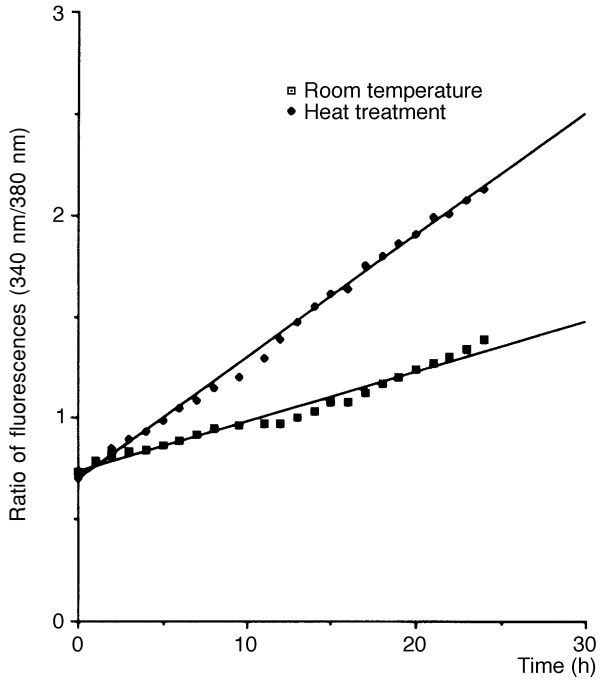

Fig. 1. Intracellular calcium concentrations (as expressed by ratio of fluorescences) in coral cells under heat treatment. Diamonds represent coral cells incubated at 33 °C (heat treatment) in calcium-free sea water. Squares represent control coral cells incubated at 25 °C (room temperature) in calcium-free seawater.

other 2 heat treatment samples with lower calcium concentrations (Student-Newman-Keuls test after Kruskal-Wallis test, $p < 0.05$) .

DISCUSSION

The experiments conducted in this study were designed to investigate the effect of 2 levels of calcium in coral bleaching. One goal was to evaluate the role of release of calcium ions from intracellular stores to the cytosol. Calcium often acts as a signal transduction element to initiate events of cell activity (Clapham 1995). But the role of its release from intracellular stores, if any, in signaling coral bleaching is unknown. The other goal was to examine whether a continuous external calcium supply is necessary for coral bleaching in addition to the initial triggering signal. FURA 2/AM only measures cytosolic calcium concentration, because once it enters the cell, it will be hydrolyzed to FURA 2 and will not penetrate into intracellular calcium stores. On the other hand, the combined use of EGTA and ionophore A23187 will deplete all the calcium in the cell. A23187 allows divalent cations to leak out from both cell membranes and intracellular calcium stores. Then, the free ions are chelated by EGTA. This approach was complemented by the experiment of inducing bleaching in sea water containing various calcium concentrations, in which the initial calcium signal and internal calcium stores were not affected, but the continuous calcium supply was greatly reduced.

The increase in intracellular calcium concentration with duration of heat exposure in the absence of an external calcium source indicates that calcium stores were gradually released during the heat-treatment period. This provides an opportunity for the increased calcium concentration to trigger other intracellular events that could result in zooxanthellae loss in those cells housing algae. In this experiment, a coral cell suspension of mixed cell types was used. It was found in our preliminary experiment that host cells with zooxanthellae had similar intracellular calcium responses under elevated temperature. However, since keeping

