ABA- and cADPR-mediated effects on respiration and filtration downstream of the temperature-signaling cascade in sponges

Elena Zocchi[1,*], Giovanna Basile[1], Carlo Cerrano[2], Giorgio Bavestrello[3], Marco Giovine[1], Santina Bruzzone[1], Lucrezia Guida[1], Armando Carpaneto[4], Raffaella Magrassi[4] and Cesare Usai[4]

[1]DIMES, Section of Biochemistry, University of Genova, Viale Benedetto XV n°1, 16132 Genova, Italy
[2]DIPTERIS, University of Genova, Corso Europa 26, 16132 Genova, Italy
[3]Istituto di Scienze del Mare, University of Ancona, Via Brecce Bianche, 60131 Ancona, Italy
[4]Institute of Cybernetics and Biophysics, National Research Council, Via De Marini 6, 16149 Genova, Italy
*Author for correspondence (e-mail: ezocchi@unige.it)

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Summary

Recently, the thermosensing pathway in sponges (Porifera) was elucidated. The thermosensor triggering this cascade is a heat-activated cation channel, with the phytohormone abscisic acid (ABA), cyclic ADP-ribose (cADPR) and calcium acting as intracellular messengers, similarly to the drought-stress signaling cascade in higher plants. Here, we investigated the functional effects downstream of the temperature-signaling pathway in *Axinella polypoides* (Porifera, Demonspongiae).

Short-term stimulation followed by long-term depression of amino acid incorporation, oxygen consumption and water filtration were observed after exposure of the sponge to a brief heat stress or to micromolar ABA. These effects could be prevented by the targeted interruption of the signaling pathway either at the level of the cation channel thermosensor or at the level of the cADPR-induced intracellular calcium increase. Moreover, release of cyclase activity into the sea water and generation of extracellular cADPR were observed following brief heat stress. Intact sponge cells were sensitive to extracellular cADPR and addition of purified cyclase increased sponge respiration similarly to heat stress.

This is the first observation of functional effects exerted on Metazoa by the phytohormone ABA: conservation of the ABA/cADPR stress-signaling cascade points to its early evolution in a common precursor of modern Metazoa and Metaphyta. The functional effects induced by extracellular cyclase/cADPR suggest an evolutionary origin of cADPR as an ancient stress hormone in Porifera.

Key words: Abscisic acid, cADPR, $[Ca^{2+}]_i$, Respiration, Marine sponges, Heat stress

Introduction

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) movements may be considered the most ubiquitous and versatile signaling system in cell physiology (Carafoli, 1987). Thus, it comes as no surprise that cyclic ADP-ribose (cADPR), a universal and potent intracellular Ca^{2+} mobilizer, is emerging as one of the most pivotal signaling molecules known so far (Lee, 2001). It is involved in such diverse cell functions as cell-cycle regulation (protists) (Masuda et al., 1997), oocyte fertilization (invertebrates) (Lee, 1996), secretion and cell proliferation (mammals) (Takasawa et al., 1993; Zocchi et al., 1998) and the drought-stress response (plants) (Wu et al., 1997).

Recently, cADPR has been shown to mediate temperature signaling in sponges (Zocchi et al., 2001a), which are members of the phylum Porifera and comprise the phylogenetically oldest multicellular animals (Metazoa) (Rodrigo et al., 1994). In *Axinella polypoides* (Demospongiae, Axinellidae), ADP-ribosyl cyclase, the enzyme responsible for cADPR synthesis from NAD^+, is activated by a temperature increase by an abscisic acid (ABA)-induced, protein kinase A (PKA)-dependent mechanism. The thermosensor triggering this signaling cascade is a heat-activated cation channel. Elucidation of the complete thermosensing pathway in *A. polypoides* highlights several features conserved in higher organisms: the cation channel thermosensor shares all the functional characteristics of the mammalian heat-activated background K^+ channel responsible for central and peripheral thermosensing, including sensitivity to mechanical stress and anesthetics (Maingret et al., 2000a; Maingret et al., 2000b; Kindler et al., 1999); involvement of the phytohormone ABA and of cADPR as its second messenger is reminiscent of the drought-stress signaling cascade in higher plants (Wu et al., 1997). Thus, these observations suggest an ancient evolutionary origin of an ABA/cADPR-mediated stress-signaling pathway in a common precursor of modern Metazoa and Metaphyta.

Here, we investigated the functional effects produced on sponge physiology by activation of the temperature-signaling cascade. As the most-simple metazoans, sponges lack a defined tissue organization and display a high degree of cell plasticity. Cells are embedded in a collagenous matrix, impregnated with siliceous or calcareous spicules, surrounding a complex network of internal canals: the main function of sponges is the filtration of water, driven by the

synchronized beat of a flagellar epithelium lining the channels. Sponges represent an attractive model system to study the ABA/cADPR interplay because of the following features: (1) the absence of organs and tissues limits the possible functional responses induced by cADPR on these organisms; (2) the absence of tight intercellular junctions enables the preparation of cell suspensions by simple squeezing of the sponge; and (3) basic biochemical mechanisms of signal transduction are present in these lower Metazoa, as exemplified by the above-described signaling pathway. Thus, results obtained with this model system could reveal biochemical pathways conserved in higher organisms.

We investigated the effects of heat stress and ABA on amino acid incorporation and oxygen consumption (as a measure of sponge metabolism) and on the filtration rate (as a measure of sponge functional activity) in *A. polypoides*. Short-term stimulation followed by long-term depression of these functional activities was observed after exposure of the sponge to heat stress or ABA. These effects could be prevented by the targeted interruption of the temperature-signaling cascade obtained with the cation channel inhibitors bupivacaine and Gd^{3+}, with the cell-permeant cADPR antagonist 8-Br-cADPR and with the intracellular Ca^{2+} chelator EGTA-AM. Moreover, release of cyclase activity into the sea water (SW) and generation of extracellular cADPR following heat stress, together with the observed effects of extracellular cADPR and cyclase on sponge $[Ca^{2+}]_i$ and respiration, point to a hormone-like function of cADPR in Porifera.

