Abscisic acid signaling through cyclic ADP-ribose
in hydroid regeneration.

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Running title: ABA and cADPR in hydroid regeneration
SUMMARY

Cyclic ADP-ribose (cADPR) is an intracellular calcium (Ca\textsuperscript{2+}) mobilizer involved in fundamental cell functions from protists to higher plants and mammals. Biochemical similarities between the drought-signaling cascade in plants and the temperature-sensing pathway in marine sponges suggest an ancient evolutionary origin of a signaling cascade involving the phytohormone abscisic acid (ABA), cADPR and Ca\textsuperscript{2+}. In *Eudendrium racemosum* (Hydrozoa, Cnidaria), exogenously added ABA stimulated ADP-ribosyl cyclase activity via a protein kinase A (PKA)-mediated phosphorylation and increased regeneration in the dark to levels observed under light conditions. Light stimulated endogenous ABA synthesis, which was conversely inhibited by the inhibitor of plant ABA synthesis Fluridone. The signal cascade of light-induced regeneration uncovered in *E. racemosum*

Light $\rightarrow$ $\uparrow$ABA $\rightarrow$ PKA $\rightarrow$ cyclase activation $\rightarrow$ $\uparrow$[cADPR]i $\rightarrow$ $\uparrow$[Ca\textsuperscript{2+}]i $\rightarrow$ regeneration

is the first report of a complete signaling pathway in Eumetazoa involving a phytohormone.
INTRODUCTION

ADP-ribosyl cyclase activity converts NAD$^+$ to the intracellular calcium mobilizer cyclic ADP-ribose (cADPR). cADPR is involved in fundamental calcium-controlled cell functions of increasing complexity along the phylogenetic tree: light-induced cell cycle regulation in Euglena [1]; oocyte fertilization in invertebrates [2]; glucose-induced insulin secretion [3], myocyte contraction [4] and cell proliferation [5] in mammals; drought-induced stomatal closure in plants [6]. cADPR-induced intracellular calcium movements thus appear to trigger the cellular response specific to each cell type, often acting together with inositol 1,3,5 triphosphate (IP3) and/or nicotinic acid adenine dinucleotide phosphate (NAADP) [7, 8].

Despite the wealth of data regarding the second messenger role of cADPR, the hormonal signals responsible for ADP-ribosyl cyclase activation are less well-defined. In mammals, acetylcholine and cholecystokinin have been shown to activate ADP-ribosyl cyclase in adrenal chromaffin cells [9] and in pancreatic acinar cells [10], respectively. In higher plants, cADPR has been shown to mediate signaling of the phytohormone abscisic acid (ABA) in the drought-stress response leading to activation of gene transcription and to stomatal closure [11, 6]. More recently, ABA and cADPR have been demonstrated to mediate temperature-signaling in sponges [12], phylogenetically the oldest metazoans, suggesting an ancient evolutionary origin of the ABA/cADPR signaling cascade in a common precursor of modern Metazoa and Metaphyta.

The position of hydroids (Coelenterata, Cnidaria) in the phylogenetic tree (they follow sponges and are considered the most ancient true animal phylum, Eumetazoa), the relative complexity of their structure (as opposed to sponges, they have well defined tissues with sensory, nerve, muscle and epithelial cells, a digestive cavity and a rudimentary nervous system, [13]) and their peculiar capacity for regeneration (a process bearing intriguing similarities with animal stem cell-mediated tissue regeneration, [13, 14] ) prompted us to investigate the presence and the functional role of the ABA/ADP-ribosyl cyclase/cADPR system in marine hydroids. Hydroids are
characterised by a metagenetic life cycle with polyp and medusa forms. A sessile, feather-like colony, formed by polyps connected by a network of gastrovascular canals (stolons) covered by a chitinous exoskeleton (perisarc), gives rise to free-swimming medusae (sexual phase). Asexual reproduction is also common: cloning can be produced accidentally, due to injury of the colony, and physiologically, by budding, autotomy and by elongation of stolons, which grow and attach to another substratum near the parent colony and then segregate to form a new colony [15]. Regeneration, defined as the capacity to replace entire sections of the body in a short time, plays an important role in the maintenance of the individual structure in hydroids, compensating for spontaneous or accidental loss of tissue [15]. Budding and regeneration involve the contribution of pluripotent stem cells, present in the stolonal network, which provide new tissue wherever growth processes take place [14, 15]. In the cosmopolitan genus *Eudendrium*, medusa generation is substituted by sessile gonophores (i.e. sexual polyps) with the function of gonads. *Eudendrium racemosum*, the species utilized in this study, is abundant in the Ligurian Sea (West Mediterranean), it is present year-round at shallow depths (0-5 m) and it shows rapid, light-induced regeneration of cut stolons “in vitro” [16].

