The Journal of Experimental Biology 204, 3443–3456 (2001)
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JEB3388

Cellular mechanisms underlying temperature-induced bleaching in the tropical sea anemone *Aiptasia pulchella*

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Accepted 13 July 2001

Summary

Temperature-induced bleaching in symbiotic cnidarians is a result of the detachment and loss of host cells containing symbiotic algae. We tested the hypothesis that host cell detachment is evoked through a membrane thermotropic event causing an increase in intracellular calcium concentration, $[Ca^{2+}]_i$, which could then cause collapse of the cytoskeleton and perturb cell adhesion. Electron paramagnetic resonance measurements of plasma membranes from the tropical sea anemone *Aiptasia pulchella* and the Hawaiian coral *Pocillopora damicornis* labeled with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) revealed no membrane thermotropic event. In addition, intracellular imaging using Fura-2AM as well as labeling anemones with ^{45}Ca revealed no significant change in $[Ca^{2+}]_i$. However, bleaching could be evoked at ambient temperature with 25 mmol l^{-1} caffeine without affecting $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ could be altered with ionomycin in isolated host cells, but ionomycin could not induce bleaching in *A. pulchella*. As caffeine can affect levels of intracellular protein phosphorylation, the ability of other agents that alter intracellular levels of protein phosphorylation to evoke bleaching was investigated. The protein phosphatase inhibitor vanadate could induce bleaching in *A. pulchella*. Two-dimensional gels of ^{32}P-labeled proteins from cold-shocked, caffeine-treated and control anemones show that both temperature shock and caffeine alter the array of phosphorylated host soluble proteins. We conclude that cnidarian bleaching is linked to a temperature-induced alteration in protein phosphorylation.

Key words: coral, bleaching, cellular dysfunction, protein phosphorylation, sea anemone, *Aiptasia pulchella*, coral, *Pocillopora damicornis*.

Introduction

Cnidarian bleaching can be defined generally as a decline in the population of the symbiotic algae, a reduction in the pigment content of the algae or both (Coles and Jokiel, 1977; Hoegh-Guldberg and Smith, 1989; Kleppel et al., 1989; Porter et al., 1989; Glynn and D'Croz, 1990; Szmant and Gassman, 1990; Lesser, 1996). Major bleaching events on coral reefs are becoming more common and appear to be caused by increased water temperature (Goreau and Hayes, 1994; Brown et al., 1996) and/or by increased exposure to ultraviolet radiation (Brown and Suharsono, 1990; Gates, 1990; Jokiel and Coles, 1990; Lesser et al., 1990; Gleason and Wellington, 1993; Le Tissier and Brown, 1996).

While there have been many ecological studies of thermal bleaching in corals and on the recovery of corals from bleaching (Brown and Suharsono, 1990; Gates, 1990; Glynn and D'Croz, 1990; Hayes and Bush; 1990; Williams and Bunkley-Williams, 1990; Fitt et al., 1993; Gleason, 1993; Brown et al., 1996), few have investigated the underlying cellular mechanisms. Several studies have demonstrated that temperature and ultraviolet radiation inhibit photosynthesis and can induce oxidative stress in the algae (Lesser et al., 1990; Lesser, 1996; Jones et al., 1998; Warner et al., 1999). Other studies report changes in enzyme activity (O'Brien and Wyttenbach, 1980; Suharsono et al., 1993) and the increased production of heat-shock proteins in the host during bleaching (Miller et al., 1992; Sharp et al., 1994; Black et al., 1995). Neither line of study offers suggestions as to how changes in temperature cause the loss of the symbiotic algae.

Gates et al. (Gates et al., 1992) demonstrated that thermal bleaching in the Hawaiian reef coral *Pocillopora damicornis* and in the sea anemone *Aiptasia pulchella* occurred predominantly by release of intact hosts cells with their contained symbiotic algae. That is, thermal stress somehow affected the adhesive properties of the host cells. After analyzing sections of bleached coral, Brown et al. (Brown et al., 1995) suggested that tissue necrosis might explain bleaching, but these authors also observed released algae still within host cells. Huang et al. (Huang et al., 1998) speculated

that heat shock might induce algal release through exocytosis after demonstrating that heat shock alters $[Ca^{2+}]_i$ in the coral *Acropora grandis*; however, these authors did not directly investigate coral bleaching. Whereas cell adhesion dysfunction may not be the only potential mechanism of temperature-induced bleaching [see (Brown et al., 1995; Le Tissier and Brown, 1996)], its occurrence is unequivocal. That is, bleaching anemones and corals release intact host cells containing their endosymbionts.

On the basis of an Arrhenius plot of the rate of release of symbiotic dinoflagellates with decreasing temperature, Muscatine et al. (Muscatine et al., 1991) suggested that a membrane thermotropic event (Melchior and Steim, 1976; Watson and Morris, 1987) might initiate bleaching. By analogy with observations on insect cell cultures (Van Bergen en Henegouwen et al., 1985; Coakley, 1987; Cress et al., 1990; Walter et al., 1990), Gates et al. (Gates et al., 1992) hypothesized that temperature stress might induce thermotropic phase transitions in host cell membranes which result in the passive influx of Ca^{2+} (Melchior and Steim, 1976; Quinn, 1989; Cheng and Lepock, 1992; Drobnis et al., 1993). As $[Ca^{2+}]_i$ controls the activity of elements of the cytoskeleton and the cell adhesion molecules to which they are linked (Hirano et al., 1987; Nagafuchi and Takeichi, 1988; Forscher, 1989; Matsumura and Yamashiro, 1993; Weeds and Maciver, 1993), changes in $[Ca^{2+}]_i$ could cause loss of adhesion by causing collapse of the cytoskeleton, which is interconnected with the cytoplasmic domain of cell adhesion molecules (Geiger et al., 1992; Takeichi et al., 1992; Sastry and Horowitz, 1993). Thus, a temperature-induced change in $[Ca^{2+}]_i$ might induce cell adhesion dysfunction.

In this study, we addressed the hypothesis that temperature stress causes a membrane thermotropic event that results in an elevated $[Ca^{2+}]_i$, causing release of host cells (bleaching). We tested this hypothesis by measuring host cell plasma membrane fluidity using electron paramagnetic resonance (EPR) in the tropical reef coral *Pocillopora damicornis* and in the tropical sea anemone *Aiptasia pulchella* and by investigating the role of $[Ca^{2+}]_i$ using pharmacological agents, the Ca^{2+}-specific fluorochrome Fura-2AM and $^{45}CaCl_2$ to investigate Ca^{2+} flux in *A. pulchella*. In addition, we investigated the role of phosphorylation of host cell proteins in cnidarian bleaching using pharmacological agents and two-dimensional gel electrophoresis.

Materials and methods

Animal collection and maintenance

Aiptasia pulchella (Carlgren) and *Pocillopora damicornis* L. were collected from Kaneohe Bay, Oahu, HI, USA, on Checker Reef adjacent to the Hawaii Institute of Marine Biology. Both were transported back to the University of California at Los Angeles (UCLA) and maintained in an aquarium at 25 °C on a 12 h:12 h light:dark cycle. *P. damicornis* colonies were used within 48 h of collection. *A. pulchella* were fed twice weekly on *Artemia* nauplia, but were starved for 24 h in an incubator (Percival Scientific, model 1-35 VL) at 25 °C on a 12 h:12 h light:dark cycle at 100 μmol quanta m^{-2} s^{-1} before being used in experiments.