Materials and Methods

Sponges

Specimens of *A. polypoides*, an arborescent sponge living on coralligenous or detritic bottoms, were collected in the Ligurian Sea (Mediterranean Sea) at Gallinara Island (Savona, Italy) at a depth between 30 and 40 m. The SW temperature was kept at 16°C during transfer of the animals to the laboratory and throughout their maintenance in an aquarium in natural SW. Sponges could be kept for several months with no apparent morphological changes: however, whole animals or cleanly cut fragments were routinely utilized 5-10 days after collection. Sponge fragments are commonly used in marine biology experimental systems, including measurements of O_2 consumption and dye filtration, as they are considered functionally representative of the entire animal (Riisgard et al., 1993; Kowalke, 2000). All manipulations involving sponges were performed underwater at 16°C.

Intracellular Ca^{2+} measurements

Intact sponge cells (approximately 8 μm diameter) could be easily obtained by gentle squeezing of cleanly cut *A. polypoides* fragments. Cell viability, as checked microscopically, was always ≥95% after mechanical dissociation and following exposure to heat stress or ABA. Loading of freshly dissociated *A. polypoides* cells with FURA 2-AM and Ca^{2+} measurements were performed as described (Zocchi et al., 2001a). Calibration to obtain the $[Ca^{2+}]_i$ from the fluorescence emission ratio E340/E380 was performed as described (Zocchi et al., 1998) except that cells were permeabilized to Ca^{2+} with 0.01% Triton. EGTA-AM (Calbiochem) loading and 8-Br-cADPR (Sigma) treatment of the cells, when needed, were performed before and immediately after FURA-loading, respectively: cells were incubated at 16°C for 30 minutes with 20 μM EGTA-AM, and 10 μM FURA 2-AM (Calbiochem) was then added for a further 90 minutes incubation or FURA 2-loaded cells were incubated with 10 μM 8-Br-cADPR for 30 minutes, then washed in SW and exposed to 24°C in the cuvette.

[^{35}S]Met/Cys incorporation

For weight determination, *A. polypoides* tissue fragments were cut underwater with a sharp scalpel, rapidly blotted on filter paper to remove excess SW and immediately transferred into a pre-weighed vial containing SW.

Freshly cut *A. polypoides* fragments (approximately 350 mg wet weight) were incubated in SW at 24°C for 3 hours in the presence of 20 μM EGTA-AM, or 10 μM 8-Br-cADPR, or without any addition. Duplicate incubations were performed at 16°C (controls). Tissue pieces were then transferred into fresh SW at 16°C and further incubated for 6 or 21 hours. [^{35}S]Met/Cys (Amersham, 2×10^6 μCi/tube) was added to the tubes for the last 6 hours before harvest. A duplicate series of samples, containing 100 μM cycloheximide (CHX, Sigma), was incubated in parallel to determine the amount of radioactivity incorporation not due to eukaryotic protein synthesis but to possible bacterial contamination. For determination of the incorporated radioactivity, tissue fragments were rinsed in SW, and cells were dissociated mechanically and washed extensively in SW by centrifugation. The residual stroma was repeatedly rinsed in SW by careful squeezing with forceps, and released cells were pooled with the previously dissociated ones. Cells and stroma were washed until the radioactivity detected in the supernatants was negligible. Cells were then lysed with 1 ml deionized water and 300 μl were bleached by the addition of 100 μl hypochlorite. Stroma was blotted dry on filter paper and cut into smaller pieces. Scintillation liquid (Packard) was then added to the cell and stroma samples, and the radioactivity was determined in a β-counter. Radioactivity incorporation was normalized to a fragment wet weight of 350 mg and subtracted of the radioactivity incorporated in the CHX-treated duplicate sample.

Dissolved O_2 measurements

The O_2 concentration was continuously monitored with a dissolved O_2 meter (HI9143, Hanna Instruments Italia) equipped with an incorporated thermometer (±0.1°C precision) and adjustable salinity setting. The O_2 consumption of the electrode was negligible compared with the sponge respiration. Experiments were performed in natural SW (salinity 3.9%) and at an ambient temperature of 16°C. Briefly, a cleanly cut *A. polypoides* fragment (approx. 3 cm length and 2.5 g wet weight) was positioned in 150 ml fresh SW in a beaker on a stainless steel tray at least 24 hours prior to the experiment, in order to let it overcome the possible stress of handling. Continuous slow stirring was obtained with a small magnetic bar positioned under the tray. Before measures of O_2 consumption, the SW was changed by means of a peristaltic pump. The electrode was inserted through a rubber cap that ensured air-tight closure of the beaker. Air bubbles inadvertently trapped below the cap during sealing were carefully removed with a syringe, inserted through the rubber cap, which also served to add chemicals during the measurements. *A. polypoides* O_2 consumption was linear in the range from 9 ppm (the O_2 concentration in non-aerated SW at 16°C) to 3 ppm at a rate of 1.1±0.34 ppm/h (n=14): however, when the O_2 concentration in the respiration chamber approached 5 ppm due to sponge consumption, the rubber cap was removed, the SW was aerated until the O_2 concentration rose again to approx. 8 ppm (10 minutes), the beaker was sealed again and the measurement resumed. After measuring the O_2 consumption of a sponge fragment for 1-2 hours, the same fragment was exposed to ABA or heat stress. ABA was added when the SW was aerated, before re-sealing. Exposure to heat stress was achieved by substituting the SW in the beaker with heated SW, by means of a peristaltic pump. Continuous stirring ensured thorough mixing of the water. When the water temperature reached 24.0°C, the beaker was transferred at room temperature. After 30 minutes (final water temperature approx. 23°C),

the beaker was transferred to the cold room, the water temperature was lowered again to exactly 14°C by mixing with chilled SW, and measurements of O_2 consumption were resumed. O_2 consumption during heat stress was not monitored due to temperature-dependent variations in O_2 solubility, which would have complicated interpretation of the results. After measuring the O_2 consumption for approximately 10 hours, the cap was removed, half of the SW was changed (ABA was added when needed), the SW was aerated overnight and measurements were resumed the following day. Under these conditions, respiration of control, unstimulated sponges was linear for 48 hours.

