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Original Article

The protein phosphatase inhibitor cantharidin induces head and foot formation in buds of *Cassiopea andromeda* (Rhizostomae, Scyphozoa)

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ABSTRACT The polyps of *Cassiopea andromeda* produce spindle shaped, freely swimming buds which do not develop a head (a mouth opening surrounded by tentacles) and a foot (a sticky plate at the opposite end) until settlement to a suited substrate. The buds, therewith, look very similar to the planula larvae produced in sexual reproduction. With respect to both, buds and planulae, several peptides and the phorbolester TPA have been found to induce the transformation into a polyp. Here it is shown that cantharidin, a serine/threonine protein phosphatase inhibitor, induces head and foot formation in buds very efficiently in a 30 min treatment, the shortest yet known efficient treatment. Some resultant polyps show malformations which indicate that a bud is ordinary polyp tissue in which preparatory steps of head and foot formation mutually block each other from proceeding. Various compounds related to the transfer of methyl groups have been shown to affect head and foot formation in larvae of the hydrozoon *Hydractinia echinata*. These compounds including methionine, homocysteine, trigonelline, nicotinic acid and cycloleucine are shown to also interfere with the initiation of the processes which finally lead to head and foot formation in buds of *Cassiopea andromeda*.

KEY WORDS: *cantharidin, trigonelline, cycloleucine, Cassiopea*

Introduction

The most important way to study pattern formation control in cnidarians is to make use of their famous ability to regenerate missing structures. *Hydra* spp. has been studied for more than 250 years by this method. *Cassiopea andromeda*, a scyphozoa, allows an additional access to that topic. These animals produce buds, which do not develop a head and a foot until they receive a certain external inducing stimulus. Such a stimulus can be provided by various chemicals and by sectioning some distance away from the region which subsequently forms the head and the foot, respectively.

The scyphistoma (polyps) of *Cassiopea andromeda* (Rhizostomae) reproduce asexually by generating buds (Fig. 1), which look similar to the three times smaller planula larvae obtained in sexual reproduction by medusae. Similar to a planula, such a bud swims by moving its cilia, settles to a suited substrate, and transforms into a polyp. As in the sexual cycle, this process is often called metamorphosis. Because the process of bud formation does not immediately lead to an immobile polyp, these species have two very similar ways of spreading in their environment. In *Cassiopea andromeda* budding proceeds in a manner different from the well known way *Hydra* spp. does, since the tip of the developing bud will not become the head, as in *Hydra* spp., but rather the foot of the future polyp (Fig. 1).

Curtis and Cowden (1971) found that buds need a suited substrate for metamorphosis to start. Under sterile conditions the buds keep swimming for days without further differentiation. Neumann *et al.* (1980) found certain marine bacteria, including *Vibrio alginolyticus,* to deliver inducing signals. Several peptides have been detected to induce the transformation into a polyp (Wolk *et al.*, 1985; Hofmann and Brand, 1987; for review see Hofmann *et al.,* 1996; Walther and Fleck, 1998). Recently, tumour promoting phorbol esters (e.g., TPA) were found to induce that process (Bischoff *et al.*, 1991; Fleck and Bischoff, 1992). Ammonium ions were found to cause a partial transformation (Berking and Schüle, 1987). Most interestingly, the same substances, which initiate the transformation of a bud into a polyp, also initiate the metamorphosis of a planula into a polyp (for review see Hofmann *et al.*, 1996; Fleck, 1997).

Neumann *et al.* (1980) proposed that in the bud, differentiation of the head is blocked by an inhibitory agent which is generated at the end where the foot will develop. That proposition was based on results of sectioning experiments first reported by Curtis and Cowden (1971): posterior fragments develop into freely floating

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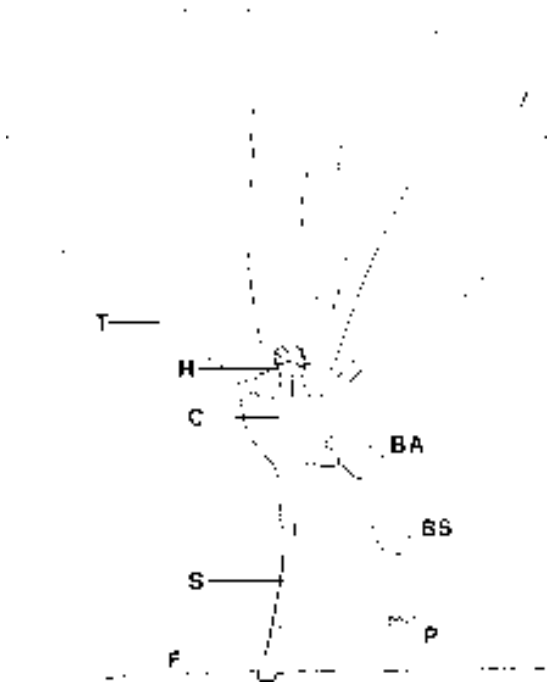

Fig. 1. The scyphistoma (polyp) of *C. andromeda* in asexual reproduction. *BA: bud, still attached to the parent, BS: bud, freely swimming, C: calyx, H: hypostome, P: polyp (scyphistoma), result of bud settlement to a suited substrate, F: foot, S: stalk, T: tentacle.*

heads without stalk and foot while the anterior ones remain in the bud stage.