Electron paramagnetic resonance

To isolate host cell membranes, *A. pulchella* were homogenized in a Teflon–glass tissue grinder in 1 ml of filtered sea water (FSW), and *P. damicornis* were scrubbed with a toothbrush in 3 ml of FSW on ice. Homogenates were centrifuged (Damon/IEC, model HN-S, for 4 min at 500 ***g***) to pellet the algae. The supernatant was decanted and centrifuged at 5000 ***g*** for 15 min at 4 °C (Eppendorf, model 5414) to pellet the mitochondria. The supernatant was then centrifuged at 100 000 ***g*** for 1 h at 4 °C (Beckman TL-100 ultracentrifuge) to pellet the host cell membranes. The pellet was retained, capped with N_2 gas, frozen at −80 °C and analyzed within 72 h.

To generate the electron paramagnetic resonance (EPR) spectrum, the membranes were thawed, and 0.1 μmol l^{-1} 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) was added in proportion to the amount of host cell membrane phospholipid. Phospholipid concentration was determined from the phosphorus content (Bartlett, 1959). The EPR spectrum was measured with a Varian E-109 EPR spectrophotometer (Varian Associates, Inc., Palo Alto, CA, USA) operating at X-band and fitted with a two-loop one-gap resonator (Hubbell et al., 1987). Measurements were made approximately every 5 °C from 0 to 40 °C. Temperature was controlled with a Varian nitrogen gas flow system and monitored with a thermocouple inserted into the resonator. The heights of the lipid and water signals were measured and their ratio calculated at each temperature (as described by McConnell et al., 1972; Shimshick and McConnell, 1973).

Bioassay for bleaching

Bleaching was evoked with an acute cold shock or heat shock in darkness following the protocol of Muscatine et al. (Muscatine et al., 1991) with some modifications. Cold shock gives bleaching results similar to those obtained with heat shock, but takes less time, so that results can be obtained within 17 h. Briefly, the anemones were held in 10 ml test tubes in 2 ml of Millipore-filtered sea water (MFSW) during the experiment. All experiments contained at least two sets of controls; one consisted of animals held at 25 °C in MFSW during the entire experiment. The second consisted of anemones that had been cold-shocked for 2.5 h at 12 °C in a water bath (Neslab model RT#110), then returned to 25 °C and allowed to release host cells containing symbiotic algae. After incubation for 14 h at 25 °C, the number of algae released was determined and expressed as a percentage of the total number of symbiotic dinoflagellates in the anemone. Algal cell number was determined either with a hemocytometer (Hausser Scientific) or with a cell imaging system. An image of the cells loaded on the hemocytometer was captured with a Sony (model DC-37) CCD video camera and stored on an IBM-PC-XT. The image was simultaneously displayed on a Sony Triniton monitor. Image analysis (i.e.

cell counts) was performed with Olympus CUE-2 image-analysis software.

Pharmacology

Different pharmacological agents were used to evoke host cell detachment in *A. pulchella* at 25 °C. Some of these agents were dissolved in dimethylsulfoxide (DMSO), so we initially employed a DMSO control. In no case did incubation with a DMSO control cause release beyond that of the MFSW control at 25 °C, and the DMSO control was omitted in subsequent experiments.

Anemones treated with a pharmacological agent were held in that agent for 2.5 h at either 25 or 12 °C, then rinsed and transferred to MFSW at 25 °C. After 14 h, the percentage of cells released was determined.

The following pharmacological agents were employed: A23187, trifluoperazine (TFP), W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide,HCl] (Calbiochem), $NiCl_2$ (Mallinckrodt), $CoCl_2.6H_2O$ (Baker), 1,2-bis(*o*-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, sodium (BAPTA, dibromo-AM) (Molecular Probes), caffeine, procaine (Sigma), nimodipine, verapamil (gifts from Dr P. O'Lague), ryanodine, 2,5-di(+)(butyl)-hydroquinone (DTBHQ) (gifts from Dr M. Barish), ionomycin, thapsigargin (Calbiochem), okadaic acid, phorbol,12-myristate,13-acetate (PMA), isobutylmethylxanthine (IBMX) (Calbiochem) and ortho-sodium vanadate (Na_3VO_4) (Fisher). All agents were dissolved in MFSW except for A23187, ionomycin, BAPTA, DTBHQ, okadaic acid and PMA, which were dissolved in DMSO.

Identification of the product of release

The product released from *A. pulchella* (i.e. isolated algae; algae within host cells) was determined by incubating the anemones in the test solution with poly-L-lysine-coated coverslips (0.1 % in distilled water, M_r>70×10^3, Sigma). After bleaching had been induced and cell products had been released, the coverslips were removed and the nature of the released product was determined. Briefly, the coverslips were rinsed with FSW and stained either with 0.01 % fluorescein diacetate (Sigma) for 20 min for detection of host cell cytoplasm or with 0.01 % Hoechst 33258 (Sigma) for 5 min for detection of host cell nuclei (Gates et al., 1992). After staining, the coverslips were rinsed with FSW, mounted on slides and viewed on an Olympus BX-40 epifluorescence microscope.

Imaging intracellular Ca^{2+}

The concentration of Ca^{2+} in isolated host endoderm cells was determined by quantitative analysis of the fluorescence image of the Ca^{2+}-specific fluorochrome Fura-2AM (Calbiochem). Endodermal cells were isolated from *A. pulchella* using the trypsin maceration technique of Gates and Muscatine (Gates and Muscatine, 1992). Following maceration, endodermal cells were concentrated on coverslips coated either with poly-L-lysine (0.1 % in deionized water; M_r>70×10^3, Sigma) or concanavalin A (10 mg ml^{-1}, Sigma Type 5). Cells were then incubated for 1 h in 5 μmol l^{-1} Fura-2AM in FSW prepared from a stock solution of 10^{-3} mol l^{-1} Fura-2AM dissolved in DMSO. The cells were then rinsed three times with MFSW, and the color was allowed to develop for 30 min to 1 h before imaging.

The fluorescence image of $[Ca^{2+}]_i$ was obtained with a computer-assisted image-analysis system for the quantification of Fura-2AM fluorescence, as described by Sanderson et al. (Sanderson et al., 1990) and Charles et al. (Charles et al., 1991). Briefly, isolated cells were observed with a Nikon Diaphot microscope with a 40× oil-immersion objective (n.a. 1.3). Fluorescent video images, captured with a modified silicon-intensifier target (SIT) video camera (Cohu, San Diego, CA, USA), were recorded on an optical memory disk recorder (Panasonic TQ2026F). Maps of $[Ca^{2+}]_i$ were constructed by substituting the 340/380 image ratio (pixel by pixel) and changes in fluorescence at 380 nm into modified equations derived from those of Grynkiewicz et al. (Grynkiewicz et al., 1985) and Monck et al. (Monck et al., 1988). Images consisted of an average of five frames recorded at 340 and 380 nm. A thermal stage fitted with a Peltier device permitted investigation of the change in fluorescence of isolated host cells as a function of temperature over the range 12–35 °C.

^{45}Ca measurements

Changes in $[Ca^{2+}]_i$ during cold shock were also measured using ^{45}Ca. Two different experiments were performed; in the first, anemones were preincubated for 24 h in 2 ml of MFSW containing $^{45}CaCl_2$ (3.7×10^4 Bq ml^{-1}) (gift of Dr E. Gonzalez). The anemones were then rinsed with MFSW and either cold-shocked at 12 °C or held at 25 °C in 2 ml of MFSW for 2 h. In the second experiment, anemones were incubated in 2 ml of MFSW containing ^{45}Ca (7.4×10^4 Bq ml^{-1}) during the 2 h experiment. In both experiments, replicate 100 μl water samples were removed every 15 min throughout the 2 h incubation. At the end of the experiment, the anemones were homogenized in 1 ml of MFSW to separate the algae from the animal tissue. The homogenate was centrifuged at 500 ***g*** for 4 min (Damon/IEC model HN-S) to pellet the symbiotic algae. The supernatant containing the animal homogenate was removed, and replicate 100 μl samples were taken for counting in a LKB RackBeta scintillation counter (Bio Safe II scintillation cocktail). Samples (100 μl) were also taken for determination of animal protein concentration, and the amount of ^{45}Ca in the anemone was expressed as disints min^{-1} mg^{-1} soluble protein.