Results obtained indicate that light-induced regeneration in *E. racemosum* is mediated by the phytohormone ABA, with cADPR as its second messenger. These observations for the first time identify the ABA/cADPR signaling pathway in primitive animals and indicate its involvement in the control of stem cell proliferation.
EXPERIMENTAL PROCEDURES

Hydroids.

E. racemosum colonies were collected in shallow water (0-5 m) inside the harbor of Santa Margherita (Genova, Italy). Hydroids were transferred to the lab in abundant sea water (SW) and were either immediately utilized or were kept in an aerated aquarium in natural SW at 17°C with 8-hours/day artificial illumination for up to four days.

For regeneration experiments, small fragments (approx. 10 mm) of stolons were cut from different colonies, randomized and incubated in sterile Petri dishes in natural, filtered SW (10 fragments per dish in 30 ml SW) at 17°C, under artificial white light (8 hours/day) unless otherwise stated, in the presence of the indicated chemicals. Regeneration, i.e. budding of new tissue at the ends of each fragment, was measured by microscopic examination with a micrometric objective (final magnification 5x).

Determination of ADP-ribosyl cyclase and cADPR hydrolase activities.

Small fragments (10 mm) were cut from several hydroid colonies, briefly sonicated (6 s) in SW containing 0.01% Triton X-100, diluted in SW to approximately 0.15 mg protein/ml and incubated at 17°C in the presence of 0.2 mM NAD$^+$ (for determination of cyclase activity) or 0.2 mM cADPR (for determination of hydrolase activity). At various times (30, 60 and 120 min) aliquots of 50 µl were withdrawn, trichloroacetic acid-extracted and analyzed by HPLC [17].

cADPR detection in hydroid extracts.

Hydroid fragments (10-15 per determination) were sonicated in 0.3 ml of 0.6 M PCA in deionized water. The intracellular cADPR concentration was determined on samples (≤ 0.3 mg protein) by a sensitive enzymatic cycling assay [18], as described in [19]. Briefly, PCA was removed by mixing the sample with 4 vol of a solution containing 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. After 1 min of vortexing, samples were centrifuged for 10 min at 1500 x g and the aqueous layer containing cADPR was recovered. Samples were then incubated overnight at 37°C with a mixture of enzymes (nucleotide pyrophosphatase, alkaline phosphatase and NAD-glycohydrolase) to hydrolyze contaminating nucleotides. Enzymes were removed by filtration with a multiscreen vacuum manifold on Immobilon-P membrane plates (Millipore, Milan, Italy) and reactions for cADPR determination were carried out in 96-well plates. A 50 µl-aliquot of reagent, containing 30 mM nicotinamide, 100 mM sodium phosphate and 0.1 µg/ml ADP-ribosyl cyclase (from Aplysia californica, Sigma, Milan, Italy) was added to 0.1 ml of sample. In parallel, the same reagent without ADP-ribosyl cyclase was added to each sample. Finally, 0.1 ml of the cycling reagent [2% (v/v) ethanol, 100 µg/ml alcohol dehydrogenase, 20 µM resazurin, 5 µg/ml diaphorase, 10 µM FMN, 10 mM nicotinamide and 100 mM sodium phosphate, pH 8.0] was added to each well. The
increase in resazurin fluorescence (544 nm excitation, 590 nm emission) was measured every 3 min over a 12-hour period, using a fluorescence plate reader (Fluostar Optima, BMG Labtechnologies GmbH, Offenburg, Germany). A cADPR standard curve was always run in parallel in each assay.

ABA detection in hydroid extracts.

Freshly cut hydroid fragments (approx. 0.5 g wet weight) were homogenized in 6 vol of methanol/water (80:20 vol/vol), methanol was evaporated and the aqueous extract was acidified (at pH 3.1) with 10% (v/v) trifluoroacetic acid and extracted with diethylether [20]. After removal of diethylether under vacuum the extract was redissolved in 0.5 ml of Tris-buffered saline (pH 7.4) and 20 and 50 µl aliquots were analyzed in duplicate by ELISA with the PGR-1 Sigma detection kit, which is specific for (+)-ABA. Occasionally, the ABA concentration was confirmed by HPLC analysis on extracts from 6 g wet weight [12].
RESULTS

ADP-ribosyl cyclase activity is present in *Eudendrium* and increases during regeneration.