Dye filtration

This assay investigates the ability of the sponge to extract nutrients from the SW by means of its filtering activity. Driven by the beat of a flagellar epithelium, water circulates in the sponge acquiferous system and molecules/unicellular organisms are retained by the cells lining the internal canals. The same experimental set-up as described above for O_2 consumption was utilized, except that the beaker (250 ml) was left uncovered and neutral red (BDH Laboratories, London) was added to the SW at a final concentration of 8 μg/ml. The optical absorbance (at 455 nm) of SW aliquots, taken every 30-60 minutes, was measured. No dye removal from the SW was observed with sponges killed by incubation with 2% formaldehyde for 30 minutes. *A. polypoides* filtration was linear for at least 8 hours, with one dye refill. The unicellular algal suspensions (*Nanochloropsis* sp.) used in the filtration experiments (Riisgard et al., 1993) performed in parallel to the dye were kindly provided by the aquarium of Genova.

ADP-ribosyl cyclase, NAD^+ and cADPR detection in SW

Whole, young *A. polypoides* animals, approx. 6 cm in height (5 g wet weight) were acclimatized for 2-3 days in 250 ml beakers, in natural SW under aeration, which also ensured mixing of the SW. The SW was changed daily with a peristaltic pump. At the beginning of the experiment, the SW was changed after 4-6 hours, and the SW was substituted with heated SW until the temperature reached 24°C. The beaker was then transferred at room temperature under aeration for 30 minutes. Samples of SW were removed at the beginning of the experiment (time_zero) immediately before and 30 minutes after the onset of stress. ADP-ribosyl cyclase activity was assayed at 16°C on 200 μl SW aliquots by addition of 2 mM NAD^+ substrate. The cADPR produced was detected by HPLC (Zocchi et al., 2001a). Detection of ADP-ribosyl cyclase in 50-fold concentrated (Microcon, Millipore) SW was performed on mildly denaturing SDS-PAGE, as described (Zocchi et al., 2001a). NAD^+ and cADPR in the SW were detected by a microfluorimetric cycling assay (Graeff and Lee, 2002), performed on 500 μl SW aliquots. Preliminarily, we verified that the assay could be performed in SW. The enzyme treatment for the degradation of endogenous NAD^+ proved to be completely effective also in SW. The cADPR standard curve in SW was linear and the sensitivity of the assay was comparable with that obtained in phosphate buffer. No cyclase activity, NAD^+ or cADPR were detected in the SW at time_zero.

Results

Effects of temperature and ABA on intracellular Ca^{2+} in *A. polypoides* cells

Exposure of freshly dissociated, FURA 2-loaded cells to 24°C (up from the aquarium temperature of 16°C) induced an immediate and progressive increase of $[Ca^{2+}]_i$ (Fig. 1), which conversely kept fairly constant in cells incubated at 16°C (not shown), in line with our previous observations (Zocchi et al., 2001a). In this study, the $[Ca^{2+}]_i$ was monitored over a longer time period (24 hours): an initial $[Ca^{2+}]_i$ increase, which was recorded over a time-span of 4-5 hours, could be fitted by a sigmoidal regression curve (i.e. it showed a tendency to reach a maximal value). This 'short-term' fluorescence increase could be prevented by pre-treatment of the cells with EGTA-AM, but not by addition of extracellular EGTA (Fig. 1), demonstrating that the $[Ca^{2+}]_i$ increase was originated by release of intracellular Ca^{2+}. Subsequently (6-24 hours), a second and higher increase of the $[Ca^{2+}]_i$ ensued, which could be completely prevented by extracellular EGTA (Fig. 1), indicating that it was caused by influx of extracellular Ca^{2+}, possibly induced by depletion of the intracellular Ca^{2+} stores. La^{3+} (100 μM) and capsaicin (100 μM) prevented (87% inhibition) or reduced (60% inhibition), respectively, the long-term EGTA-sensitive $[Ca^{2+}]_i$ increase induced by heat stress, suggesting that it occurred through Ca^{2+}-release-activated Ca^{2+} channels (CRACs). If the temperature was decreased again to 16°C during or after the first sigmoidal $[Ca^{2+}]_i$ rise, the subsequent Ca^{2+} increase was reduced or unaffected, respectively (not shown). Pre-treatment of the cells with either EGTA-AM or with the cell-permeant cADPR antagonist 8-Br-cADPR (Walseth and Lee, 1993) prior to heat exposure prevented both the short- and the long-term $[Ca^{2+}]_i$ increases (Fig. 1), demonstrating (1) a causal relationship between the initial intracellular Ca^{2+} release and the subsequent extracellular Ca^{2+} influx and, (2) the role of cADPR in this temperature-induced perturbation of the Ca^{2+} homeostasis.