Determination of cyclic AMP concentration

The anemones (*N*=4) were heat-shocked for 16 h at 30 °C, cold-shocked for 2.5 h at 12 °C, incubated in 25 mmol l^{-1} caffeine for 2.5 h at 25 °C or held at 25 °C for 2.5 h in 2 ml of FSW. After the treatment, the anemones were quickly rinsed in FSW, blotted on weighing paper, placed in a 1.5 ml microfuge tube, flash-frozen in liquid nitrogen and then

homogenized in 1 ml of 6% trichloroacetic acid. The homogenate was centrifuged at 500 ***g*** for 4 min (Damon/IEC, model HN-S) to pellet the symbiotic algae. The supernatant was retained for use in the cyclic AMP (cAMP) assay using the Biotrak cAMP enzyme immunoassay system (Amersham). The pellet was retained for protein determination. The results are expressed as fmol cAMP mg^{-1} protein.

Labeling proteins with ^{32}P

To label anemone proteins with ^{32}P-labeled orthophosphate (1 mCi; 3.7×10^7 Bq ml^{-1}, Amersham), the anemones were rinsed three times in phosphate-free artificial sea water (ASW), placed in 1 ml of phosphate-free ASW and briefly allowed to expand. The anemones were then irrigated with 100 μCi (3.7×10^6) Bq ml^{-1} of ^{32}P and incubated for 4 h in the dark at 12, 25 or 30 °C or incubated in 10 mmol l^{-1} caffeine at 25 °C. After incubation, the anemones were homogenized in a low-salt buffer (40 mmol l^{-1} Tris, 10 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF, 10 mmol l^{-1} NaF, 2 mmol l^{-1} EGTA and 1 mmol l^{-1} vanadate) containing 1 μg ml^{-1} of the protease inhibitors pepstatin, leupeptin and chymostatin (Sigma). The homogenate was centrifuged at 500 ***g*** for 4 min (Damon/IEC model HN-S) to pellet the symbiotic algae, and the supernatant was retained for protein determination and determination of radioactivity (10 μl samples were counted in a LKB RackBeta scintillation counter; Bio Sage II scintillation cocktail).

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed with a Pharmacia Biotech Multiphor II apparatus following the manufacturer's instructions. An equal number of counts of ^{32}P was loaded on the immobiline DryStrips (pH 3–10, Pharmacia Biotech) for the isoelectric focusing dimension. When the second dimension (gel electrophoresis) was finished, the gels (Excel Gel SDS, gradient 8-18, Pharmacia Biotech) were dried and autoradiographed on X-ray film for 14 days. Broad-range standards (BioRad) were used for molecular mass determination. The autoradiographs were scanned into Adobe PhotoShop, the images were colored and landmark proteins were superimposed to assist in determining which proteins were unique to each treatment.

Protein measurements

The soluble protein concentration of the anemone homogenate was determined by the method of Bradford (Bradford, 1976) or using the DC Protein Assay (BioRad). In addition, any protein released into the medium by cold-shocked aposymbiotic anemones was also determined by the Bradford method.

Statistical analyses

The mean and standard deviation of the mean (S.D.) were calculated for the data. In addition, significant differences between treatments were determined either by *t*-test or by analysis of variance (ANOVA) using the program Statview.

Results

Electron paramagnetic resonance measurements of membrane fluidity

To determine whether there was a phase transition in isolated host cell plasma membranes from *A. pulchella* and *P. damicornis* in response to temperature stress, we measured the EPR signal in isolated membranes from *A. pulchella* and from *P. damicornis* labeled with 0.1 μmol l^{-1} TEMPO. The transformed data from animal plasma membranes from *A. pulchella* labeled with the spin probe TEMPO yield a linear relationship from 0 to 40 °C (Fig. 1). As the membrane preparation from *P. damicornis* consistently reduced the spin probe and the unknown reducing agent was not responsive to addition of 5 mmol l^{-1} ferric cyanide, measurements were limited to temperatures from 0 to 15 °C (Fig. 1). These data also yield a linear relationship (r^2=0.98 for each) indicating no membrane phase transition in plasma membranes from either *A. pulchella* or *P. damicornis* (Fig. 1).

The effects on intact animals of pharmacological agents that perturb [Ca^{2+}]

We tested the possibility that $[Ca^{2+}]_i$ could be altered by temperature stress by applying pharmacological agents to intact animals at 25 °C and observing the effect on release of host cells. These results were compared with results obtained from anemones held at their growth temperature (25 °C) and with results from cold-shocked anemones (12 °C). The results of treatment of the anemones with pharmacological agents are summarized in Table 1. To determine whether cold shock evoked entry of Ca^{2+} through Ca^{2+} channels, we treated intact anemones before and during cold shock with the Ca^{2+} channel blockers nimodipine, verapamil, Ni^{2+} and Co^{2+}. None of these agents prevented release of host cells. Next, we treated intact anemones at 25 °C with the Ca^{2+} ionophores A23187 and ionomycin. Neither caused a significant release of host cells, nor did the calmodulin antagonists W-7 and TFP (Uyeda and Furuya, 1986).

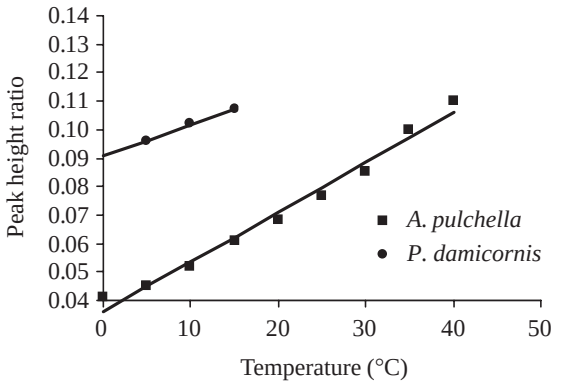

Fig. 1. Peak height ratio (height of the lipid signal/height of the water signal) of the electron paramagnetic resonance spectrum generated from membranes from *Aiptasia pulchella* and *Pocillopora damicornis* and labeled with 0.1 μmol l^{-1} TEMPO over the temperature range 0–40 °C. r^2=0.98 for each.

Table 1. *The ability of various pharmacological agents to evoke host cell detachment in* Aiptasia pulchella

Agent	Concentration range (mol l^{-1})	Added during cold shock	Effect
Ca^{2+} channel blockers			
Nimodipine	10^{-7} to 10^{-6}	Yes	Release not prevented (P=0.42)
Verapamil	10^{-7} to 10^{-5}	Yes	Release not prevented (P=0.31)
Ni^{2+}	10^{-5} to 10^{-3}	Yes	Release not prevented (P=0.19)
Co^{2+}	5×10^{-4} to 5×10^{-2}	Yes	Release not prevented (P=0.24)
Ca^{2+} ionophores			
A23187	10^{-7} to 10^{-5}	No	No release (P=0.45)
Ionomycin	10^{-7} to 10^{-5}	No	No release (P=0.70)
Second messenger-pathway agents			
W-7	10^{-6} to 10^{-5}	No	No release (P=0.93)
TFP	10^{-7} to 10^{-5}	No	No release (P=0.25)
Ca^{2+}-ATPase inhibitors			
Thapsigargin	10^{-8} to 10^{-6}	No	No release (P=0.54)
DTBHQ	10^{-6} to 10^{-4}	No	No release (P=0.92)
Ca^{2+} store releasing agents			
Caffeine	10^{-5} to 2.5×10^{-2}	No	Caused release (P=0.01)
Ryanodine	10^{-5} to 3×10^{-4}	No	Release not prevented (P=0.33)
Procaine	10^{-5} to 3×10^{-3}	Yes	Release not prevented (P=0.42)
Ca^{2+} chelator			
BAPTA	10^{-5} to 10^{-3}	Yes	Release not prevented (P=0.82)

To determine whether cold shock evoked release of Ca^{2+} from intracellular stores, we used the Ca^{2+}-ATPase inhibitors thapsigargin and DTBHQ (Moore et al., 1987; Kass et al., 1989; Thastrup et al., 1989; Thastrup, 1990; Thastrup et al., 1990; Holliday et al., 1991; Lytton et al., 1991). Neither thapsigargin nor DTBHQ caused a significant release of host cells.