In several hydrozoan species, metamorphosis is induced by TPA as well (Müller, 1985; for review see Walther *et al.*, 1996; Fleck, 1997). In particular, for *Hydractinia echinata* there is a long list of additional inducers (for review see Walther *et al.*, 1996; Berking, 1998). However, most of these inducers failed to start transformation of the planula or the bud into a polyp in the scyphozoa *Cassiopea andromeda*. Here we report that cantharidin, a serine/threonine protein phosphatase type 1 and 2a (PP-1 and PP-2A) inhibitor (Li and Casida, 1992), induces that process in buds of *C. andromeda* very efficiently. In contrast, cantharidin failed to induce metamorphosis from the larva to the polyp in the hydrozoan *Hydractina echinata* (unpublished result). However, cantharidin strongly interferes with foot formation at the bud's base in *Hydra vulgaris* (Pérez, 1996) and causes premature supernumerary head formation in a mutant strain of *Hydra magnipapillata* (Zeretzke and Berking, 1996).

Results

Control of the position where a bud forms

The experiments shown here were generally performed on *C. andromeda* and most were confirmed with *C. xamachana*. Polyps of both *C. andromeda* and *C. xamachana* produced buds in the basal third of the calyx, usually at only two positions just opposite to each other (Fig. 1,2a). Often, the buds formed a chain with the oldest one at the tip. Up to four buds linked together were observed at the same time. Because the body of a mature polyp is incompletely separated by four longitudinal septa, we wondered whether this symmetry was responsible for the observed symmetry of the positions where buds are formed. In 15 polyps of *C. xamachana,* one quarter of the body was excised, which bore a chain of buds (Fig. 2a). In both tissue pieces the wound closed within one day. Three out of 15 regenerated the missing quarter, the others displayed a threefold symmetry with respect to the septa even 15 days after sectioning. All polyps produced buds at two sites just opposite to each other and thus, the position of the bud did not correlate with the position of a septum. Therewith, the positioning of buds at the calyx appeared to be largely the result of a mutual inhibition of the two bud anlagens. The removed quarters did not regenerate septa and did not regenerate a stalk, but started to produce a bud seven days after sectioning. In all cases the bud was produced at the most distal site, i.e., at the position where the stalk was expected to form. Budding at that position was obtained in two other cases as well: (1) when polyps regenerated after the stalk and the basal part of the calyx had been removed (Fig. 2b) and (2) when oral parts of sectioned buds were cultivated (Fig. 3a). With respect to the mentioned isolated polyp quarters, a stalk with a foot was formed later at the place where initially buds had formed. This took place several weeks after sectioning and after several buds had formed there. The results indicate a similarity between bud and stalk formation and that budding can start and proceed in the absence of a foot and a stalk.

Cantharidin induces head and foot formation in buds

Untreated buds remained in the bud stage for about two weeks. Then, in the absence of applied inducing conditions, they developed a head and a foot within a few days (Fig. 4). A 30 min pulse treatment of buds with 20 µM/l cantharidin caused induction in all buds (Fig. 5). As compared to induction due to contact to an inducing substrate, IS (see 'Materials and Methods'), the visible onset of head and foot formation, was delayed for one to two days.

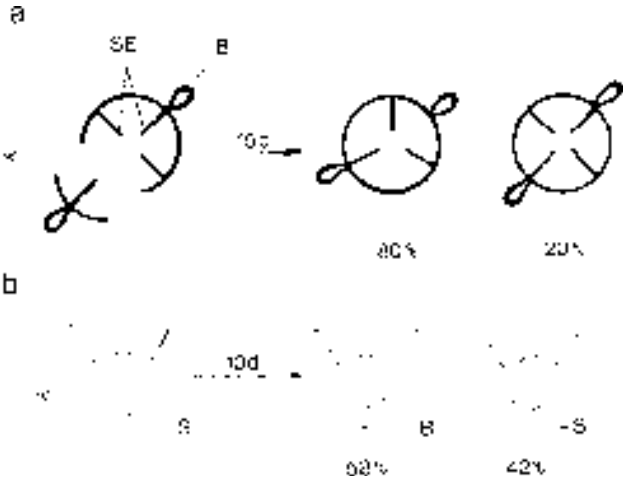

Fig. 2. Polyps of *C. andromeda* regenerate the budding region. (a) *Shown is an optical section of the budding region of a polyp. Two buds (B) are shown which have formed at maximal distance to each other close to a septum (SE). A quarter of the calyx including a septum and the budding region was removed (n= 15). The fate of the remainder was scored 15 days following sectioning.* **(b)** *The aboral part of the calyx including budding region and stalk (S) was removed (n= 19). The fate of the oral fragment was scored 10 days following sectioning.*