Heat stress had been previously shown to induce ABA synthesis in *A. polypoides*, with ABA in turn stimulating ADP-ribosyl cyclase through PKA (Zocchi et al., 2001a). Indeed, the

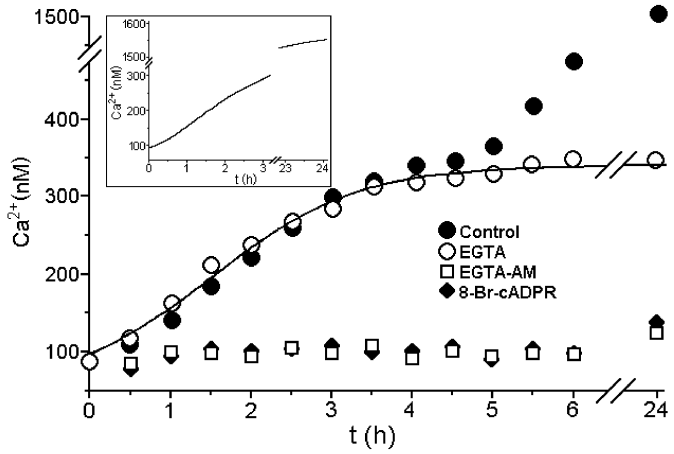

Fig. 1. Short- and long-term effects of temperature and ABA on $[Ca^{2+}]_i$ in *A. polypoides* cells. FURA 2-loaded cells were incubated at 24°C in SW without (●) or with 4 mM EGTA (○) in a thermostated microfluorimetric cuvette, under continuous stirring. EGTA-AM loading (□) or 8-Br-cADPR treatment (◆) of the cells were performed before and after FURA-loading, respectively. The fluorescence emission ratio E340/E380 was acquired every 30 minutes and calibrated to obtain the corresponding $[Ca^{2+}]_i$; each point is the mean of four values. The double bars indicate interruptions in the x- and y-axis. Inset: trace showing the increase in $[Ca^{2+}]_i$ as continuously recorded after addition of 50 nM ABA at a constant temperature of 16°C. Traces are from one of five comparable experiments, performed on different animals and giving similar results.

concentration of ABA in sponge tissue increased rapidly following exposure of *A. polypoides* fragments to 24°C from a basal value of 5.8 pmoles/g to 25, 140 and 180 pmoles/g after 2 minutes, 60 minutes and 4 hours, respectively (results from a representative experiment), with kinetics similar to those already described (Zocchi et al., 2001a). Thus, we also investigated the short- and long-term effects of ABA exposure on $[Ca^{2+}]_i$. Indeed, addition of 50 nM ABA at 16°C resulted in an increase in $[Ca^{2+}]_i$ similar in extent and kinetics to that registered after heat stress (Fig. 1, inset).

The presence of short- and long-term effects of heat stress and ABA on $[Ca^{2+}]_i$ in *A. polypoides* cells prompted us to investigate their functional consequences on sponge physiology at different times after stress induction: during the initial sigmoidal $[Ca^{2+}]_i$ increase (i.e. 4-8 hours after heat stress) and after 24 hours.

$[^{35}S]$Met/Cys incorporation after heat stress

We explored amino acid incorporation into *A. polypoides* fragments over two different 6-hour time spans: immediately after heat stress, roughly overlapping the short-term $[Ca^{2+}]_i$ increase, and 24 hours after heat stress, during the 'long-term' $[Ca^{2+}]_i$ increase. $[^{35}S]$Met/Cys incorporation into *A. polypoides* cells and stroma increased six- and fourfold, respectively, in the 6 hours immediately following heat stress, compared with controls kept at 16°C. Conversely, a marked decrease in amino acid incorporation into cells and stroma compared with controls was observed 24 hours after heat stress. Both effects were prevented by pre-treatment of the sponge fragments with EGTA-AM or with 8-Br-cADPR (Fig. 2). Thus, a brief temperature increase induces a short-term stimulation, followed by a long-term inhibition of protein synthesis over the subsequent 6 and 24 hours, respectively. Both effects are mediated by a cADPR-induced increase in $[Ca^{2+}]_i$. Incubation of sponge fragments at 16°C with 10 μM 8-Br-cADPR alone for 24 hours resulted in a 36% decrease of amino acid incorporation compared with untreated controls (n=3). This effect suggests that the antagonist might interfere with a stimulatory effect of endogenous cADPR on basal sponge protein synthesis.

O_2 consumption and dye filtration after exposure to heat stress or ABA

Preliminarily, both O_2 consumption and dye filtration were explored in control, unstimulated *A. polypoides* fragments. O_2 consumption was found to be linear over a range between 9 ppm and 3 ppm, and could be completely inhibited with 10 mM NaN_3, indicating that it was indeed the result of mitochondrial respiration. When consumption by the sponge reduced O_2 concentration in the SW of the respiration chamber below 5 ppm, the SW was aerated until the O_2 concentration again reached 8 ppm and measurements were resumed. Thus, a series of parallel lines was obtained over a period of 48 hours (not shown). Neutral red filtration proved to occur linearly for approximately 8 hours, with a single dye refill after 4 hours incubation: thereafter, clearance of the dye decreased, possibly because of sponge saturation. A similar result was also obtained when an algal suspension was used instead of the dye. In this case, the decrease of cell number per ml was

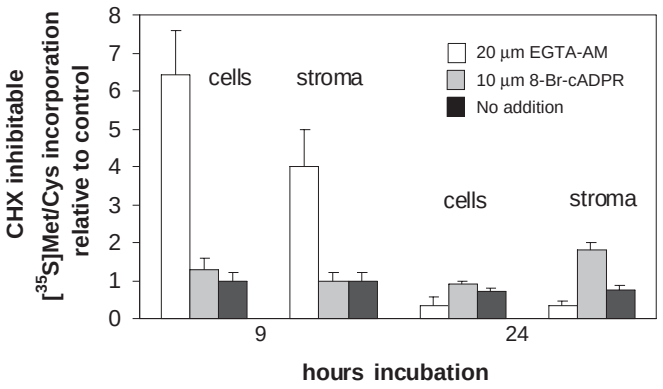

Fig. 2. $[^{35}S]$Met/Cys incorporation into *A. polypoides* tissue fragments after exposure to a temperature rise. Freshly cut *A. polypoides* fragments were incubated in SW at 24°C for 3 hours with 20 μM EGTA-AM, or with 10 μM 8-Br-cADPR, or without any addition (see key). Tissue pieces were then transferred into fresh SW at 16°C and $[^{35}S]$Met/Cys was added to the tubes for the last 6 hours before harvest. Radioactivity incorporation, determined as described in Materials and Methods, is expressed as variations relative to respective controls, i.e. fragments incubated at 16°C throughout the experiment. x-axis numbers indicate the total length of the incubations. Results are the mean±s.d. of four experiments, performed on different animals.

