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Original Article

Ca^{2+}-ions and pattern control in *Hydra*

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ABSTRACT The fresh water polyp *Hydra* forms buds which develop a foot at their base and separate from the parent. In the strain *H. vulgaris* (Zürich), various compounds including phorbolesters, diacylglycerols, cantharidin and Li^+-ions were found to prevent foot formation at the bud's base. Therewith, the bud transforms into a branch which persists at the parent. Other strains were found to be unaffected by such treatments. Here we show that a reduced Ca^{2+}-ion concentration of the culture medium causes branch formation in the *H. vulgaris* (Zürich) strain but not in the other strains tested. However, all strains tested transformed their buds into branches when the medium was enriched with Ba^{2+} and Sr^{2+} ions. We suggest that the various treatments either reduce the internal concentration of Ca^{2+}-ions by stimulating Ca^{2+}-ion export or compete with Ca^{2+}-ions at their target. *H. vulgaris* (Zürich) is the most sensitive strain tested and appears to have the most efficient Ca^{2+}-pumps. This appears to be necessary for these animals derived from a lake which is extremely rich in Ca^{2+} ions.

KEY WORDS: *hydrozoa ecology, Ba^{2+}, Sr^{2+}, TPA, cantharidin*

Introduction

In Hydrozoa, one approach to study the control of pattern formation is to treat the animals with compounds known to interfere with signal transduction in bilaterians. The compounds applied include activators of protein kinase C (PKC), like diacylglycerol (DAG) 1,2-dioctanoyl-*rac*-glycerol (1,2-diC_8) and the tumour promoting phorbolester 12-o-tetradecanoylphorbol-13-acetate (TPA), inhibitors of protein kinases like K-252a, the tyrosine kinase inhibitors staurosporine, genistein and H-7, the phosphatase inhibitor cantharidin, xanthate D609 which is suspected to inhibit phospholipase C (phosphoinositidase), and Li^+ ions. The processes studied include head, foot and bud formation in *Hydra* (Müller, 1989, Müller, 1990, Hassel and Berking, 1990, De Petrocellis *et al.*, 1993, Hassel *et al.,* 1993, Pérez and Berking, 1994, Pérez, 1996) and the control of metamorphosis in various marine cnidarians (Spindler and Müller, 1972, Müller, 1985, Leitz and Müller, 1987, Henning *et al.*, 1991, Fleck, 1997, Thomas *et al.*, 1997, Kehls *et al.*, 1999, Siefker *et al.*, 2000, for review see Berking, 1998).

The present study concerns budding in *Hydra*. *Hydra* has a tube shaped body with a mouth / anus opening surrounded by tentacles at one end, called head, and a foot which ends in a basal disc at the other end. *Hydra* can reproduce asexually by forming buds. The development visibly starts with a small protrusion of the body wall, and the bud grows by cell multiplication and recruitment of tissue of the parent animal (Tripp, 1928, Burnett, 1961, Sanyal, 1966, Campbell, 1967). The tip of the bud develops into the head. At the bud's base a foot forms in a ring-shaped manner. This finally causes the separation of the bud from the parent. In the best studied strain, *Hydra vulgaris* (Zürich), the formation of the basal disc and the separation from the parent can be prevented by most of the agents noted. The point however is that only this strain responds to a short treatment, the others so far tested, do not. With respect to the other developmental processes mentioned, the results obtained are similarly diverse.

In this article we describe experiments which indicate that Ca^{2+} ions are essential for basal disc formation and bud separation.

Results

In H. vulgaris *(Zürich) the formation of a foot at the bud's base is missing when cultured in medium with a low concentration of Ca^{2+} ions*

Members of the strain *H. vulgaris* (Zürich) bearing a young bud were cultured in water containing 10 µmol l^{-1} Ca^{2+} ions (and 2 mmol l^{-1} Na^+/K^+- phosphate buffer, pH 7.4). In about one third of the treated animals (17 out of 55) the bud was found to develop into a side branch which did not detach from the parent. Controls kept in standard medium (among others 1 mmol l^{-1} Ca^{2+} ions, see Materials and Methods) formed a foot and detached within three

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; TPA, 12-o-tetradecanoylphorbol-13-acetate.