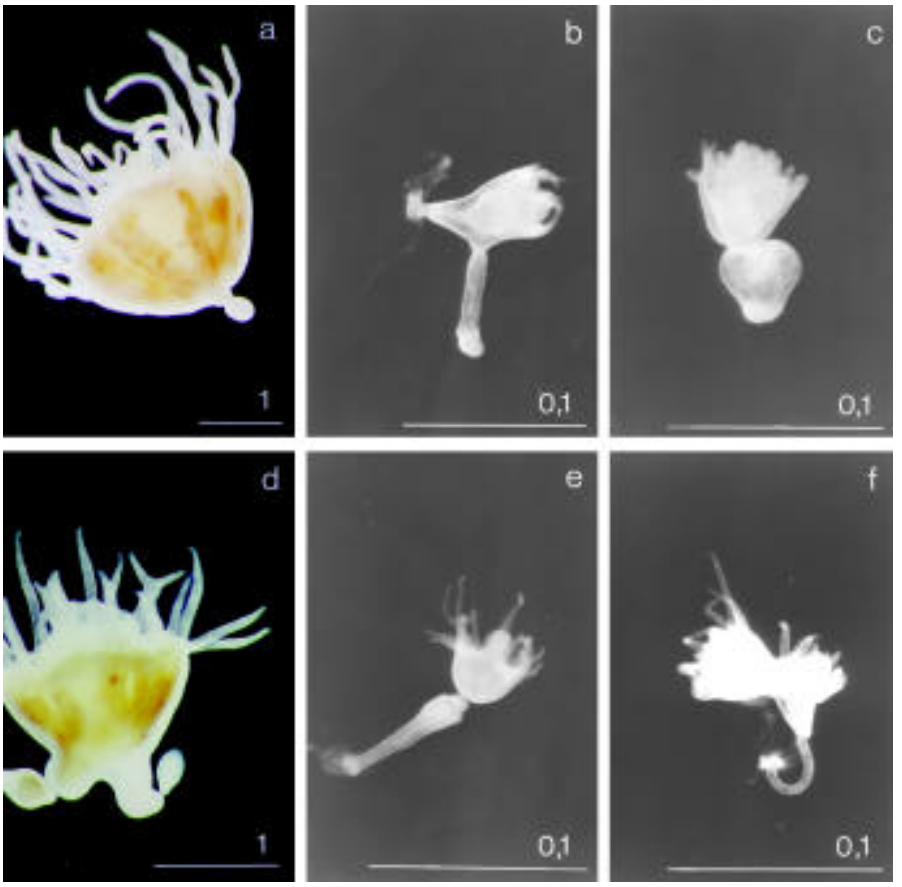

Fig. 3. Examples of malformations obtained by sectioning or treatment of polyps (scyphistoma) or of buds with various concentrations of cantharidin for 30 min. **(a)** *An oral fragment of a bud of C. xamachana cultivated for two months producing a bud at its aboral end.* **(b)** *C. andromeda polyp with two stalks. Photo: ten days after treatment of buds with 20 μM/l cantharidin.* **(c)** *C. andromeda bud in the process of separation into a calyx without foot (oral end) and a bud. Photo: four days after treatment with 20 μM/l cantharidin.* **(d)** *C. andromeda polyp with a bud at the place of the stalk. Photo: nine days following treatment with 30 μM/l cantharidin.* **(e)** *C. andromeda polyp separating into a calyx without foot (oral end) and a complete polyp. Photo: fourteen days after treatment (30 μM/l cantharidin) of a polyp bearing a young bud. Eight days following treatment the bud separated from the parent.* **(f)** *C. andromeda polyp with two heads. Photo: eight days after treatment of a bud with 20 μM/l cantharidin. Bars in the figure correspond to mm.*

About one third of the cantharidin induced developments resulted in normal shaped polyps, the others showed abnormalities (see below). Induction due to contact with IS resulted in normal shaped polyps only. A concentration of 30 μM/l cantharidin caused head and foot formation as well, but some suffering occurred, such as a transient partial separation of the ectoderm from the mesogloea (i.e., the extracellular matrix which separates the ectoderm from the entoderm). A concentration of 10 μM/l was less efficient than 20 μM/l, but almost all induced buds developed into normal polyps (not shown).

Abnormalities obtained by treatment of buds with cantharidin

The abnormalities observed following a cantharidin treatment (30 min, 20 μM/l) can be classified as follows: (1) formation of an additional stalk half way between the head and the foot (Fig. 3b). (2) Formation of an indentation at about one half of the body length and later on a separation at that site (Fig. 3c). The oral end developed into a freely floating calyx with head structures. The basal end developed into a bud, as it was observed following treatment with ammonium chloride (Berking and Schüle, 1987). (3) In some few cases an additional head was formed half way between the head and the foot. In some of these cases, a separation was observed into an oral part with a head only and an aboral part with a head and a foot (Fig. 3e). In other cases separation did not take place (Fig. 3f).