We then tested the effect of caffeine, which causes the release of Ca^{2+} from intracellular stores (Uyeda and Furuya, 1986; Klein et al., 1992; Lee, 1993). Fig. 2 shows that 10 mmol l^{-1} caffeine evokes release of symbiotic algae at 25 °C, and at 25 mmol l^{-1} the percentage release is equal to that of the cold-shock control (P<0.01). Moreover, the longer the anemones are exposed to caffeine, the more cells are released (Fig. 3). In addition, caffeine evokes the release of entire host cells

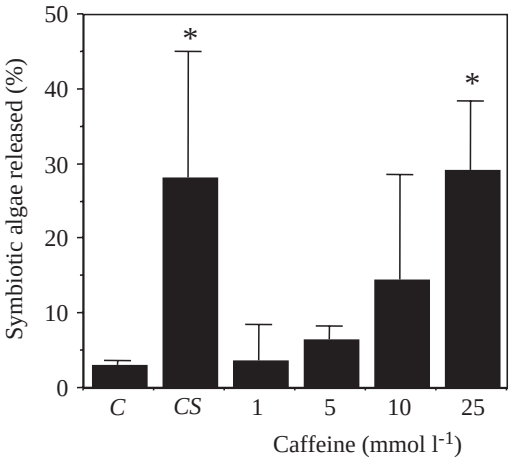

Fig. 2. The effect of a 2.5 h treatment with different concentrations of caffeine on release of symbiotic algae from *Aiptasia pulchella*. The control (*C*) anemones were held at 25 °C, while the cold-shocked anemones (*CS*) were held at 12 °C for 2.5 h. Cell release is expressed as a percentage of the total number of symbiotic dinoflagellates in the anemone [(released/released+retained)×100] released into the medium. Values are means + S.E.M., N=15 in each condition. *Significantly different from the control value, $P\leqslant0.01$.

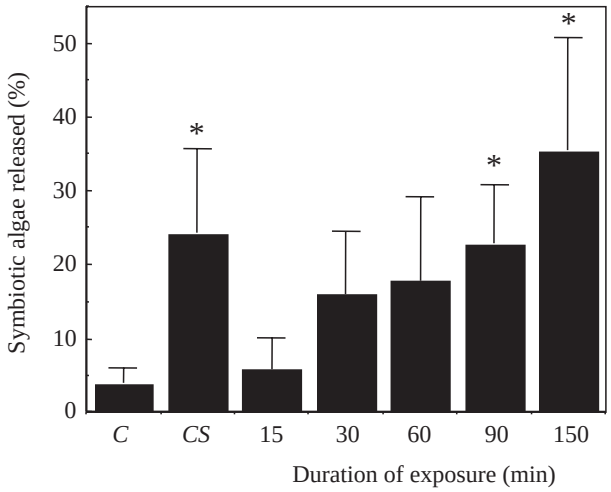

Fig. 3. The effect of varying the duration of exposure to 25 mmol l^{-1} caffeine on release of symbiotic algae from *Aiptasia pulchella*. The control anemones (*C*) were held at 25 °C, while the cold-shocked anemones (*CS*) were held at 12 °C for 2.5 h. Cell release is expressed as a percentage of the total number of symbiotic dinoflagellates in the anemone [(released/released+retained)×100] released into the medium. Values are means + S.E.M., N=5 in each condition. *Significantly different from the control value, $P\leqslant0.01$.

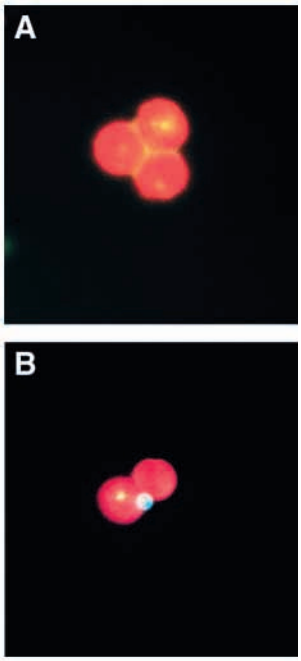

Fig. 4. Photomicrographs of the product of release from *Aiptasia pulchella* after treatment with 25 mmol l^{-1} caffeine. In A, the product was stained with 0.01 % fluorescein diacetate (×4000). Host cell cytoplasm is visible at the interstices of the algae; in B, the product was stained 0.01 % Hoechst 33258 (×4000). The host cell nucleus is visible adjacent to the algal doublet.

containing the endosymbiotic algae (Fig. 4). When the cells released from anemones treated with 25 mmol l^{-1} caffeine are stained with fluorescein diacetate, the esterases in the host cell cytoplasm cleave the dye to its active state (Fig. 4A). The host cell cytoplasm is revealed as yellow fluorescence in the interstices between the red fluorescent algae. When the released cells are stained with the DNA-specific dye Hoechst 33258, the host cell nucleus can be seen adjacent to the algal doublet (Fig. 4B), with the algae occupying most of the interior of the host cell. This unusual architecture is described in detail elsewhere (Gates et al., 1992). Finally, aposymbiotic anemones treated with caffeine tended to release more protein to the surrounding sea water than the 25 °C controls, although this difference was not statistically significant (Fig. 5, P<0.094). Increased protein release is interpreted as the result of release and subsequent disintegration of endodermal cells.

Because caffeine releases Ca^{2+} from ryanodine-sensitive stores (Uyeda and Furuya, 1986; Klein et al., 1992; Lee, 1993), we tested whether ryanodine could block the caffeine-induced release of cells. Ryanodine was not able to block the caffeine-induced release of cells (P=0.33). Cell release from anemones cold-shocked in the presence of procaine, which can block Ca^{2+} release from caffeine-sensitive stores (Kitamura et al., 1986; Klein et al., 1992), was also not affected (P=0.42) (Table 1).

Finally, to test generally whether Ca^{2+} is involved, we added the Ca^{2+} chelator BAPTA during cold shock. BAPTA binds Ca^{2+} before it can exert a physiological effect (Dubinsky, 1993; Snow and Nuccitelli, 1993), and BAPTA did not prevent the release of host cells evoked by cold shock (P=0.82) (Table 1).

Observation of isolated host cells by Ca^{2+} imaging

To gain further insight into the role of caffeine in evoking host cell release, we used the Ca^{2+}-sensitive fluorochrome Fura-2AM to monitor $[Ca^{2+}]_i$ in isolated host cells during the application of selected pharmacological agents. Addition of ionomycin, which does not cause bleaching in *A. pulchella*, increased $[Ca^{2+}]_i$ from the resting level of 68±30 to 570.0±191.4 nmol l^{-1} within 60 s of its application to isolated host cells (Fig. 6). In contrast, 12 min after the addition of caffeine, $[Ca^{2+}]_i$ was 114.7±61.5 nmol l^{-1} (Fig. 6). These data provide direct evidence that caffeine, while able to induce bleaching in *A. pulchella*, does not cause an increase in $[Ca^{2+}]_i$ in isolated host cells from *A. pulchella*.