microscopically determined on samples of SW removed at different times. We routinely utilized the dye to investigate effects of heat stress and ABA on sponge filtration: however, results obtained with the dye were always confirmed with the algal clearance method. Owing to the above-mentioned saturation, filtration was measured for not more than 8 consecutive hours, followed by a 12-hour dye- or algal-free interval before the next measurement was taken (Fig. 3B,D). Finally, no dye or algal filtration was detected with sponges killed by incubation in 2% formaldehyde for 30 minutes.

Respiration and filtration proved remarkably comparable between different specimens; nonetheless, each fragment served as its own control, prior to heat stress or ABA exposure. Both functional activities increased immediately after exposure of sponge fragments to heat stress or ABA and comparable effects were produced by the two treatments (Fig. 3): 2.2- and 1.9-fold increases of O_2 consumption and 1.6- and 2.0-fold increases in dye filtration were recorded over control values, registered on the same sponge fragment before heat stress and ABA treatment, respectively (values from one representative experiment out of five, giving closely comparable results). The initial stimulation of O_2 consumption and filtration (2-6 hours after treatment) was followed by a progressive decrease, down to approx. 50% of control values 24 hours after stress induction (Fig. 3A,C), similar to what was observed for amino acid incorporation.

The effect of inhibitors of the temperature-signaling cascade was then investigated on short-term stimulation and long-term inhibition of respiration and filtration induced by heat stress and ABA. The cation channel thermosensor inhibitors bupivacaine and Gd^{3+} (Zocchi et al., 2001a) both prevented the stimulatory effect of a transient heat stress on respiration (Fig. 4A) and on filtration (Fig. 4B). Pre-treatment of sponge fragments with 20 μM EGTA-AM or with 10 μM 8-Br-cADPR

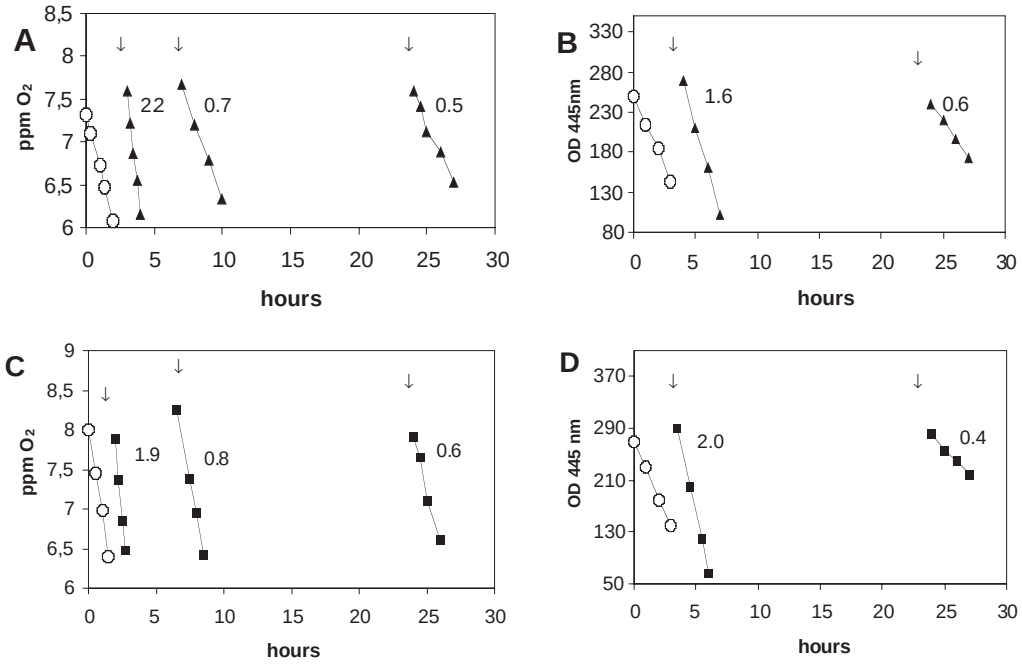

Fig. 3. Effects of heat stress and ABA on O_2 consumption and dye filtration in *A. polypoides*. O_2 consumption (panels A and C) and dye filtration (panels B and D) were measured at 16°C on *A. polypoides* fragments (approx. 5 cm length) before (control, ○) and after exposure to 24°C for 30 minutes (▲) or addition of 20 μM ABA (■). Results shown in each panel are from one representative experiment out of five, performed on different animals. The calculated slope of the linear regression curves (R≥0.98), relative to the respective control, is shown. The arrows indicate aeration of SW (panels A and C) or dye addition to the SW (panels B and D).

also completely prevented the temperature-induced effects (not shown) as well as the ABA-induced stimulation of either functional activity (Fig. 4). Incubation of sponge fragments with 10 μM 8-Br-cADPR alone, in the absence of any stress induction, resulted in a progressive reduction of O_2 consumption compared with untreated controls, with a 20% and 30% decrease being recorded after 2 and 4 hours incubation, respectively. This result, together with a similar extent of inhibition by the cADPR antagonist on amino acid incorporation, suggests involvement of endogenous cADPR in the regulation of basal O_2 consumption and protein synthesis in *A. polypoides* cells. Finally, incubation of sponge fragments for 2 hours with 20 μM EGTA-AM or with 10 μM 8-Br-cADPR prior to exposure to heat stress or ABA completely prevented the long-term decrease of O_2 consumption observed 24 hours after stress induction.