Cyclase activity was detected in lysates from freshly collected *E. racemosum*, at $0.15 \pm 0.06 \text{nMoles cADPR/min/mg protein}$ (mean $\pm$ SD of 20 determinations), together with an eight-times higher cADPR hydrolase activity ($1.20 \pm 0.18$). No seasonal variations of cyclase activity were observed in *E. racemosum*, which is present year-round without significant variations of its biomass [16]. Conversely, a sharp increase of both cyclase and hydrolase activities was observed during spontaneous tissue regeneration, a light-stimulated process in *E. racemosum* [12], which can be induced by cutting of stolon fragments. Cyclase and hydrolase activities increased in the regenerating tissue budding from the stolons’ cut ends as well as in the center of the fragments (Table 1). Under light conditions, the cyclase activity of the fragments’ extremities was similar to that of the center and more than ten-fold higher compared to that at the start of incubation. In the fragments regenerating under dark conditions, cyclase activity increased to a somewhat lesser extent and was higher in the extremities compared to the central part of the fragment (Table 1). Hydrolase activity was also increased during regeneration, although to a lesser extent compared to the cyclase, leading to a significant decrease of the hydrolase-to-cyclase ratio (Table 1).

These results suggested involvement of cyclase activity in the process of *E. racemosum* regeneration.

Abscisic acid stimulates regeneration and ADP-ribosyl cyclase activity of *Eudendrium* fragments.

The known stimulatory effect of abscisic acid (ABA) on sponge cyclase and metabolism (respiration, filtration and protein synthesis, [21]) prompted us to investigate the effect of ABA on cyclase activity and on regeneration in *E. racemosum* fragments. Outgrowth at the ends of control fragments was detectable after 24 hours (Fig. 1A, white bars) and was preceded (at 18 hours) by a ten-fold increase of cyclase activity over values measured at the start of incubation (Fig. 1B, white
Addition of 50 µM ABA accelerated regeneration, which was already detectable after 18 hours (Fig. 1A, grey bars) and induced an earlier (3 hours) and higher increase of the cyclase activity (Fig. 1B, grey bars). The increase of the cyclase activity preceded tissue outgrowth in both ABA-treated and control fragments. At 5 µM, ABA stimulated cyclase activity and regeneration compared to control, however at a quantitatively lower extent than ABA 50 µM (Fig. 1 A, B). During both spontaneous and ABA-stimulated regeneration, the increase of cyclase activity was accompanied by an increase of the hydrolase, which showed a similar time-course, but a somewhat lesser extent of activation compared to the cyclase (Fig. 1 C and D). As a result, the hydrolase-to-cyclase ratio (R) decreased during regeneration, from a “resting” value of 9.0 at time = zero down to 1.7 and 0.7 during light- or ABA-induced regeneration (Fig. 1 C and D).

In sponges, ABA was shown to activate ADP-ribosyl cyclase by means of a protein kinase (PK)-dependent phosphorylation [12]. Thus, we investigated the effect on cyclase activity and on the intracellular cADPR content ([cADPR]i) of a brief exposure of E. racemosum fragments to ABA, in the absence or presence of K252a, a general cell-permeable PK inhibitor. Incubation with 50 µM ABA for 60 min induced a sharp increase of the cyclase activity in E. racemosum (Fig. 2A, grey bar), which was completely prevented by 2 µM K252a (Fig. 2A, black bar). Hydrolase was also activated by ABA, but to a significantly lesser extent compared to the cyclase (1.12 ± 0.1, 1.44 ± 0.2, 1.75 ± 0.2 and 1.0 ± 0.1 nMoles/min/mg for time = zero, control, ABA- and K252a-treated fragments, respectively), leading to a marked ABA-induced decrease of the hydrolase-to-cyclase ratio (R), which was prevented by K252a (Fig. 2A). Cyclase activation was paralleled by a four-fold increase of the [cADPR]i, which was likewise abrogated by the PK inhibitor (Fig. 2B). To identify the PK involved in cyclase activation we tested the effects of two different PK-specific, cell permeant (myristoylated) inhibitors: peptide 14-22, specific for PKA and peptide 20-28, specific for PKC (both from Calbiochem). The PKA-specific inhibitor almost completely prevented the ABA-induced, early (1 hour) cyclase activation, as well as the belated (24 hours) cyclase increase.
observed in unstimulated fragments during regeneration. Conversely, the PKC-specific inhibitor was without significant effect in both cases (Fig. 3). These results indicate that in E. racemosum ADP-ribosyl cyclase activation during ABA-stimulated or spontaneous regeneration occurs through a PKA-mediated phosphorylation.