Treatment of buds not yet separated from the parent polyp did not result in head or foot formation while still attached to the parent (30 min, 30 μM/l cantharidin). Young buds regressed; their tissue appeared to be reintegrated into the calyx of the parent. Older buds continued their development and separated from the parent, but displayed an unusual blown-up-shape. In several cases the ectoderm appeared partially separated from the mesogloea due to the high concentration of cantharidin. Buds of such treated animals produced a head and a foot when transferred to IS just after separation from the parent. Most were of normal shape, some showed the same types of malformation buds displayed and were treated with cantharidin following separation from their parents. In a few cases, the stalk of the parent polyp showed an indentation which finally led to a separation of the basal part. Those pieces looked very similar to buds, moved like buds, settled like buds and produced a head and a foot like buds (Fig. 3d).

Interference with initiation of head and foot formation

We tested substances which have been shown to interfere with metamorphosis induction in the hydrozoon *Hydractinia echinata*. Endogenous compounds were found to antagonise metamorphosis induction of planula larvae to polyps when applied simultaneously for 24 h with the artificial inducer Cs^+. The compounds include homarine (N-methylpicolinic acid), trigonelline (N-methylnicotinic acid), betain (N-trimethylglycine) and taurine. The first three were argued to act by delivering a methyl (or an aminopropyl) group. Consistently, methionine antagonises induction as well, and cycloleucine, which competes with methionine, antagonises the inhibitory influence of these compounds (for review see Walther *et al.*, 1996; Berking, 1998). In the following lines we describe how these substances affect initiation of the processes which finally

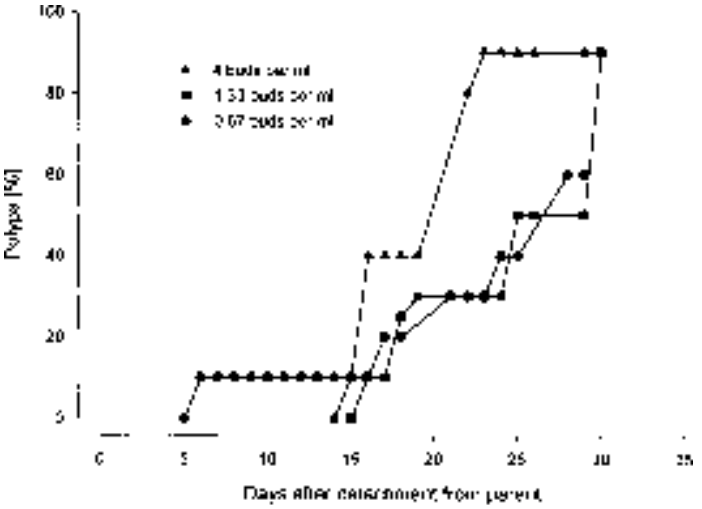

Fig. 4. Buds of *C. andromeda* produce head and foot spontaneously about two weeks following detachment from the parent. *Buds were kept in polystyrene dishes in various densities.* ▲, *4 buds/ml, n= 20;* ■, *1.3 buds/ml, n= 20;* ●, *0.67 buds/ml, n= 10.*

lead to head and foot formation in *C. andromeda* and *C. xamachana*.

Trigonelline and nicotinic acid

Buds of *C. andromeda* were allowed to make contact to the inducing substrate, IS (see 'Materials and Methods') while the sea water was enriched with trigonelline (N-methylnicotinic acid) for a period of 24 h. Following treatment, the buds were separated from the IS and transferred to a fresh medium without trigonelline. Concentrations of 1000 and 100 μM/l strongly antagonised induction (Fig. 6, Table 1), while 1 μM/l did not (not shown). During and following treatment, the buds moved like healthy buds. The initiation of head and foot formation was also antagonised in oral and aboral fragments. A concentration of 1000 μM/l trigonelline prevented the initiation of head and foot formation completely for at least two days (oral halves: 13 out of 13). The finding that oral fragments were hindered to form a head, indicates that not the substrate but rather the pattern forming system, is affected by the treatment because isolated oral fragments need no IS for the initiation of head formation. In parallel experiments with *C. xamachana,* treated oral fragments also failed to form a head within the period of observation (n=18, 10 days). This indicates that the 24 h pulse treatment does not result in a slowing down of structure forming processes but rather prevents the onset of the developments usually triggered by sectioning. The later observed increase of the frequency of oral fragments bearing a head in the various test groups may result from insterile conditions.

Trigonelline also antagonises the induction of head and foot formation caused by TPA (12-0-tetradecanoylphorbol-13-acetate) (Table 2). TPA harms the animals (see also Bischoff *et al.*, 1991). A concentration of 1000 μM/l trigonelline strongly reduced the efficiency of TPA induction while 100 μM/l displayed a lower but still significant reduction (Table 2). Results obtained with *C. xamachana* were similar (not shown).