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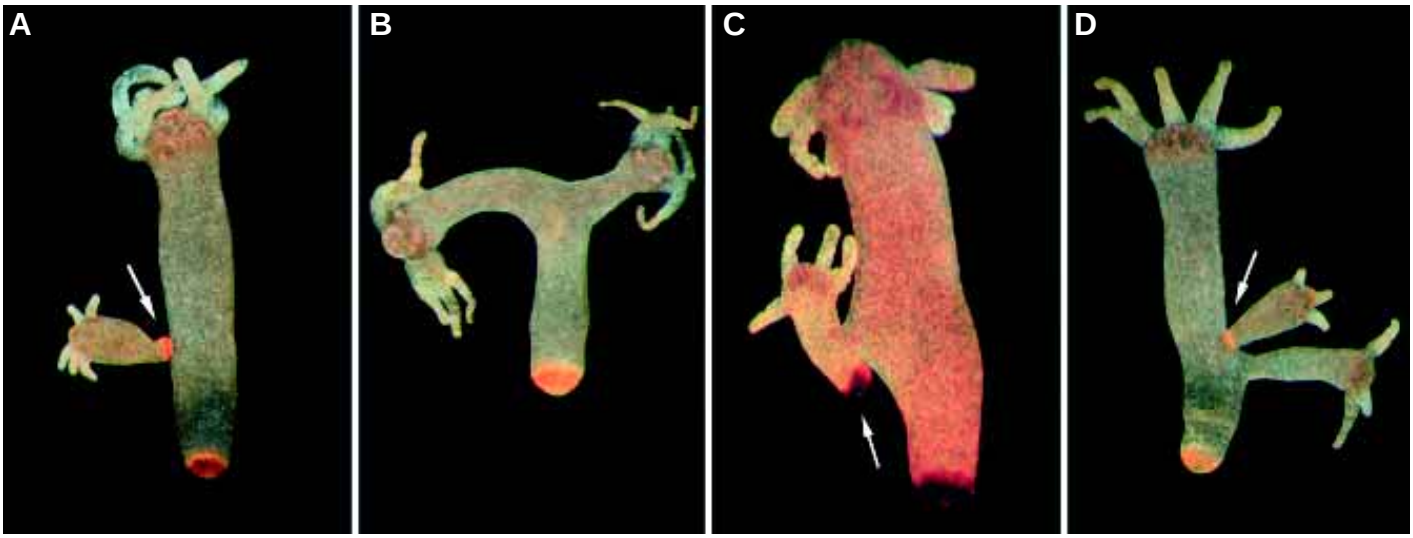

Fig. 1. Normal and branched animals of *Hydra vulgaris* (Zürich). *Polyps bearing a 3 to 6 hour old bud were treated for 2 days with 10 µmol l^{-1} Ca^{2+}-ions. Three days after treatment the animals were stained according to Hoffmeister and Schaller (1985).* **(A)** *Untreated control animal bearing a bud just before separation from the parent (4 days old bud). The foot is indicated by an arrow.* **(B)** *Y-shaped animal: the bud has transformed into a branch.* **(C)** *H-shaped animal: the branch formed a foot-patch in lateral position (indicated by arrow, 10 day old branch).* **(D)** *Y-shaped animal with a branch (10 days old) and a normal bud (5 days old, foot indicated by an arrow). This bud developed following treatment.*

to four days. Examples of such malformed animals and an untreated control are shown in Fig. 1. When the Ca^{2+} ion concentration was adjusted to 100 µmol l^{-1} all animals (47 out of 47) developed normal buds. In a further experiment buds of different age were kept in Ca^{2+} ion reduced culture medium. One to six hour old buds developed into branches. Older ones were much less sensitive (Table 1).

The effect of a Ca^{2+} ion reduced culture medium is strain dependent

The experiments shown above were done with a strain of *H. vulgaris* which had been collected from the Lake Zurich (Switzerland) by P. Tardent in 1966. In the past, experiments with this strain appeared in most publications dealing with *Hydra*. We also tested *H. vulgaris* collected from a lake close to Basel (Switzerland) by Thomas Honneger (Technau and Holstein, 1996). In addition, we used the standard wild type strain 105 of *H. magnipapillata* (which is also one of the best studied strains world-wide) and the multiheaded strain (mh-1), an inbred strain obtained from wild *H. magnipapillata* (Sugiyama and Fujisawa, 1977). Further, *H. viridissima* the common green *Hydra* was treated. We treated animals (*H. magnipapillata* strain 105: n = 121, *H. magnipapillata* strain mh-1: n = 188, *H. vulgaris* (Basel): n = 18, *H. viridissima*: n = 18) which had just started visibly to form a bud and found none of the polyps of these different strains to produce side branches, neither in Na^+/K^+ phosphate buffer depleted of Ca^{2+} ions nor in this buffer enriched by small amounts of Ca^{2+} ions (1 µmol l^{-1} to 1 nmol l^{-1}).