**ABA is involved in light-stimulated E. racemosum regeneration.**

Regeneration of E. racemosum is sharply light-dependent [16]. In control fragments, regeneration was indeed markedly light-sensitive, as outgrowth from the stolons’ cut ends was visibly higher under light conditions compared to that in the dark (Fig. 4 A and B). Addition of ABA dramatically stimulated regeneration in the dark, inducing a similar outgrowth as observed under light conditions (Fig. 4 C). The total outgrowth, as measured microscopically after 48 hours incubation of E. racemosum fragments, was four-fold higher under light conditions compared to that in the dark (Fig. 5). ABA 5 µM stimulated regeneration in the dark 4.5-fold compared to controls, up to values similar to those observed in control fragments in the light, suggesting that this concentration may induce stimulatory effects on regeneration similar to those afforded by endogenous ABA produced under light exposure. This seems to be supported also by the similar [cADPR]i measured in fragments regenerating in the light (without additions) or in the dark, but in the presence of 5 µM ABA (0.52 ± 0.08 vs. 0.59 ± 0.09 pMoles/g wet weight, respectively; n = 3). Finally, the similar growth of ABA-treated fragments under light and dark conditions indicated that the effects of ABA and of light on growth were not additive (Fig. 5).

These results suggested involvement of ABA in the signal transduction pathway leading to light-stimulated regeneration. To test this hypothesis, we investigated presence of ABA in E. racemosum tissue and we measured the hormone concentration under light- and dark conditions. Indeed, ABA was detected in E. racemosum tissue extracts at a concentration of 18 ± 4 pMoles/g wet tissue (n = 5). After 24 hours in the dark, endogenous ABA decreased slightly but its concentration dramatically increased shortly (15 min) after re-exposure to light (Fig. 6). After 4 hours light, hormone levels decreased down to near-basal values (Fig. 6), indicating that an effective ABA
turnover occurs in *E. racemosum*, similarly to what observed in plants [22]. Fluridone, an inhibitor of ABA synthesis from carotenoids in plants [23], prevented the light-induced increase of ABA concentration (Fig. 6). The amount of ABA found in hydroids and the kinetics of increase following light exposure closely parallel those observed in sponges after heat-stress [12]. On a fresh weight basis, the amount of ABA found in hydroids (20-100 pMoles/g) is similar to that reported in the tissues of higher plants (40-400 pMoles/g) [20, 24, 25] and is 2-12 times higher than that found in mammalian tissues (1-8 pMoles/g) [20].

These results demonstrate presence of the phytohormone ABA in *E. racemosum* and rapid stimulation of its endogenous neo-synthesis by light. Carotenoids, ABA precursors in plants, were indeed detected spectrophotometrically [26] in acetone extracts from freshly collected *E. racemosum* colonies. At 42 ± 8 µg/g wet weight, this concentration is approx. half of that reported in carrots [27].

Fig. 7 summarizes the time-courses of light-induced changes on cyclase activity, ABA and cADPR levels in *E. racemosum* fragments exposed to light after a 12-hour period in the dark. ABA levels increase within minutes of light exposure and subsequently return to near-basal values after 18-24 hours. Cyclase activation follows the increase of [ABA]i and reaches maximal values after 18 hours, similarly to the [cADPR]i. The hydrolase-to-cyclase ratio changes from 9.0 at time = zero to approx. 3.0 after 24 hours.