Attempts to measure $[Ca^{2+}]_i$ as a function of temperature were thwarted because the fluorescence of Fura-2AM itself is temperature-sensitive (Fig. 7). Any change in $[Ca^{2+}]_i$ we

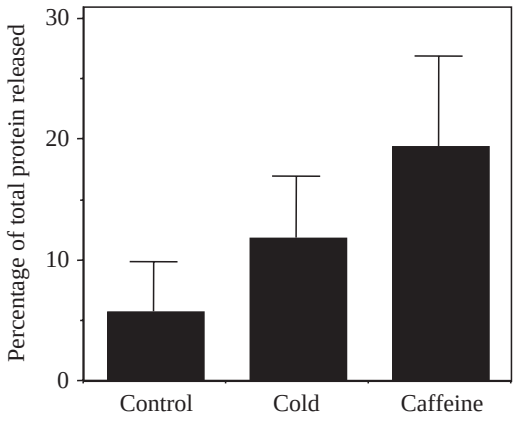

Fig. 5. The effect of a 2.5 h treatment with 25 mmol l^{-1} caffeine on the percentage of total protein released from aposymbiotic *Aiptasia pulchella*. The control anemones were held at 25 °C, while the cold-shocked anemones were held at 12 °C for 2.5 h. Values are means + S.E.M., N=6 in each condition.

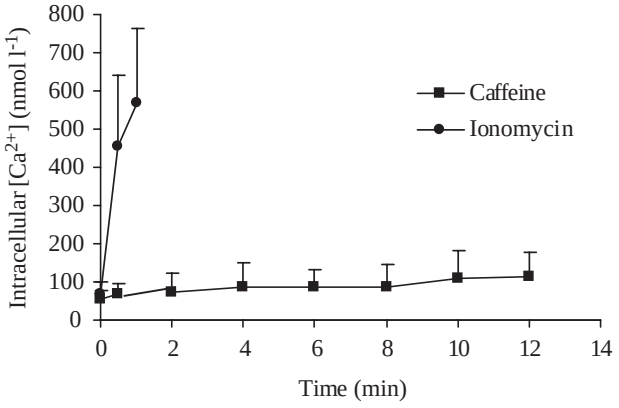

Fig. 6. The intracellular Ca^{2+} concentration in isolated host cells from *Aiptasia pulchella* treated with either 10^{-6} mol l^{-1} ionomycin or 25 mmol l^{-1} caffeine. Ca^{2+} values were obtained from computer analysis of stored images of cells labeled with Fura-2AM. Values are means + S.E.M., N=20 for ionomycin-treated cells; N=12 for caffeine-treated cells.

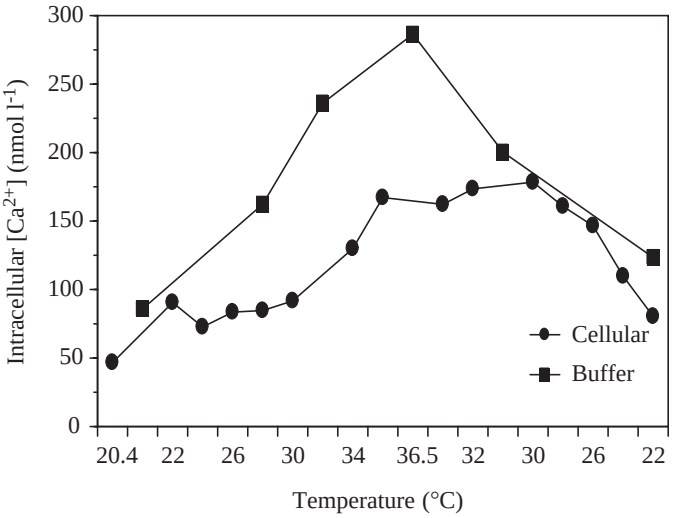

Fig. 7. The intracellular Ca^{2+} concentration in isolated host cells (N=30) from *Aiptasia puchella* and the Ca^{2+} concentration of a buffer containing 150 µmol l^{-1} Ca^{2+} obtained by Ca^{2+} imaging using the dye Fura-2AM during temperature stress. The cells were subjected to a temperature stress from 20.4 to 36° C and back to 22 °C, while the buffer was subjected to a temperature change from 21.6 to 36.5 °C and back to 22 °C.

observed in isolated host cells was within the temperature-sensitive curve for Fura-2AM.

^{45}Ca measurements on intact animals

Using the dye Fura-2AM, we were able to image Ca^{2+} fluxes only over periods of 15 min or less because of quenching of the fluorescence. We used ^{45}Ca to investigate Ca^{2+} fluxes over longer periods. In anemones incubated in ^{45}Ca during cold shock, the flux of ^{45}Ca into the anemones did not differ from that of the control anemones. In the control animals, there was a 3.5 % decrease in $[Ca^{2+}]$, while in the cold-shocked anemones there was a 4 % decrease. In addition, the total amount of ^{45}Ca in the cold-shocked anemones (animal tissue) was $5.7\times10^5\pm5.5\times10^5$ disints min^{-1}, which is not significantly different from that in the control anemones ($3.4\times10^5\pm2.1\times10^5$ disints min^{-1}; P=0.28; unpaired t-test). In anemones preincubated in ^{45}Ca, there was no difference between control and cold-shocked anemones either in release of Ca^{2+} or in the amount of Ca^{2+} in the anemones at the end of the cold shock (data not shown).

Measurement of cAMP concentration

Caffeine can affect protein phosphodiesterase activity as well as intracellular Ca^{2+} levels, so we next tested whether caffeine induced host cell detachment in the sea anemone *A. pulchella* by inhibiting phosphodiesterases, leading to an increase in the concentration of cAMP. The cAMP concentration in the animal homogenate was measured after a 2.5 h incubation in 25 mmol l^{-1} caffeine and compared with the cAMP concentration in control anemones held at 25 °C, their growth temperature. Fig. 8 shows that caffeine induced an

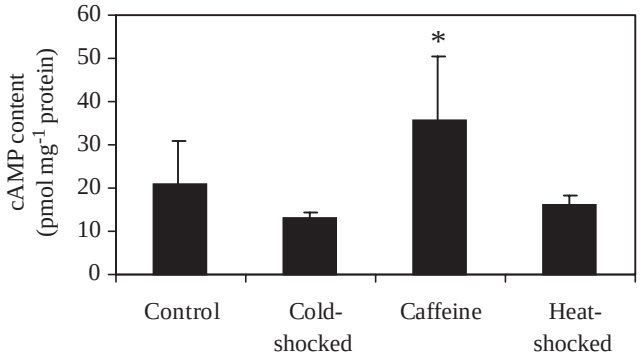

Fig. 8. The amount of cAMP (pmol mg^{-1} protein) in the sea anemone *Aiptasia pulchella* cold-shocked at 12 °C, held at 25 °C (Control), incubated in 25 mmol l^{-1} caffeine at 25 °C for 2.5 h or heat-shocked at 30 °C for 14 h. Values are means + S.E.M., N=4. *Significantly different from the control value, $P\leqslant0.05$.

increase in cAMP concentration compared with the control (P<0.05). To test whether temperature shock acted like caffeine and increased cAMP concentration, anemones were either heat-shocked (30 °C) nor cold-shocked (12 °C), and the cAMP concentration in the animal homogenate was assessed and compared with the cAMP concentration in control anemones. Fig. 8 shows that neither heat shock nor cold shock increased cAMP concentration compared with the control. Both the cold- and heat-shocked anemones had a lower intracellular concentration of cAMP than did the controls, although these differences were not statistically significant (P=0.38). This suggests that cAMP is not involved in the induction of host cell detachment by thermal stress.

The effects of pharmacological agents that affect protein phosphorylation in intact anemones

Caffeine, which increases cAMP concentrations by inhibiting phosphodiesterases, has a general effect on intracellular protein phosphorylation. We exposed *A. pulchella* to other pharmacological agents that affect protein phosphorylation (Table 2). IBMX, a phosphodiesterase inhibitor, had no effect on cell release from *A. pulchella* (P=0.90), nor did the protein kinase C activator PMA (P=0.79).