In order to test whether the methyl group of trigonelline (N-methylnicotinic acid) is responsible for the observed inhibition, buds were treated with 100 μM/l nicotinic acid in a dish with IS. No difference was observed between treated and untreated buds within 10 days (Table 1). The same result was obtained when oral and aboral fragments of buds of *C. xamachana* were treated in the same way (not shown).

Methionine and homocysteine

A concentration of 100 μM/l L-methionine strongly antagonised the initiation of head and foot formation of buds of *C. andromeda* in the presence of the IS (Table 1) and TPA (Table 2), respectively. Results obtained with *C. xamachana* were similar (not shown). A concentration of 100 μM/l L-homocysteine applied for 24 h antagonised induction by IS (Table 1) and by TPA (Table 2). Lower concentrations displayed no significant influence. Homocysteine appears to display a much lower inhibitory influence on head and foot formation than methionine.

Cycloleucine

Cycloleucine (1-aminocyclopentane-1-carboxylic acid) competes with methionine due to its structural similarity. Therewith, it is able to antagonise the production of S-adenosylmethionine (SAM) from methionine and ATP. SAM is the most important donor of methyl and aminopropyl groups in organisms. In metamorphosis of *Hydractinia echinata,* 60 μM/l cycloleucine antagonised the inhibitory influence of trigonelline in the presence of Cs^+ as a metamorphosis inducer. In *C. andromeda* a much lower concentration, 1 μM/l cycloleucine, still displayed an inhibitory influence when applied for 24 h, either together with IS (Table 1) or with TPA as the inducer (Table 2).

Discussion

The scyphozoon *Cassiopea andromeda* produces buds which bear no head and no foot when they detach from the parent animal. They need an external stimulus for head and foot formation to start.

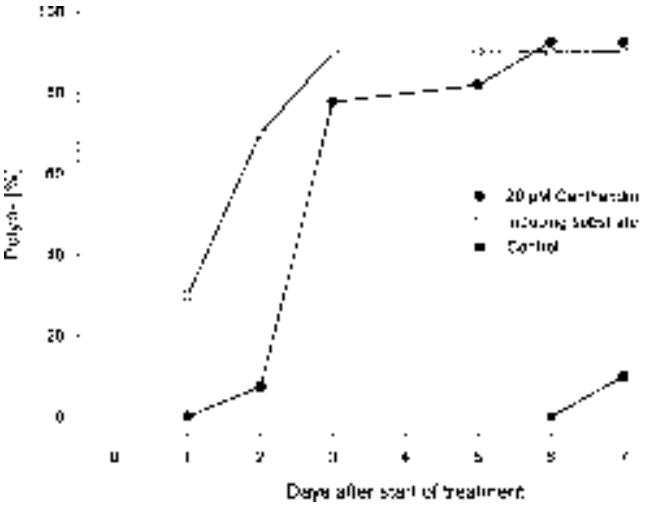

Fig. 5. Cantharidin initiates head and foot formation in *C. andromeda.* *Buds were either treated for 30 min with 20 μM/l cantharidin* (●, *n= 27) or incubated for 24 h with inducing substrate, IS, (see 'Materials and Methods')* (○, *n= 20) or incubated without cantharidin and IS* (■, *n= 20).*

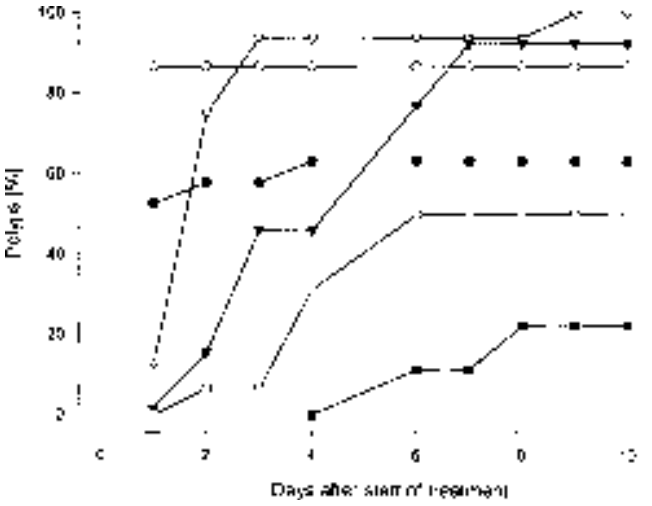

Fig. 6. Trigonelline antagonises induction of head and foot formation in *C. andromeda.* *Filled symbols indicate trigonelline treatment (24 h, 0.1 mM/l). Intact buds incubated for 24 h with IS (▽, n= 16; ▼, n= 13). Aboral halves incubated for 24 h with IS (❑, n=16; ■, n=18). Oral halves without IS (❍, n= 15; ●, n= 19).*