**Effect of various inhibitors on light-stimulated *E. racemosum* regeneration.**

To confirm involvement of ABA, ADP-ribosyl cyclase and intracellular calcium in the signal transduction pathway of light-induced regeneration in *E. racemosum*, we used a number of inhibitors to interrupt signaling at specific sites (Fig. 8). Over the concentration range used to inhibit ABA synthesis in plants [24, 25], Fluridone reduced regeneration in a dose-dependent way, confirming involvement of ABA in the induction of hydroid regeneration. The PK inhibitor K252a, which prevented ABA-induced ADP-ribosyl cyclase activation and the consequent [cADPR]i increase (Fig. 2), induced a 70% growth reduction after 24 hours exposure (not shown) and a
somewhat lower inhibition (50%) after 48 hours exposure (Fig. 8). The PKA-specific inhibitor (myristoylated peptide 14-22) reduced *E. racemosum* regeneration by a similar extent (not shown). Addition of the membrane-permeant cADPR antagonist 8-Br-cADPR [28] decreased regeneration by 70%, while the IP3 antagonist U73122 was less active (35 % reduction). The combined effect of both antagonists together was not additive, reducing regeneration to an extent similar to that observed with 8-Br-cADPR alone (not shown). Finally, chelation of intracellular calcium with membrane-permeant EGTA-AM resulted in the almost complete inhibition of *E. racemosum* regeneration. Neither of these treatments affected hydroid vitality, as judged by microscopic examination of the tissue protruding from the fragments’ cut ends.

Taken together, these results demonstrate that regeneration in *Eudendrium* is an ABA-stimulated, calcium-dependent process primarily mediated by cADPR.
DISCUSSION

Abscisic acid (ABA) is a phytohormone involved in plant response to abiotic stress and in the regulation of seed dormancy and germination [29]. In vegetative tissue, where ABA controls stomatal closure and transcription of several genes, ABA levels increase in response to environmental conditions, such as drought, high salinity, light and low temperature [29]. Intracellular calcium is known to be a signaling intermediate of ABA: of the principal regulators of intracellular calcium levels known to date, inositol (1,4,5)-triphosphate (IP3), cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP⁴⁺), IP3 and cADPR have been shown to be involved in ABA signaling [see ref. 30 for a recent review]. ABA increases IP3 in Arabidopsis [31] and cADPR can substitute for ABA in stimulating transcription of two ABA-regulated stress-responsive genes [8] and in inducing stomatal closure [6]. In sponges (phylum Porifera), ABA is involved in temperature signaling, inducing ADP-ribosyl cyclase activation, intracellular calcium increase and stimulation of filtration and respiration [12, 21]. The striking biochemical similarities between the ABA/cADPR signaling pathway in plants and in sponges suggest its ancestral role in relating cell functions to environmental conditions in a common precursor of modern Metazoa and Metaphyta.

Here we demonstrate the involvement of ABA, and of cADPR as its second messenger, in light-induced regeneration in hydroids, the most ancient metazoan phylum possessing typical animal tissues (Eumetazoa). Thus, this is the first report of a complete signaling pathway in Eumetazoa involving a phytohormone. In E. racemosum, light exposure activates ABA synthesis, which stimulates ADP-ribosyl cyclase via a PKA-mediated phosphorylation and the consequent increase of the intracellular cADPR concentration then leads to an increase of the [Ca²⁺]i, which stimulates proliferation (Fig. 9). The fact that cyclase activation by light and ABA is also accompanied by an increase of hydrolase activity (Fig. 1 C, D) may suggest that a bifunctional enzyme is responsible for both activities, similarly to what observed in mammals, where the
hydrolase-to-cyclase ratio (R) is 10 [7]. Both light and ABA induce a decrease of the ratio, from a “resting” value of 8.0 down to 0.5-3.0 in the “activated” condition, which, together with the activation of the cyclase, concurs to increase the [cADPR]i (Fig. 7). Cyclase activation via PKA has been observed in sponges, where it is triggered by heat-stress [12] and also in bovine adrenal chromaffin cells, where it is triggered by acetylcholine and is also accompanied by activation of cADPR-hydrolase activity [9].

It may be of interest to observe that the increase of cyclase activity induced by light is similar in the regenerating extremities and in the center of *E. racemosum* fragments, while under dark conditions the increase of cyclase activity is higher in the extremities compared to the central part of the fragments (Table 1). The reason for this may lie in the fact that the hydroid exoskeleton (perisarc) is transparent to light, as witnessed by the fact that the internal tissue can be observed upon trans-illumination (Fig. 4). Thus, light exposure may stimulate ABA synthesis and cyclase activity throughout the tissue inside the perisarc; indeed, budding of new tissue occurs from the ramifications, as well as from the extremities of cut fragments (Fig. A, C). Regeneration in the dark is probably induced by the mechanical and/or chemical stress elicited by cutting of the fragments and exposure of the tissue to external SW and is accompanied by a higher cyclase activation in the tissue exposed to the environmental stress (extremities) compared to the central part of the fragment.