Table 2. *The effects of pharmacological agents on cell host release from the sea anemone* Aiptasia pulchella

Agent	Concentration (mol l^{-1})	Result
Phosphodiesterase inhibitor		
Isobutylmethylxanthine	10^{-6} to 10^{-3}	No release (P=0.90)
Protein kinase C activator		
Phorbol ester (PMA)	10^{-10} to 10^{-7}	No release (P=0.79)
Phosphatase inhibitors		
Okadaic acid	10^{-7} to 10^{-6}	No release (P=0.42)
Vanadate	10^{-5} to 10^{-3}	Caused release (P=0.004)

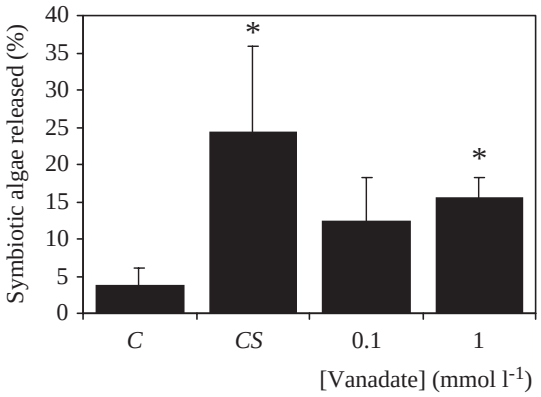

Fig. 9. The effect of a 2.5 h treatment with two concentrations of vanadate on the release of symbiotic algae from *Aiptasia pulchella*. The control anemones (*C*) were held at 25 °C, while the cold-shocked anemones (*CS*) were held at 12 °C for 2.5 h. Cell release is expressed as a percentage of the total number of symbiotic dinoflagellates in the anemone [(released/released+retained)×100] released into the medium. Values are means + S.E.M., N=4 in each condition. *Significantly different from the control value, $P \leqslant 0.0004$.

Of the two phosphatase inhibitors tested, okadaic acid did not affect release of cells (P=0.42), but 1 mmol l^{-1} vanadate caused release of 15.5±2.7 % (P=0.004) of the symbiotic algae (Fig. 9). Higher concentrations of vanadate had no further effect, and vanadate was never as effective as temperature or caffeine in causing release of cells.

^{32}P-phosphorylated proteins

To test directly whether temperature alters intracellular protein phosphorylation in *A. pulchella*, anemones were held at 25 °C, cold-shocked (12 °C), heat-shocked (30 °C) or incubated in 10 mmol l^{-1} caffeine in the presence of ^{32}P, and the animal protein was extracted and subjected to two-dimensional gel electrophoresis (Fig. 10). To facilitate comparison among treatments, gels were superimposed using landmark proteins. Although the superimposition was not always perfect, many ^{32}P-labeled proteins unique to each treatment were easily distinguished.

Comparison of the ^{32}P-labeled proteins between control and cold-shocked, control and caffeine-treated and cold-shocked and caffeine-treated anemones revealed a number of proteins unique to each treatment (Fig. 10) (Sawyer, 1998). The pattern of protein phosphorylation in the control anemones differs from that in the cold-shocked anemones, and the patterns in both control and cold-shocked anemones differ from that in the caffeine-treated anemones. The protein phosphorylation pattern from the heat-shocked anemones was not determined because of consistently poor incorporation of ^{32}P by these anemones. The molecular mass of the proteins that differed between the treatments ranged from 5 to 100 kDa (Fig. 10), and the number of unique proteins in the control and cold-shocked anemones ranged from 17 to 34. This is a conservative estimate; because of the slight offset in landmark proteins between gels, the number of unique proteins was deliberately underestimated.

Discussion

In the present study, we investigated the mechanism by which increased or decreased temperature causes loss of host cells and results in cnidarian bleaching. We initially tested the hypothesis that increased or decreased temperature induced a membrane thermotropic event in host cell membranes. This thermotropic event would cause an increase in $[Ca^{2+}]_i$, and this increase would induce cell adhesion dysfunction of the host cells (Gates et al., 1992). We saw no evidence of a phase transition, as measured by EPR, in isolated host cell membranes from the sea anemone *A. pulchella* and the coral

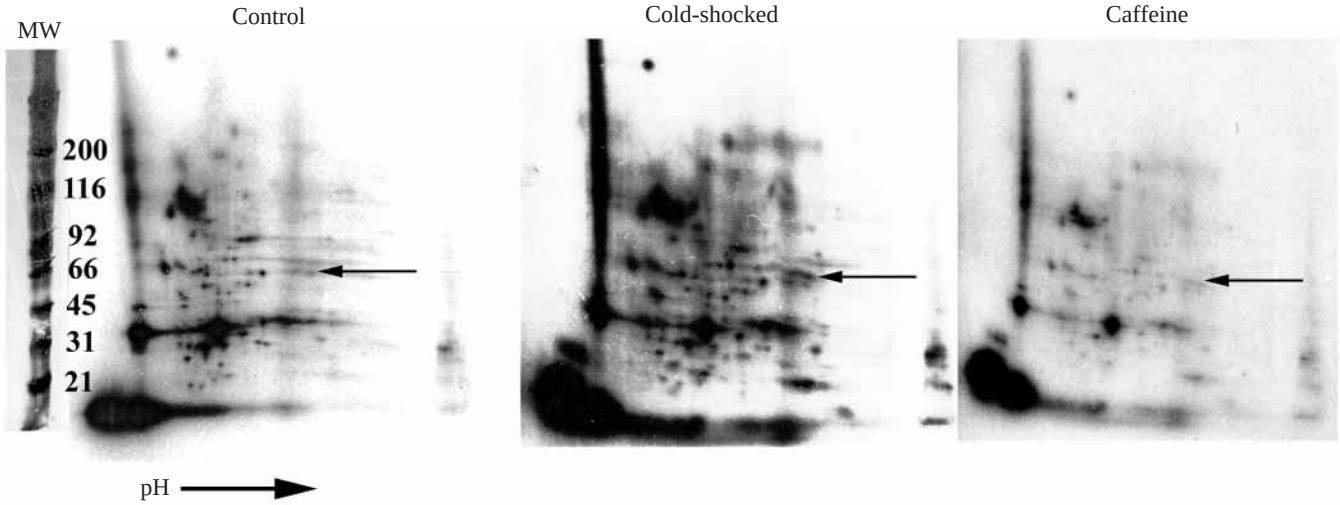

Fig. 10. Autoradiographs of ^{32}P-phosphorylated proteins separated by two-dimensional gel electrophoresis from the control value anemones held at 25 °C, anemones cold-shocked at 12 °C and anemones treated with 25 mmol l^{-1} caffeine for 2.5 h. The arrows point to a group of proteins that are of equal molecular mass (MW) but are differentially phosphorylated.

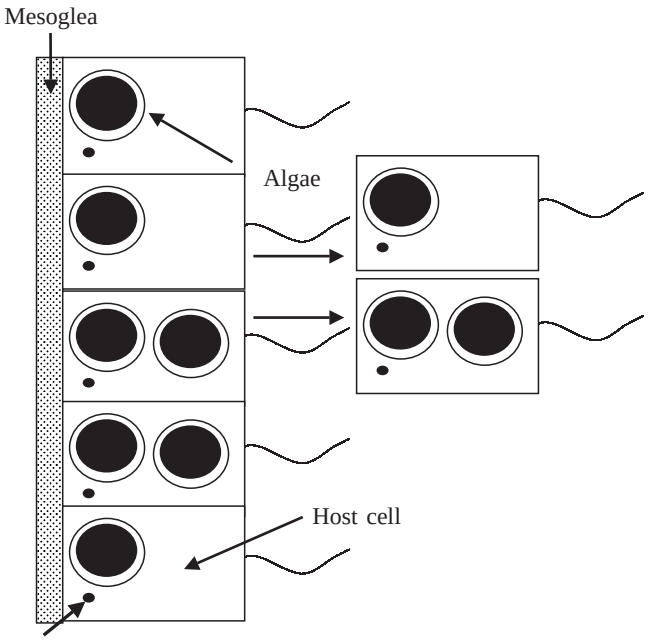

Fig. 11. Schematic representation of how cell adhesion is controlled in the tropical sea anemone *Aiptasia pulchella*, indicating that increased and decreased temperature as well as altered protein phosphorylation induce host cell detachment.