H. vulgaris (Zürich) was found to suffer most severely from such a treatment: 50 % of these animals disintegrated after 5-9 days of treatment. After 19 days of treatment 95 % (119/125) of the Basel strain were still alive. All members of the Zürich strain had disintegrated. 50 % of the treated *H. magnipapillata* disintegrated within about 10 days.

DMSO treatment caused the formation of branched animals in all strains tested

The detergent DMSO is often used to make membranes permeable to molecules, which would never get into or out of cells on their own. Further, DMSO can be expected to facilitate molecules present within the intercellular space to leak out. The aim of this experiment was to facilitate the leakage of Ca^{2+} ions. We found that

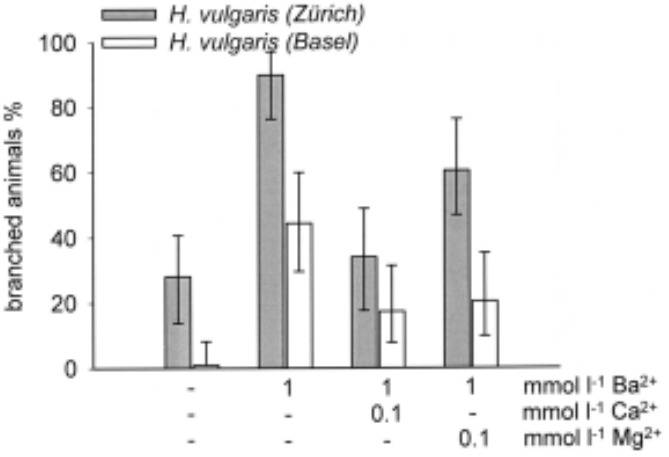

Fig. 2. Treatment with Ba^{2+} ions causes the formation of branched animals. *Polyps of strain* H. vulgaris *(Zürich) (grey bars) and polyps of strain* H. vulgaris *(Basel) (white bars) bearing each a 3 to 6 hour old bud were treated either for 2 days with 1 mmol l^{-1} $BaCl_2$. In addition the medium contained 2 mmol l^{-1} Na^+/K^+-phosphate buffer and optional 100 µmol l^{-1} of Ca^{2+} and Mg^{2+} ions. Thereafter the animals were transferred to standard culture medium. The result was scored 5 days after onset of treatment. None of the control animals in standard culture medium formed a branch (not shown). Each bar represents three independent experiments with 26 to 43 animals. The vertical bars indicate the respective 95% confidence interval.*

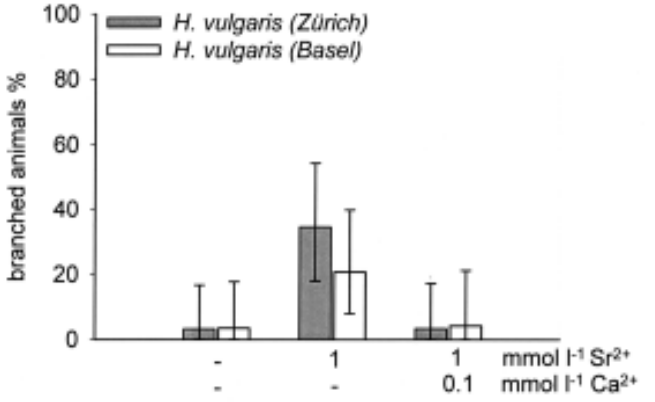

Fig. 3. Treatment with Sr^{2+} ions causes the formation of branched animals. *See legend to Fig. 2. The difference is the replacement of Ba^{2+} ions by Sr^{2+} ions.*

a DMSO treatment caused the animals of all strains to branch. However, in the various *Hydra* strains the efficiency was different: *H. vulgaris* (Zürich) was the most sensitive one (Table 2).