In contrast, the well known hydrozoon *Hydra* spp. produces buds which are complete small animals bearing a head and a foot when they drop off. A further difference is, that in *C. andromeda* the tip of the bud will eventually become the foot of the polyp while in *Hydra* spp. it will become the head. It is generally accepted that budding in *Hydra* spp. starts with an autocatalytic process which is restricted to a small patch by lateral inhibition (Gierer and Meinhardt, 1972). The latter is indicated by the distance a new bud keeps from an existing one in the circumference, as shown for both species (see Fig. 2a). The open question is what autocatalysis and lateral inhibition look like. It is also generally accepted that the polarity of the polyp is determined by a rather stable quantity (Gierer *et al.* 1972), a tissue parameter, termed positional value (Wolpert, 1969) which changes gradually along the longitudinal axis. The head contains the maximal value, the foot the minimal one. Applied to buds of *C. andromeda,* the lowest positional value is in its tip. In the tip, the value has decreased with respect to its original value. This is indicated by the polar organisation of the bud. In particular, the cilia of the bud ectoderm show a coordinated orientation to the oral pole as they do in the polyp, and as indicated by the swimming behaviour. Thus, a rearrangement of at least some of the cilia must have taken place in the course of bud formation. This indicates that, in the bud, pattern formation has started but is somehow blocked to proceed. In the scyphozoon C*ephea cephea,* a close relative of *Cassiopea* spp., the tip of the bud will also become the foot of the polyp, however, most buds form a head and a foot before separating from the parent (Sugiura, 1966). The blockage of pattern formation appears to be less in this species.

Transversal sectioning of a bud of *Cassiopea* spp. allows head formation in the posterior fragment while foot formation does not take place. This occurs in most cases, the others regenerate into complete buds, as the anterior fragments do as a rule. Based on these results Neumann *et al.* (1980) proposed that from the bud's anterior end a signal emerges which prevents head formation at the posterior end. Upon settlement to a suited substrate or due to treatment with inducing substances, foot formation is proposed to be initiated and that in turn it should reduce the head inhibiting influence of that very tissue. In addition, a foot inhibiting signal may be assumed to emerge from the posterior end. This is indicated by the observation that a bud does not form a foot at its anterior end until settlement and, in particular, that a posterior half obtained by sectioning forms a head but not a foot, as well. If the postulated inhibitors act structure specific, as proposed by Meinhardt (1993) for *Hydra* spp., we are confronted with a rather complex situation: from the anterior pole not only the foot inhibitor emerges but a head specific one, as well. The opposite may occur at the opposite pole. Such additional signal substances are not proposed to exist in *Hydra* spp. There is yet no proposition how it can be organised that in *Cassiopea* spp. they are generated at the respective sites at the correct time.

The malformations obtained by treatment of buds with cantharidin allow a further view into the pattern forming system: cantharidin caused: (1) normal polyps, (2) transversal separation of buds in which the posterior part forms a head, the anterior part regenerates the bud's architecture. This result is identical to that obtained by transversal sectioning Curtis and Cowden (1971), Neumann et *al.*, 1980) and to that obtained by treatment with ammonia (Berking and Schüle, 1987), (3) polyps with a head and a foot at the respective ends and in addition a head in the middle part or (4) a foot in the middle part. In particular, cases (3) and (4) indicate that cantharidin directly or indirectly stimulates an autocatalysis. But this enhancement displays no structure specificity.

When from normal sized polyps of *C. andromeda* and *C. xamachana* the stalk and part of the lower calyx is removed, the stalk, including the foot, will not be regenerated for a long time;

TABLE 1

SUBSTANCES WHICH ANTAGONISE INITIATION OF HEAD AND FOOT FORMATION IN BUDS OF C. ANDROMEDA BY A 24 H TREATMENT IN THE PRESENCE OF INDUCING SUBSTRATE (IS)

Applied Substance	Number of buds n	head and foot formation 2nd day n (%)	head and foot formation 3rd day n (%)
-	16	12 (75)	15 (94)
100 µM/ trigonelline	13	2 (15) *	6 (46) *
-	20	14 (70)	17 (85)
1000 µM/ trigonellinel	20	1 (5) *	6 (30) *
-	20	7 (35)	15 (75)
100 µM/l nicotinic acid#	20	10 (50)	13 (65)
-	20	13 (65)	15 (75)
10 µM/ L-methionine	46	9 (20)*	14 (70)
100 µM/lL-methionine	0 *	1 (5) *	
-	60	56 (93)	57 (95)
100 µM/lL-homocysteine	70	36 (51) *	57 (71) *
-	30	23 (77)	28 (93)
0.1 µM/ cycloleucine	29	22 (76)	28 (96)
-	30	25 (83)	26 (87)
1 µM/lcycloleucine	19	5 (26) *	8 (42) *
10 µM/ cycloleucine	20	1 (5) *	2 (10) *