The effect on O_2 consumption of 8-Br-cAMP, a membrane-permeant activator of PKA, was also investigated. Previously, we had demonstrated that ADP-ribosyl cyclase activation following heat stress or ABA occurs by PKA-mediated phosphorylation (Zocchi et al., 2001a). Incubation of *A. polypoides* fragments with 200 μM 8-Br-cAMP, a membrane-permeant PKA activator, at a constant temperature of 16°C induced a 30% increase of O_2 consumption (Fig. 4A) and a 50% increase of dye filtration (Fig. 4B) over controls during the first 60 minutes after addition.

Effects of extracellular cADPR on $[Ca^{2+}]_i$ and O_2 consumption

ADP-ribosyl cyclase activity was detected in the SW (250 ml) surrounding control, unstimulated *A. polypoides* sponges. Its levels increased approx. 20-fold following a brief heat stress (30 minutes at 24°C), from 0.16±0.02 to 3.1±0.25 nmoles cADPR/ml/minute (mean values from three experiments). A parallel increase of the cADPR concentration in the SW was

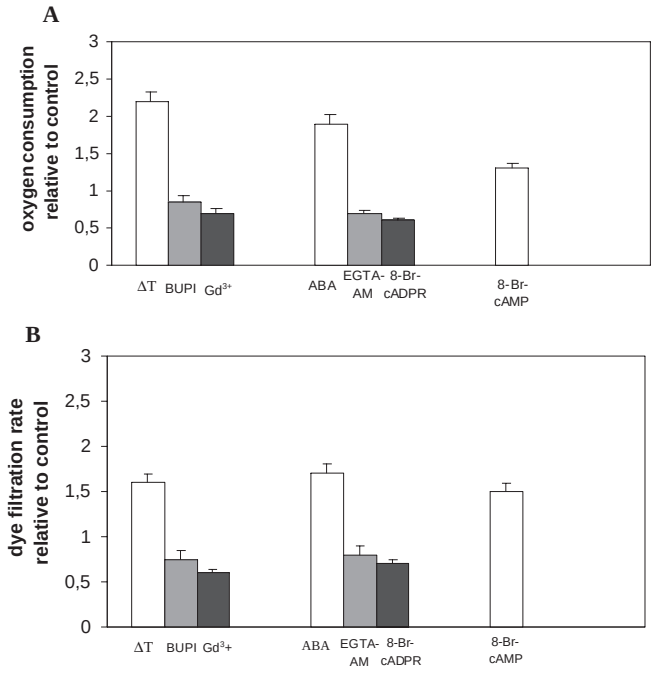

Fig. 4. Effect of inhibitors of the temperature-signaling cascade in *A. polypoides* on temperature- and ABA-induced short-term stimulation of O_2 consumption and filtration rate. O_2 consumption and dye filtration were recorded for 2 hours before (control) and after exposure of *A. polypoides* fragments to heat stress, ABA or 8-Br-cAMP. EGTA-AM (15 μM) and 8-Br-cADPR (10 μM) were added 30 minutes before ABA (20 μM). Bupivacaine (BUPI; 1 mM) and $GdCl_3$ (50 μM) were added during heat stress (24°C for 30 minutes). The calculated slope of the linear regression curve (R≥0.98) is compared with that of the respective control, i.e. the same fragment prior to stress induction or addition of chemicals. Data are the mean±s.d. of five experiments.

observed, from 0.2±0.06 nM to 4.02±0.6 nM, along with a decrease of the NAD^+ concentration from 20±3 nM to 3.0±0.5 nM (mean values from three experiments). The observed efflux of NAD^+ from intact sponge cells (E.Z., unpublished), similar in extent and kinetics to that reported in mammalian cell lines (Zocchi et al., 1999), might account for the presence of the dinucleotide in the SW. Moreover, absence in the SW of HPLC-detectable (sensitivity limit, 50 nM) ATP (or of adenylic nucleotides derived therefrom), despite the fact that its intracellular concentration in *A. polypoides* is approximately 30 times higher than that of NAD^+ (12.3±1.2 versus 0.4±0.05 nmoles/mg protein, as detected by HPLC on TCA extracts of freshly dissociated sponge cells), rules out cell lysis as a possible explanation for the release of NAD^+ and of cyclase activity in the SW.

Several biochemical characteristics of the released cyclase were investigated: (1) the ratio between cyclase activity on NAD^+ and hydrolase activity on cADPR was comparable with that observed in the cell lysate (≥100) (Zocchi et al., 2001a) and similar to that reported for the *Aplysia* cyclase (Munshi et al., 1999); (2) cyclase activity on NAD^+ was approximately 20 times higher than that on the analog nicotinamide guanine dinucleotide (NGD^+), again similar to the cell lysate and to *Aplysia* cyclase; (3) the molecular mass of the released cyclase, as detected on mildly denaturing SDS activity gel, was approximately 20 kDa, i.e. lower than the phosphorylated cytosolic enzyme purified by affinity chromatography on antiphosphoserine antibody-coated agarose (Zocchi et al., 2001a). Release of cyclase activity and generation of cADPR in the SW after heat stress prompted us to investigate a possible effect of extracellular cADPR on the $[Ca^{2+}]_i$ of intact sponge cells. Permeabilized *A. polypoides* cells were shown to respond to cADPR and to ryanodine with an immediate $[Ca^{2+}]_i$ increase, which could be completely prevented by pretreatment of the cells with 8-Br-cADPR (Zocchi et al., 2001a). Upon addition of 10 μM cADPR to intact, FURA 2-loaded, *A. polypoides* cells, at a constant temperature of 16°C, a slow and progressive increase of the E340/E380 fluorescence ratio was observed (Fig. 5A); increasing the concentration of cADPR (up to 200 μM) resulted in an increase of the slope of the Ca^{2+} rise (Fig. 5A). Pre-incubation of the cells with 30 μM 8-Br-cADPR (Fig. 5A) or with 30 μM EGTA-AM for 30 minutes completely prevented the Ca^{2+} rise.