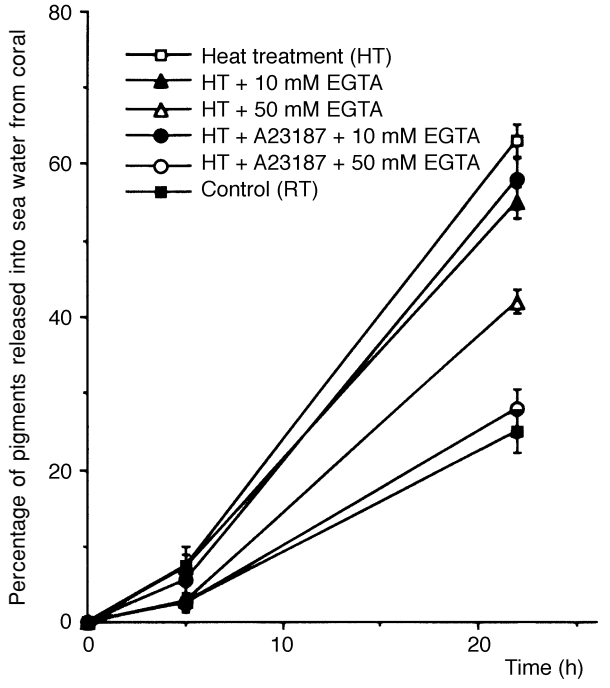

Fig. 2. The effect of EGTA and ionophore A23187 on coral bleaching. Open and solid squares represent pigments released in the sea water at 33 °C (HT) and 25 °C (RT), respectively. Solid triangles and solid circles represent pigments released in sea water containing 10 mM EGTA with or without ionophore A23187, respectively. Open triangles and open circles represent pigments released in sea water containing 50 mM EGTA with or without ionophore A23187, respectively.

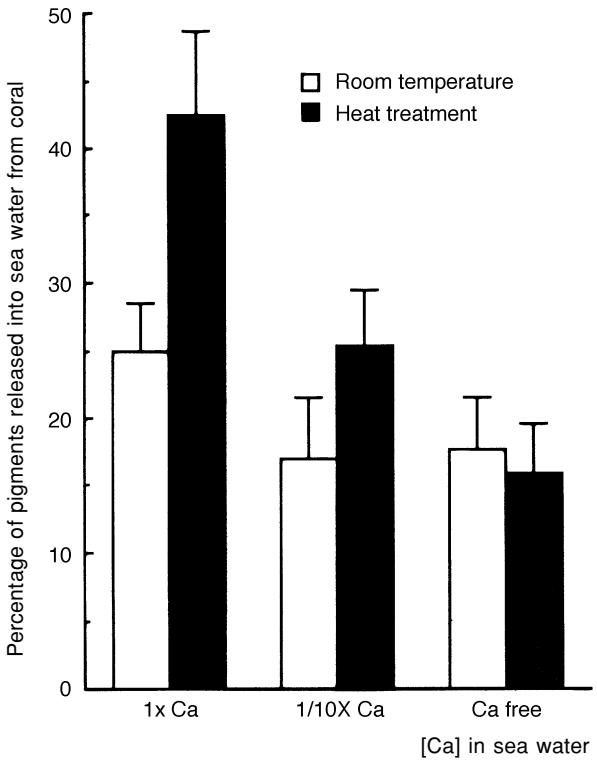

Fig. 3. The effect of extracellular calcium concentration on coral bleaching. The 1x Ca, 1/10x Ca, and Ca-free represent the normal calcium concentration (9 mM) , 1/10 calcium concentration (0.9 mM), and calcium-free condition (0 mM) but with 0.1 mM EGTA seawater, respectively.

these cells in suspension requires constant stirring, which is another stress in addition to temperature elevation, mixed cell preparations were used in this study as the system for investigation of cellular response of coral.

Stevenson et al. (1986) showed that calcium concentration of Chinese hamster ovary (CHO) cells increased within 3 min of heat treatment. The rate of increase in calcium concentration of coral cells in this study was much lower than that of CHO cells. Fang et al. (1997) found not only that an increase of calcium was involved in the production of heat shock protein of coral cell as a cellular signal, but also that the time scale for the increase of calcium in coral cells was similar to the time scale for inducing coral bleaching (Hoegh-Guldberg and Smith 1989). This was probably because, while bleaching is occurring, each host cell launches its own triggering of the calcium signal to release zooxanthellae. Therefore, as the degree of bleaching extends, an accumulative increase in calcium concentration can be observed in the same time scale. The calcium concentration also increased at a much lower rate in cells of the control group. This is because calcium can be released from intracellular stores under stressful conditions (Pozzan et al. 1994). Also the prepared coral cells, unlike those cultured cells that are physiologically stable in vitro, were under stress even at the control temperature.