Each test includes a group (of corresponding size) of untreated buds (not shown). No one of them produced a head or a foot up to the third day following treatment. * Indicates a significant difference to the respective control (χ^2, Fisher-Yates test, respectively, 5% level). Numbers given in italics indicate that in this group the frequency of animals bearing a head and a foot is less than half of that of the corresponding control. #The experiment was performed with C. xamachana.

rather, at the position of the stalk a bud develops (Fig. 2b). On the other hand, following cantharidin treatment a few animals fragmented their stalk such that the distal ends transformed into a bud like piece of tissue which displayed all properties of a bud, including its ability to transform into a polyp following settlement. This fits the proposition (1) that cantharidin initiates autocatalysis, in this case even in stalk tissue and (2) it indicates that budding and stalk formation have features in common: both start with a local decrease of the positional value. The difference is that a bud separates from the parent possibly due to the initiation of the processes which finally lead to head formation. The switch from bud to stalk formation at the base of the growing stalkless calyx may be caused by a change of the tissue property in this very tissue. We suggest that autocatalysis does not start because the positional value eventually has become too low at the aboral end of the growing calyx.

We know neither the postulated inhibitors nor the activators. However, in order to find substances (morphogens) and biochemical processes involved in that control, it seems helpful to have a consistent hypothesis of the properties of the control system. Thus, one approach is to try to explain the data obtained by as few assumptions as possible. We assume that, in the first instance, one and the same autocatalytic process is responsible for pattern formation. Secondary influences decide the direction of a quantitative change of the positional value at the site of autocatalysis. In this model not two but only one activator is proposed to exist, which as proposed by Gierer and Meinhardt (1972), stimulates its own generation, and that of an inhibitor which antagonises that generation. The local autocatalytic generation of the activator can result in a decrease or an increase of the positional value depending on the local concentration of a second inhibitor. The generation of this inhibitor is proposed to be also stimulated by the activator. According to the model in an animal and also in a bud, the activator and both inhibitors are generated at both ends. As in the model of Gierer and Meinhardt (1972), the activator has the shortest range in the tissue. In addition, it has been proposed that the generation of the morphogens depends on the positional value: where the value is high there is a high unregulated basal generation and in the case of autocatalysis a high maximal generation. The opposite happens at a low positional value (Berking, 1998).

In *Hydra* spp. and *Cassiopea* spp. budding starts with autocatalysis of the single hypothetical activator. When the concentration of the second inhibitor is low in that very tissue the positional value increases, as observed in *Hydra* spp. When it is high, the positional value decreases as observed in *Cassiopea* spp. (Berking, 1998). The stable inhibition of head and foot formation in buds of *C. andromeda* and *C. xamachana* may be understood as follows. The bud separates from the parent in a stage at which at its posterior end the maximal positional value is not yet attained, as indicated by the absence of the head. It will not reach the maximal value later on because, based on the model, increasingly more morphogens have to be generated including more inhibitors for a further increase. Thus, their concentrations increase and eventually they antagonise autocatalysis and a further increase of the positional value. Both processes will be resumed if the concentration of the inhibitors is artificially reduced (or if their action is somehow antagonised). If there is a strong reduction (e.g., due to cantharidin treatment) autocatalysis may even start in the middle part. Small local quantitative differences in the concentration of the second inhibitor cause the positional value either to increase or decrease, which eventually causes qualitatively different structures: head and foot, respectively.

In summary, we suggest that the pattern forming systems of *Hydra* spp. (Hydrozoa) and *Cassiopea* spp. (Scyphozoa) are very similar, though the processes of budding display strong differences. Further, we suggest that in *Cassiopea* spp. (as proposed for *Hydra* spp.) a bud specific pattern forming system does not exist, rather the general system which controls the patterning of the polyp, its growth and regeneration does also control budding. A bud in *Cassiopea* spp. is an ordinary polyp tissue as buds of *Cephea cephea* and *Hydra* spp. are, but in buds of *Cassiopea* spp. both the highest and the lowest positional values are not attained until induction. The mentioned peptides are proposed to initiate the processes which cause the transformation into a polyp by contact to specific receptors (for review see Fleck, 1997; Walther and Fleck 1998). TPA (Bischoff *et al.*, 1991; Fleck and Bischoff, 1992) and cantharidin can be argued to act by affecting protein phosphorylation (Li and Casida, 1992). If the propositions made are correct, then these agents somehow interfere with the primary pattern forming system of the animal such that autocatalysis is stimulated. Possibly, chemicals reduce the level of inhibition. The similarity of buds and planulae of *Cassiopea* spp. with respect to shape, behaviour and response to inducing agents is almost not understood but indicates strong similarities of their pattern forming systems.