Finally, we investigated the effect of the addition of cyclase (purified from *Aplysia californica*; Sigma) on O_2 consumption and on dye filtration of *A. polypoides* fragments. Addition of 2.5 mU/ml (2.5 nmoles cADPR produced/ml/minute), i.e. a cyclase activity similar to that detected in the SW after heat stress (see above), increased the O_2 consumption by 40%. NAD^+ (100 μM) supplementation to the cyclase or addition of 100 μM cADPR as such induced a similar 50% increase of the respiration (Fig. 5B, left panel). Dye filtration was also stimulated by the presence of extracellular cyclase activity, with or without NAD^+ supplementation, or by the addition of pre-formed cADPR (Fig. 5B, right panel).

Discussion

In this study, we investigated the functional effects produced by activation of the recently described temperature-signaling cascade in *A. polypoides* (Zocchi et al., 2001a). These effects included short-term stimulation followed by long-term depression of amino acid incorporation, O_2 consumption and water filtration, and they were induced or prevented by the targeted activation or interruption, respectively, of the signaling cascade. Specifically: (1) ABA induced similar effects as heat stress, confirming its role in the temperature-signaling cascade; (2) the cation-channel thermosensor inhibitors bupivacaine and Gd^{3+} prevented the effects induced by heat stress; and (3) the cADPR antagonist 8-Br-cADPR prevented both the temperature- and ABA-induced effects, confirming the role of cADPR as the ultimate messenger in this signaling pathway.

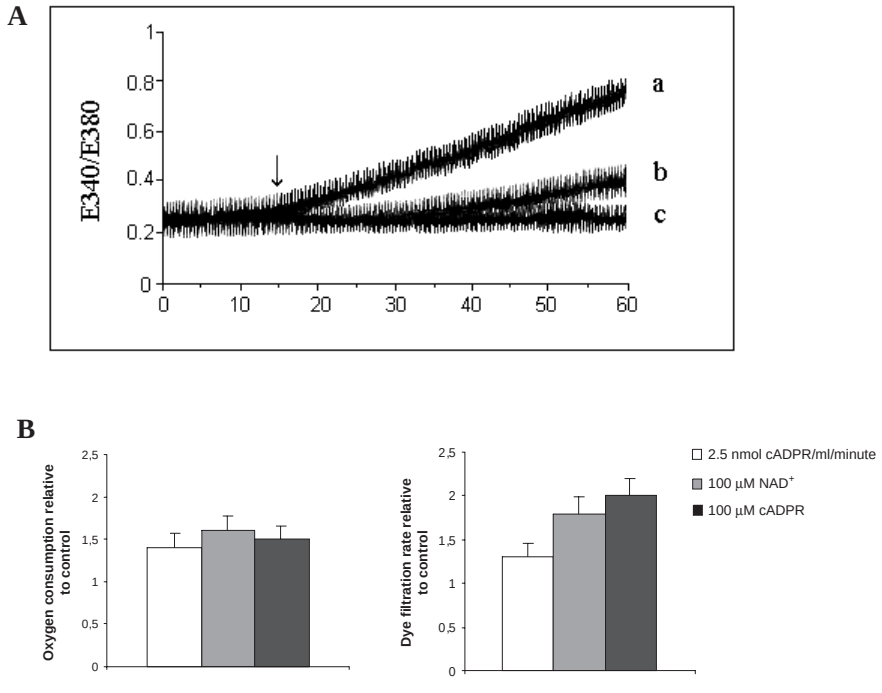

Fig. 5. Effects of extracellular cADPR on $[Ca^{2+}]_i$ and O_2 consumption in *A. polypoides*. (A) Fura 2-loaded sponge cells were incubated at 16°C in Ca^{2+}/Mg^{2+}-free artificial SW in a thermostated microfluorimetric cuvette, under continuous stirring. The arrow indicates addition of cADPR. Trace a: 200 μM cADPR. Trace b: 10 μM cADPR. Trace c: cells pre-incubated with 30 μM 8-Br-cADPR for 30 minutes prior to addition of 10 μM cADPR. The fluorescence emission ratio E340/E380 is shown. (B) O_2 consumption (left panel) and dye filtration (right panel) of *A. polypoides* fragments (approx. 5 cm length) were recorded for 2 hours before (control) and after addition of cyclase activity (2.5 nmoles cADPR/ml/minute), 100 μM NAD^+ or 100 μM cADPR (see key). The calculated slope of the linear regression curves (R≥0.98) relative to control is shown. Data are the mean±s.d. of four experiments.

Finally, activation of PKA by 8-Br-cAMP induced a short-term increase of O_2 consumption, as would be expected by its stimulatory effect on ADP-ribosyl cyclase (Zocchi et al., 2001a).

Temperature- and ABA-induced stimulation of respiration and filtration were also observed on *Chondrosia reniformis* (Demospongiae, Hadromerida) (not shown), a sponge that, from a phylogenetic viewpoint, is very far from *A. polypoides* (Alvarez and Crisp, 1994). Thus, results obtained on *A. polypoides* should be considered representative of a wide spectrum of Porifera.

This is the first observation of functional effects exerted on Metazoa by micromolar concentrations of the phytohormone ABA, which is involved in drought- and temperature-stress signaling in plants (Leckie et al., 1998; Xiong et al., 1999): conservation of the ABA/cADPR signaling cascade from sponges to higher plants (Wu et al., 1997; Leckie et al., 1998) suggests its ancient evolutionary origin in a common precursor of modern Metazoa and Metaphyta. Indeed, ABA has also been detected in the mammalian brain (Le Page-Degivry et al., 1986), although its functional effects and the conditions modulating its synthesis are still unknown. The stimulation of sponge O_2 consumption (this report) and of plant root elongation (Spollen et al., 2000) induced by ABA might indicate a role of this hormone, and of cADPR as its second messenger, in the induction of anabolic processes in response to environmental stimuli. Interestingly, the cation channel thermosensor in *A. polypoides* shares several pharmacological and functional properties with the mammalian two-pore domain K^+ channels involved in pH and O_2 sensing in the mammalian brain (Maingret et al., 2000a; Maingret et al., 2000b; Patel et al., 1998).