P. damicornis over the temperatures measured. Perturbing intracellular Ca^{2+} stores with pharmacological agents did not affect the bleaching response, and we did not observe an increase in $[Ca^{2+}]_i$ using either the calcium dye Fura-2AM or ^{45}Ca during temperature shock. Caffeine, which can release Ca^{2+} from intracellular stores as well as affect protein phosphorylation levels (Uyeda and Furuya, 1986; Klein et al., 1992; Lee, 1993), can cause host cell detachment in *A. pulchella* without affecting $[Ca^{2+}]_i$. In addition, vanadate, a protein phosphatase inhibitor, can induce bleaching in *A. pulchella*. Measurement of protein phosphorylation using two-dimensional gel electrophoresis of ^{32}P-labeled proteins demonstrates that cold-shocked, caffeine-treated and control anemones have different levels of phosphorylated host proteins. This suggests that temperature-induced cnidarian bleaching alters the protein phosphorylation of the host and, by this mechanism, induces the loss of host cells (Fig. 11).

EPR measurement of membrane fluidity

We tested the hypothesis that temperature-induced host cell detachment in symbiotic cnidarians begins with a membrane thermotropic event. Muscatine et al. (Muscatine et al., 1991) observed a break at 15 °C in the Arrhenius plot of rate of release of symbiotic dinoflagellates *versus* temperature. We saw no evidence for a membrane phase transition between 0 and 40 °C (Fig. 1) in plasma membranes from *A. pulchella*. Isolated plasma membranes from *P. damicornis* labeled with TEMPO also show no evidence of a membrane phase transition (Fig. 1), although the temperature test range was restricted to 0–15 °C because of the unexplained reduction of the spin probe TEMPO. The addition of ferric cyanide did not halt this reduction, so we conclude that the reduction of the probe was not the result of mitochondrial activity, but of an unknown agent. The absence of a phase change in *P. damicornis* over this limited range of temperature is consistent with similar data from *A. pulchella*.

The absence of a phase transition in our data could be explained in several ways. First, phase transitions in plasma membranes are common only at very low temperatures (Blazyk and Steim, 1972; Quinn, 1988), so we may not have used a low enough temperature; however, 0 °C is below the lethal cold temperature for both *A. pulchella* and *P. damicornis*, so a phase transition at a temperature below that would be ecologically meaningless. In addition, plasma membranes often contain significant amounts of cholesterol, which stabilize membrane fluidity below the gel-to-liquid-crystalline transition temperature (Chapman et al., 1979; Yeagle, 1987). As cholesterol makes up approximately 17 % of the lipids in *A. pulchella* (Doino, 1991), it may help to stabilize the membranes during cold shock. Further, temperature-induced phase transitions in membranes have been reported mainly in specialized membranes, such as those from spermatozoa, chloroplasts and mitochondria (Raison et al., 1971; Blazyk and Steim, 1972; Quinn, 1989; Jurado et al., 1991; Ruiz-Sanz et al., 1992; Parks and Lynch, 1992; Drobnis et al., 1993). These specialized membranes contain a higher proportion of protein and also large amounts of specific phospholipids, making them more susceptible to a temperature-induced membrane phase transition (Quinn, 1988; Quinn, 1989). Thus, the absence of a phase transition in plasma membranes from *A. pulchella* and *P. damicornis* is typical of other plasma membranes.

The effects on intact animals of pharmacological agents that perturb $[Ca^{2+}]_i$

To determine whether changes in $[Ca^{2+}]_i$ were involved in temperature-induced host cell detachment in *A. pulchella*, we used a variety of pharmacological agents to alter intracellular Ca^{2+} pathways. If we could cause host cell detachment with an agent known to affect a Ca^{2+} pathway, we might gain insight into how temperature causes loss of host cell adhesion. We used six different types of pharmacological agents: Ca^{2+} channel blockers, Ca^{2+} ionophores, second-messenger pathway agents, Ca^{2+}-ATPase inhibitors, Ca^{2+} chelators and Ca^{2+} store releasing agents (Table 1). All have been used successfully to implicate Ca^{2+} involvement in cells as diverse as melanoma metastatic cells to flagellates (Meissner, 1986; Uyeda and Furuya, 1986; Grega et al., 1987; Kass et al., 1989; Thastrup, 1990; D'Ancona et al., 1992; Klein et al., 1992; Dubinsky, 1993; Simasko and Yan, 1993; Snow and Nuccitelli,

1993). In *A. pulchella*, the only pharmacological agent that induced host cell release was caffeine, an agent that can induce Ca^{2+} release from stores in the endoplasmic reticulum (Uyeda and Furuya, 1986; Klein et al., 1992; Lee, 1993). The action of caffeine on *A. pulchella* is similar to that of temperature shock in several ways. Increasing concentrations of caffeine increased cell release (Fig. 2) just as more extreme (farther from the growth temperature) temperature shocks increase cell release (Muscatine et al., 1991). In addition, increased exposure time to caffeine increased cell release (Fig. 3) as does increased duration of temperature shock. Caffeine treatment induces release of host cells identical in appearance to those released after temperature shock (see Gates et al., 1992). Finally, aposymbiotic anemones are sensitive to caffeine (Fig. 4) just as they are to acute changes in temperature.

Besides causing Ca^{2+} release from the endoplasmic reticulum, caffeine can also inhibit the enzyme phosphodiesterase (Uyeda and Furuya, 1986; Klein et al., 1992; Lee, 1993). To determine caffeine's action on *A. pulchella*, we used two different pharmacological agents, procaine and ryanodine, that can interact with caffeine-induced Ca^{2+} release. Procaine can block Ca^{2+} release from caffeine-sensitive stores (Kitamura et al., 1986; Klein et al., 1992), but did not affect cell release during cold shock. Ryanodine is capable of blocking Ca^{2+} stores opened by caffeine (Meissner, 1986), but did not inhibit caffeine-induced cell release. These data, as well as the failure to see a change in release in response to any of the other Ca^{2+} pathway altering agents, call into question whether caffeine is inducing host cell detachment by inducing changes in intracellular Ca^{2+} concentrations.

Ca^{2+} imaging of isolated host cells

Using isolated host cells loaded with the intracellular Ca^{2+} dye Fura-2AM, we directly investigated the involvement of Ca^{2+} in temperature-induced bleaching. Cells treated with ionomycin (Fig. 6) showed a fivefold increase in $[Ca^{2+}]$ within 30 s, indicating that this is a viable method for measuring $[Ca^{2+}]_i$ in isolated host cells, yet ionomycin did not induce bleaching. When cells were treated with 25 mmol l^{-1} caffeine, no change in $[Ca^{2+}]_i$ was detected (Fig. 6) even after 12 min. This again suggests that caffeine does not act on *A. pulchella* by inducing Ca^{2+} release from the endoplasmic reticulum.

We also used Fura-2AM to investigate how temperature affected $[Ca^{2+}]_i$; however, these results were inconclusive (Fig. 7) because the binding constant of Ca^{2+} to the dye is affected by temperature (Groden et al., 1991; Owen, 1991).