IP3 is also involved along with cADPR in the intracellular calcium increase during *Eudendrium* regeneration, as demonstrated by the significant decrease (35%) caused by the IP3 antagonist U73122 on ABA-induced growth (Fig. 8). However, the fact that 8-Br-cADPR and U73122 together did not exert a higher inhibitory effect compared to 8-Br-cADPR alone (70%) suggests that cADPR and IP3 act sequentially rather than synergistically. A sequential action of cADPR and IP3 has been recently observed in murine fibroblasts, where a cADPR-induced calcium release is necessary to start localised IP3-mediated calcium signals in response to purinergic receptor stimulation [32]. Failure of 8-Br-cADPR to completely prevent *Eudendrium* regeneration,
as observed with EGTA-AM, may indicate involvement of NAADP⁺, a potent calcium mobilizer co-produced with cADPR by invertebrate and mammalian ADP-ribosyl cyclases [7], or may be due to the higher difficulty of 8-Br-cADPR as compared to EGTA-AM to cross the perisarc, which, at the start of regeneration, completely covers the hydroid tissue except for the fragment cut ends.

The capacity for extensive regeneration upon loss of tissue in hydroids is reminiscent of the process of stem cell proliferation/differentiation in higher animals, which in recent years is being reported in several tissues other than those historically known to possess a stem cell reservoir (epithelial and hemopoietic tissue). Recently, stroma-produced cADPR has been shown to stimulate human hemopoietic stem cell proliferation [33]: the signal(s) inducing ADP-ribosyl cyclase activation in the bone marrow stroma, however, are as yet unidentified. As ABA has been detected in mammalian tissues [20], the present data may suggest a role for the phytohormone in the regulation of human stem cell proliferation/differentiation similar to that unveiled in our distant metazoan ancestors.
REFERENCES


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FIGURE LEGENDS

Fig. 1. Time course of fragment elongation and ADP-ribosyl cyclase/cADPR hydrolase activities during *Eudendrium racemosum* regeneration.

Small fragments (10 mm each) cut from different hydroid colonies and randomised were incubated in Petri dishes (10 pieces per dish) in SW at 17°C under artificial light (8 hours/day) in the absence (control) or in the presence of ABA. Results shown are the mean ± SD from three different experiments.

A. Regeneration at the fragments’ cut ends was determined microscopically: total outgrowth as measured on all fragments per Petri dish was divided by the number of fragments. Control, white bars; ABA 50 μM, grey bars; ABA 5 μM, striped bars. Regeneration was detectable in control and in ABA-treated fragments starting at 24 and 18 hours incubation, respectively.

B. ADP-ribosyl cyclase activity was determined on lysates prepared from 10 fragments per experimental point: results are expressed as activity relative to time = zero (at the start of the incubation). Control, white bars; ABA 50 μM, grey bars; ABA 5 μM, striped bars.

C. ADP-ribosyl cyclase (white squares) and cADPR-hydrolase (black squares) of control fragments (≥10 fragments per experimental point).

D. ADP-ribosyl cyclase (white squares) and cADPR hydrolase (black squares) of 50 μM ABA-treated fragments (≥10 fragments per experimental point).

Fig. 2. ADP-ribosyl cyclase activation by ABA is prevented by K252a.

*E. racemosum* fragments were incubated in SW at 17°C under artificial light, in the absence (control, white bars) or in the presence of 50 μM ABA, without (grey bars) or with (black bars) 2 μM K252a. After 60 min, ADP-ribosyl cyclase and cADPR-hydrolase activities and the intracellular cADPR concentration ([cADPR]i) were measured as described under Experimental Procedures (10 fragments per experimental point). R indicates the hydrolase-to-cyclase ratio.

Values shown are the mean ± SD from five (A) and three (B) different experiments.

Fig. 3. Effect of PKA- and PKC-specific inhibitors on ADP-ribosyl cyclase activation.