The inhibitory effect of the cell-permeant cADPR antagonist 8-Br-cADPR on basal sponge protein synthesis and on O_2 consumption suggests a role for endogenous cADPR in the regulation of basal cell metabolism as well as in its modification in relation to environmental conditions. In mammals, from which Porifera are separated by 500 million years of evolution, cADPR has been recently shown to stimulate cell proliferation of cyclase-transfected murine fibroblasts (Zocchi et al., 1998) and of human hematopoietic stem cells incubated with exogenously added or with stroma-produced cADPR (Podestà et al., 2000; Zocchi et al., 2001b). Moreover, cADPR antagonists have been shown to decrease $[Ca^{2+}]_i$ and to inhibit cell growth in both cell systems. cADPR-induced stimulation of cell proliferation necessarily implies an increased energy production by the cells to meet the higher demand. An effect of cADPR on mammalian cell respiration has not been reported so far, although cyclase activity has been observed in mitochondria (Yamada et al., 1997; Liang et al., 1999), its function still unknown.

The long-term inhibitory effects on sponge physiological functions induced by heat stress or ABA are caused by the profound derangement of the intracellular Ca^{2+} homeostasis started by the initial cADPR-induced intracellular Ca^{2+} mobilization, which in turn triggers a protracted extracellular Ca^{2+} influx, possibly through CRACs (Reichling and Levine, 1997). This seems to be supported by the observed inhibitory effect of micromolar La^{3+} (Pizzo et al., 2001) and capsaicin on the EGTA-inhibitable Ca^{2+} influx. Capsaicin is being reported as a specific CRAC inhibitor (Fischer et al., 2001). La^{3+} is also active on voltage-dependent Ca^{2+} channels (Carbone and Swandulla, 1990); however, their absence in *A. polypoides* cells (Zocchi et al., 2001a) suggests inhibition of CRACs. The cADPR antagonist 8-Br-cADPR prevents both the short-term increase and the long-term inhibition of O_2 consumption following heat stress or ABA, underlying the causal role of cADPR in the induction of both responses. The long-lasting effect of a transient, although sharp, temperature increase provides a sort of cell 'memory' via $[Ca^{2+}]_i$, which negatively affects sponge physiology long after the return of temperature to lower values. This mechanism might be responsible for the recently observed mass mortality episode attributed to an exceptional SW temperature increase in the Northwestern Mediterranean (Cerrano et al., 2000). In this respect, the kinetics of the temperature rise, and of the consequent cADPR-induced $[Ca^{2+}]_i$ increase, might be important: a sharp, though transient, temperature increase, as investigated here, results in a sudden increase of $[cADPR]_i$ (Zocchi et al., 2001a) and of $[Ca^{2+}]_i$ (Fig. 1), which triggers extensive Ca^{2+} influx and negatively affects sponge physiology. By contrast, a slow and progressive temperature increase, as occurs during seasonal cycles, could result in a limited increase of $[cADPR]_i$ and $[Ca^{2+}]_i$, thus stimulating sponge functional activities.

Finally, an intriguing observation described here is the release of a low-molecular-weight cyclase (20 kDa) into the SW following heat stress. A similar effect was registered with the contractile sponge *Chondrosia reniformis* following gentle mechanical stimulation (not shown). The mechanism underlying release of cyclase activity and of NAD^+ does not involve cell lysis, as ATP (or adenylic nucleotides derived therefrom) was undetectable in the SW despite its intracellular concentration being 20 times higher than that of NAD^+. Cyclase activity has been shown to be compartmentalized in large granules in *Aplysia* ovotestis (Hellmich and Strumwasser, 1991) and granule-rich cells have been observed in *A. polypoides* (Van de Vyver and Buscema, 1985) and in *C. reniformis* (Bonasoro et al., 2001). In *C. reniformis*, these cells are frequently observed around the water canals, where they appear to actively discharge their contents under conditions of increased sponge plasticity ('creeping') (Bonasoro et al., 2001). Whatever the mechanism involved, release of cyclase activity into the SW following heat stress suggests a possible role of extracellular cADPR in the sponge response to environmental stress. Efflux of NAD^+ from sponge cells (E.Z., unpublished), similar to that observed from mammalian cells (Zocchi et al., 1999), might provide the required substrate for the extracellular generation of cADPR. Indeed, both NAD^+ and cADPR were detected in the limited SW volumes used in these experiments, their levels changing in opposite directions after heat stress, indicating consumption of the former and production of the latter by the released cyclase.

Notably, intact sponge cells respond to extracellular cADPR with a progressive $[Ca^{2+}]_i$ increase (Fig. 5A) and addition of cyclase activity or of pre-formed cADPR to the SW induces an increase of sponge O_2 consumption (Fig. 5B): these results are reminiscent of the functional effects (increase of $[Ca^{2+}]_i$ and of proliferation) induced by extracellular cADPR on murine fibroblasts (Franco et al., 2001) and on human hemopoietic progenitors (Podestà et al., 2000). In this case, similar effects were obtained with micromolar concentrations of exogenously added nucleotide or with nanomolar concentrations provided by ectocyclase-expressing, adjacent stromal cells, highlighting

the importance of close cell contacts for efficient cADPR delivery (Zocchi et al., 2001b; Franco et al., 2001). These observations suggest that cADPR might have originated as an ancient stress hormone, with both intracellular and extracellular functions, and has maintained these unusual properties in higher organisms, where it can function both as an intracellular signal molecule and as a cytokine.

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