Fang et al. (Fang et al., 1997), using Fura-2AM, observed an increase in Ca^{2+} concentration in isolated cells from the coral *Acropora grandis* during heat treatment; however, they used cells that did not contain symbiotic algae. Huang et al. (Huang et al., 1998) demonstrated that bleaching in *A. grandis* required intracellular Ca^{2+}. Thus, while it is possible that some cell types in corals release Ca^{2+} from intracellular stores with heat stress, in our hands, isolated host cells from the sea anemone *A. pulchella* showed no measurable change in $[Ca^{2+}]_i$ during temperature stress.

^{45}Ca measurements on intact animals

The duration of Ca^{2+} imaging with Fura-2AM is limited because of quenching of Ca^{2+} fluorescence, so we used ^{45}Ca to investigate long-term fluxes of Ca^{2+} in cold-shocked anemones. ^{45}Ca has been used successfully to evaluate Ca^{2+} fluxes in both sea anemones and corals (Tambutte et al., 1995; Allemand and Benazet-Tambutte, 1996; Benazet-Tambutte et al., 1996). When anemones were incubated in ^{45}Ca, to load Ca^{2+} stores with isotope, and then cold-shocked, release of ^{45}Ca by the cold-shocked anemones did not differ from that of control anemones. In addition, when anemones were cold-shocked in the presence ^{45}Ca to test for uptake of Ca^{2+}, no difference between control and cold-shocked anemones was detected. These data suggest that temperature shock (either warm or cold) does not induce an alteration in intracellular Ca^{2+} levels.

Measurement of cAMP

Temperature shock induces host cell detachment, i.e. bleaching, in the sea anemone *A. pulchella* by an unknown mechanism. Caffeine can also induce host cell detachment in this anemone, suggesting that caffeine and temperature may act on cell adhesion at a common locus. Inhibition of phosphodiesterase activity by caffeine can increase the concentration of cAMP, which can induce loss of cell adhesion in various mammalian cells (Hsie and Puck, 1971; Ortiz et al., 1973; Steinbach and Schubert, 1975; Miller et al., 1976; Li et al., 1977; Spruill et al., 1981; Kreisberg and Venkatachalam, 1986; Lamb et al., 1988; Glass and Kreisberg, 1993). Thus, temperature and caffeine could induce host cell detachment in *A. pulchella* by causing an increase in cAMP concentration. However, the data in Fig. 8 show that an increase in cAMP concentration in *A. pulchella* is evoked by caffeine, but not by heat or cold shock, suggesting that the modes of action of caffeine and temperature stress in causing cnidarian bleaching differ.

The effects on intact anemones of pharmacological agents that affect protein phosphorylation

An increased cAMP concentration induced by caffeine would stimulate protein kinase A activity and alter protein phosphorylation. Caffeine can enhance the tyrosine phosphorylation of proteins in cell culture (Aharoni et al., 1993). As phosphorylation of cell adhesion molecules controls adhesion (Volberg et al., 1991; Eriksson et al., 1992; Matsuyoshi et al., 1992; Romer et al., 1992; Volberg et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993) and phosphorylation of proteins is temperature-sensitive (Maher and Pasquale, 1989; Keyse and Emslie, 1992), temperature and caffeine may both act by altering the phosphorylation of cell adhesion proteins.

The pharmacological agent IBMX was unable to induce release of cells from *A. puchella* (Table 2). IBMX, like caffeine, is a phosphodiesterase inhibitor, but the two agents have different potencies. IBMX may be more effective at evoking Ca^{2+} release from intracellular stores than inhibiting

phosphodiesterase (Ishitani et al., 1995; Usachev and Verkhratsky, 1995), while caffeine may be a better phosphodiesterase inhibitor. The protein phosphatase inhibitor vanadate did induce release of cells (Fig. 9), while okadaic acid and PMA did not (Table 2). Vanadate is an inhibitor of tyrosine phosphatase, while both okadaic acid and PMA affect serine/threonine phosphorylation (Brautigan, 1992; Matsuyoshi et al., 1992; Walter and Mumby, 1993; Tsunoda, 1993). Thus, it is possible that vanadate and caffeine (Aharoni et al., 1993) can induce algal cell loss from *A. pulchella* by affecting tyrosine phosphorylation levels.

Patterns of ^{32}P protein phosphorylation

The pattern of incorporation of ^{32}P into proteins is different in cold-shocked and control anemones as determined by two-dimensional gel electrophoresis. This difference supports the hypothesis that temperature-induced host cell detachment results from altered protein phosphorylation. In addition, the labeling patterns of the ^{32}P-labeled proteins in the two-dimensional gels from the caffeine-treated anemones are different from those from control anemones, supporting the hypothesis that caffeine can affect the phosphorylation of protein. However, the pattern of ^{32}P-labeled proteins in the two-dimensional gels of the cold-shocked anemones is not the same as that obtained from the caffeine-treated anemones. This suggests that, while caffeine may induce host cell detachment by altering the phosphorylation of proteins, it may affect different proteins from those affected by temperature shock. On the basis of the pharmacological studies and the patterns of protein phosphorylation revealed by two-dimensional gel electrophoresis, it is clear that temperature-induced host cell detachment in *A. pulchella* is correlated with altered protein phosphorylation. It is not known which proteins are differentially phosphorylated during temperature shock in *A. pulchella*.

We were unable to resolve phosphorylated proteins in heat-shocked anemones because incorporation of ^{32}P during high-temperature stress was poor, nor did we determine whether the ^{32}P was incorporated on tyrosine or serine/threonine residues. Caffeine, vanadate and temperature affect protein phosphorylation on tyrosine residues in other systems (Maher and Pasquale, 1989; Brautigan, 1992; Aharoni et al., 1993). Altered tyrosine phosphorylation of the cadherin and catenin cell adhesion molecules can perturb their adhesiveness (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). Thus, temperature shock in *A. pulchella* may alter the levels of tyrosine phosphorylated cell adhesion proteins.

Temperature could affect protein phosphorylation by affecting kinase and phosphatase activity. There is little information about the temperature-sensitivities of these enzymes; smooth muscle phosphatase and myosin-associated muscle phosphatase have Q_{10} values of 5.3 and 5.2, respectively (Mitsui et al., 1994). Such a high Q_{10} indicates that these enzymes would be very temperature-sensitive (Hochachka and Somero, 1982). Heat shock also induces the synthesis of a tyrosine phosphatase in cultured human skin cells, suggesting that the control of phosphorylation levels is important during environmental stress (Keyse and Emslie, 1992).

Temperature stress is not the only environmental factor that induces bleaching in symbiotic cnidarians; ultraviolet radiation can also cause bleaching (Lesser et al., 1990; Gleason and Wellington, 1993; Brown et al., 1994; Le Tissier and Brown, 1996). Ultraviolet radiation can induce oxidative stress in sea anemones and corals (Shick et al., 1991; Dykens et al., 1992; Nii and Muscatine, 1997). Oxidative stress can alter protein phosphorylation and impair cell adhesion in cell culture (Heffetz et al., 1990; Gailit et al., 1993; Barchowsky et al., 1994; Sullivan et al., 1994; Zhang et al., 1994; Feng et al., 1995; Whisler et al., 1995), so it may do so in symbiotic cnidarians. Oxidative stress stimulates both tyrosine and serine/threonine phosphorylation by inhibiting the respective protein phosphatases (Heffetz et al., 1990; Sullivan et al., 1994; Whisler et al., 1995). In addition, oxidative stress stimulates the expression of a tyrosine phosphatase (Keyse and Emslie, 1992), which is also induced by temperature shock in cell culture. That protein phosphatases are differentially affected by oxidative stress and temperature stress in cell culture lends support to the hypothesis that these enzymes may be involved in both temperature and ultraviolet-radiation-induced cnidarian bleaching. Altered protein phosphorylation could be the common mechanism by which both temperature and ultraviolet radiation induce cnidarian bleaching.

The authors would like to thank Dr G. Baghdasarian and Dr R. D. Gates for their many helpful discussions. This research was supported by NSF grant 9115834.

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