When this intracellular calcium concentration change was suppressed by sufficient EGTA (50 mM) in the presence of ionophore A23187, the extent of pigment loss drastically decreased (Fig. 2). In addition, when all extracellular calcium was chelated by 50 mM EGTA, so that the supply of calcium into coral cells was cut off, the extent of pigment loss was also inhibited. This implies that bleaching is not the result of an initial calcium concentration increase, but requires a continuous supply of calcium to complete the process. The results of 10 mM EGTA with A23187 and 10 mM EGTA alone indicate that 10 mM EGTA could not chelate all the calcium ions in the sea water plus that in the cell, while 50 mM EGTA could. However, even when both 50 mM EGTA and the ionophore A23187 were used simultaneously to deplete extracellular calcium as well as intracellular stores, pigment loss was blocked profoundly but not completely (Fig. 2).

In addition, the result of this experimental group (reduced to only 28% pigment loss) was very similar to that of the control group in normal artificial sea water at room temperature (about 25% pigment loss). This shows that under effective depletion of calcium, bleaching can almost be reduced to a level near that of a normal temperature condition. It also suggests that there is involvement of other mechanisms which do not require the participation of calcium, such as pigment disintegration in coral bleaching, because after calcium was depleted, the phenomenon of bleaching could still be observed.

The extent of pigment loss was influenced by the amount of calcium in sea water (Fig. 3). This further demonstrates that calcium plays a larger role than that of a single internal signal to initiate zooxanthellae release. Calcium is essential for exocytosis of cortical granules from fertilized oocytes (Knight et al. 1989) and the exocytosis of synaptic vesicles from nerve cells (Heidelberger et al. 1994). Calcium ions also play a pivotal role in the control of cytoskeleton dynamics during neurotransmitter release (Trifar'o and Vitale 1993). Thus, it seems possible that a calcium-dependent exocytotic process is involved in heat-induced coral bleaching. Symbiotic algae enter host cells by phagocytosis (Rands et al. 1993). If the zooxanthellae in coral cells can leave the host under stress by a similar non-destructive cell activity such as exocytosis, then, the symbiotic relationship is of much more adaptive value for coral than would be a more rigid relationship. This may provide indirect evidence for the speculation that coral bleaching may provide an opportunity for the recombining of hosts and its symbiotic algae that might be better adapted to altered circumstances (Buddemeier and Fautin 1993, Ware et al. 1996). However, the cellular mechanism of coral cell exocytosis still needs to be studied. Indeed, coral bleaching is more than an anomalous ecological phenomenon. It is a very special cellular model not only allowing us to examine how a plant cell, whether actively or passively, leaves an animal cell, but also may eventually help us understand the evolution of coral and zooxanthellae symbiosis.

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鈣離子參與珊瑚受熱白化過程之研究

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珊瑚會因高溫而導致白化，然而白化過程中共生藻從珊瑚細胞釋放的機制，則尚不明瞭，我們發現珊瑚細胞會因高溫而使得其細胞內鈣離子濃度提高，而且隨加熱時間增長而蓄積，同時使用鈣離子螯合物 EGTA 及 cation ionophore A23187 去除珊瑚細胞內外之鈣離子，使得珊瑚因高溫而白化之程度降低，因此海水中鈣離子的濃度與高溫導致的白化有密切關係；研究結論顯示鈣離子訊號在珊瑚白化細胞層次中扮演樞要的角色。

關鍵詞：珊瑚白化，鈣離子濃度，胞吐作用。

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