*E. racemosum* fragments were incubated as described in the legend to Fig. 1, in the presence of 50 μM ABA for 1 hour (striped bar) or in the absence of ABA for 24 hours (white bar). The PKA-specific inhibitor (myristoylated peptide 14-22, black bars) and the PKC-specific inhibitor (myristoylated peptide 20-28, grey bars), both at 5 μM, were added 30 min before the start of
incubation. ADP-ribosyl cyclase activity was measured on lysates prepared from 10 fragments per experimental determination. Results are expressed as activity relative to time = zero (at the start of incubation). Values shown are the mean ± SD from three different experiments.

**Fig. 4. Regeneration of *E. racemosum* fragments observed under low power microscopy.**

*E. racemosum* fragments (10 mm) were cut from different colonies, randomized, divided into three Petri dishes and incubated in SW at 17°C under artificial light (A) or in the dark, without (control, B) or with 50 µM ABA (C). After 3 days, fragments were observed under low power microscopy (5x magnification). Results from a representative experiment are shown.

**Fig. 5. Effect of ABA on *E. racemosum* regeneration under light and dark conditions.**

*E. racemosum* fragments were incubated in SW at 17°C under artificial light (8 hours/day) (white bars), or in the dark (black bars), in the absence (control) or in the presence of ABA 5 or 50 µM. After 3 days, outgrowth at the fragments’ cut ends was measured microscopically. Total outgrowth was divided by the number of fragments (10 fragments per experimental point).

Values shown are the mean ± SD from three different experiments.

**Fig. 6. ABA synthesis in *E. racemosum* is stimulated by light.**

ABA content was measured in extracts from *E. racemosum* immediately after sampling (t = 0), after 24 hours incubation at 17°C in the dark (24 h dark), and at different times after exposure to white light (15 min, 1 h and 4 h). Duplicate samples were incubated under the same conditions in the absence (white bars) or in the presence (grey bars) of 50 µM Fluridone.

Results shown are the mean ± SD from three different experiments.

**Fig. 7. Time-course of light-induced changes in cyclase activity, ABA and cADPR levels in *E. racemosum* fragments.**

Fragments cut from different colonies and randomized were incubated in Petri dishes in SW at 17°C in the dark for 12 hours, then exposed to artificial light for the first 8 hours of a 24 hour-incubation time. Cyclase activity (black squares), ABA (black triangles) and cADPR (white squares) levels are compared to values measured at the end of the dark period (T₀). Mean results from three experiments are shown (≥ 20 fragments per experimental point); SD ≤ 20% of the mean value.
Fig. 8. Effect of various inhibitors on light-induced regeneration in *E. racemosum*.

*E. racemosum* fragments were incubated in SW at 17°C under artificial illumination (8 h/day) in the absence (control) or presence of the indicated chemicals. After 48 hours, tissue outgrowth budding from the fragments’ cut ends was measured (10 pieces per experimental point). Results are expressed as growth relative to control, untreated, fragments. Values are the mean ± SD from three different experiments. Flu, fluridone. U731, IP3 inhibitor U73122.

Fig. 9. The signal transduction pathway of light-stimulated regeneration in *E. racemosum*. 
Table 1. Cyclase and hydrolase activities measured on *E. racemosum* fragments during regeneration.

<table>
<thead>
<tr>
<th>Condition</th>
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<th>Cyclase (nMoles/min/mg)</th>
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Small hydroid fragments (10 mm each) were cut from different colonies, randomized and incubated in SW in Petri dishes at 17°C under artificial light (8 hours/day) or in the dark. After 4 days, the regenerated tissue protruding from the fragments’ ends was cut and cyclase and hydrolase activities were determined, as described under Experimental Procedures, on lysates prepared from the regenerating part (extremities) and from the central section (center) of the fragments. Values are compared to those measured on fragments at time = zero (at the start of incubation). Results shown are the mean from three experiments; SD ≤ 18% of the mean value.
Fig. 1

Fig. 2
**Fig. 3**

**Fig. 4**
Fig. 5

Fig. 6
Fig. 7

Fig. 8

Fig. 9

Light → ABA → PKA → cyclase activation → ↑[cADPR]i → ↑[Ca^{2+}]i → regeneration

Fluridone, P14-22, 8-Br-cADPR, EGTA-AM
Abscisic acid signaling through cyclic ADP-ribose in hydroid regeneration
Stefania Puce, Giovanna Basile, Giorgio Bavestrello, Santina Bruzzone, Carlo Cerrano,
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