In this paper we described a general approach to find agents which antagonise the transformation of a bud into a polyp: inducing substrate and TPA. We applied two inducers which are expected to use different initial steps in their way to cause induction. And we simultaneously applied various potential agonists and antagonists of induction. Our choice was guided by observations made with metamorphosis induction in the hydrozoon *Hydractinia echinata*. The agents were argued to either stimulate or to antagonise methylation and/or aminopropylation (for review see Walther *et al.*, 1996; Berking, 1998). In *C. andromeda* and *C. xamachana,* the potential methyldonors methionine and trigonelline antagonise

TABLE 2

SUBSTANCES WHICH ANTAGONISE INITIATION OF HEAD AND FOOT FORMATION IN BUDS OF C. ANDROMEDA BY A 24 H TREATMENT IN THE PRESENCE OF THE PHORBOLESTER TPA AS INDUCER

Applied Substance	Inducer TPA	n	head and foot formation 2nd day n (%)	head and foot formation 3rd day n (%)
-	5 µM/l	32	17 (53)	17 (53)
100 µM/l trigonelline	5 µM/l	31	7 (23) *	8 (26) *
-	5 µM/l	20	15 (75)	17 (85)
1000 µM/l trigonelline	5 µM/l	19	0 *	1 (5) *
-	1 µM/l	29	19 (66)	20 (69)
100 µM/l L-methionine	1 µM/l	29	2 (7) *	10 (34) *
-	1 µM/l	20	10 (50)	15 (75)
10 µM/l L-homocysteine	1 µM/l	20	7 (35)	20 (100)
100 µM/l L-homocysteine	1 µM/l	20	0 *	6 (30) *
-	5 µM/l	31	20 (65)	22 (71)
10 µM/l cycloleucine	5 µM/l	68	0 *	1 (1) *

See legend to table 1.

induction as they do in *Hydractinia echinata.* In both animals nicotinic acid, the demethylated trigonelline, did not antagonise the transformation into a polyp. However, homocysteine, the demethylation product of methionine, antagonises induction, as well. The inhibitory influence of homocysteine may result from its transformation into methionine. Consistently, its influence is weaker than that of methionine. In summary, the results indicate that in *Hydractinia echinata* and in the studied *Cassiopea* species a certain high concentration of potential methyldonors antagonises induction by the natural substrate and by TPA respectively.

However, in contrast to *H. echinata* cycloleucine, which competes with methionine due to its structural similarity, antagonises induction in buds of *C. andromeda*. There are two puzzling points: (1) induction appears to be more sensitive to cycloleucine than to methionine. (2) In *Echerichia coli*, yeast and rats, the concentration required for 50% inhibition of adenosyltransferase activity *(in vitro)* is between 2 and 6 mM/l cycloleucine (Chou *et al*., 1977), while 1 µM/l cycloleucine effectively antagonises metamorphosis induction in *C. andromeda.* Thus, one may argue that a low concentration of methionine is necessary to maintain biochemical processes necessary for induction, while a high concentration of methionine antagonises the initiation of the processes leading eventually to head and foot formation.

Materials and Methods

Animals and culture conditions

Polyps of *C. andromeda* and *C. xamachana* were a kind gift of Dr. K.D. Hofmann, University of Bochum, Germany. The polyps were reared in artificial sea water (Tropic Marin), pH 8.2, 950-1050 mOsmol, at 23°C in moderate darkness and fed 5 times a week with nauplii of *Artemia salina*. In order to obtain buds, the stalk and part of the calyx of the polyps were removed, because a contact of dropped off buds with the stalk and debris adhering to the stalk immediately initiates settlement and transformation into a polyp. Polyps that visibly started to regenerate a stalk were resectioned. The buds were collected once a day before feeding the polyps. They can be reared for about two weeks in artificial sea water (without additional antibiotics).

Chemicals

Cantharidin (Calbiochem), 1-aminocyclopentane-1-carboxylic acid (Cycloleucine) (Sigma), 12-0-tetradecanoylphorbol-13-acetate (TPA), (Sigma), Trigonelline (Sigma).

Initiation of head and foot formation in buds

Curtis and Cowden (1971) observed that a suited substrate initiates polyp formation within one day. Such a substrate is the material usually found at the bottom of culture dishes or adhering to the stalk of polyps. The material consists of organic debris, including bacteria. This material was collected, slightly disintegrated if necessary, and applied to buds in order to initiate polyp formation. This material is termed IS, inducing substrate. Tests were performed in polystyrene dishes containing the buds, 3 ml sea water, optionally IS, and the chemicals to be tested. At the end of treatment, the buds were removed and washed two times with 10 ml fresh sea water without IS and then transferred to a new dish with 3 ml fresh sea water. In a further group of tests IS was replaced by TPA.

The dishes were incubated at 23°C in moderate darkness. Sectioning of buds (optionally) was performed in the solution of the chemical to be tested. Following sectioning, the oral parts were treated in a fresh medium of that solution for 24 h, while to the aboral parts in addition IS or TPA was given for that period. The experiments were performed in triplicate. Significance (5% level) was tested by application of the χ^2-test and the Fisher-Yates test, respectively.

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