Ba^{2+} or Sr^{2+} treatments caused the formation of branched animals in all strains tested

Ions of the alkaline earth metals Barium and Strontium are well known to compete with Ca^{2+} ions at their various targets. If an internal low level of Ca^{2+} ions is the cause for the production of branches, a treatment with these ions should be expected to initiate branching not only in members of *H. vulgaris* (Zürich) but in members of other strains as well. This indeed was found. Figure 2 shows that a treatment with Ba^{2+} ions caused members of the Basel strain to form side branches. Further, the treatment increased the frequency of branch formation in the Zürich strain. Ca^{2+} ions and to a lower extent also Mg^{2+} ions were found to antagonise the influence of Ba^{2+} ions. An identical test with Sr^{2+} ions showed a similar result, but the efficiency of Sr^{2+} ions was lower than that of Ba^{2+} ions (Fig. 3). A treatment with 300 µmol l^{-1} Ba^{2+} ions for one day caused the formation of branched animals in *H. magnipapillata* (23%) (not shown).

Mg^{2+} ions display a dose dependent influence

In the absence of Ca^{2+} ions buds of *H. vulgaris* (Zürich) develop into side branches. When the culture medium is enriched with low amounts of Mg^{2+} ions (100 µmol l^{-1}) the frequency of side branch formation is reduced and the animals look much better than those of a control without Mg^{2+} ions (Fig. 4). However, when the concentration was increased to 1 mmol l^{-1} the frequency of branch formation increased (in long treated animals). We conclude that low concentrations of Mg^{2+} ions stabilise the tissue while high concentrations are able to antagonise Ca^{2+} ions at their targets.

Branching due to TPA and cantharidin treatments is antagonized by Ca^{2+} ions

In *H. vulgaris* (Zürich) a single pulse treatment with 12-o-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, was found to cause animals to branch (Pérez and Berking, 1994). The experiments were done in culture medium which contains 1 mmol l^{-1} Ca^{2+} ions. Here we show that in the presence of 100 µmol l^{-1} more than one half of the animals produced a branch instead of a bud while only some few did so in the control without TPA. When the Ca^{2+} ion concentration was decreased the effect increased. When the Ca^{2+} ion concentration was increased the effect of TPA decreased down to 10 % branched animals. Thus, the effect of TPA is antagonised by an external high level of Ca^{2+} ions (Fig. 5).

In the presence of Ca^{2+} ions (1 and 10 mmol l^{-1} $CaCl_2$, respectively) animals of the strain *H. vulgaris* (Zürich) bearing a young bud (about 3 hours old) were treated for 1h 45 min with 0.5 µmol l^{-1} cantharidin. The cantharidin treatment was preceded and followed by a treatment with medium containing the same Ca^{2+} ion concentration but no cantharidin (45 and 3 h 15 min, respectively). The group of animals treated with 1 mmol l^{-1} $CaCl_2$ represents the control for standard culture medium which contains among other ions 1 mmol l^{-1} $CaCl_2$. It turned out that a high concentration of Ca^{2+} ions antagonised the influence of the phosphatase inhibitor cantharidin to transform a bud into a branch (Table 3).

H. vulgaris *(Zürich) kept in distilled water reduces it's Ca^{2+} and K^+ ion content whereas* H. vulgaris *(Basel) does not*

Adult animals of *H. vulgaris* (Zürich) and *H. vulgaris* (Basel) have a different body size. Based on the protein content (measured after Lowry *et al.*, 1951) members of the Basel strain are about 2.5 times larger than members of the Zürich strain. The content of K^+ ions reflects this difference: the amount per animal was found to be three time higher in the Basel strain that in the Zürich strain (Table 4).

TABLE 1

LOW AMOUNTS OF CA^{2+} IONS IN MEDIUM CONTAINING 2 MMOL L^{-1} NA^+/K^+-PHOSPHATE BUFFER CAUSE A YOUNG BUD OF *HYDRA VULGARIS* (ZÜRICH) TO TRANSFORM INTO A BRANCH (5 DAY CULTIVATION)

bud age (h)	Ca^{2+} (µmol l^{-1})	animals (n)	branched animals n (%)
1 - 9	0.1	57	22 (39)
1 - 9	10	55	15 (27)
9 - 18	0.1	60	5 (8)
9 - 18	10	58	0

TABLE 2

TREATMENT WITH DMSO IN DISTILLED WATER CAUSES BRANCHED ANIMALS IN ALL STRAINS TESTED

Hydra strains	animals (n)	incubation time (d)	DMSO %	branched animals, n (%)
H. v. (Zürich)	30	5	0.1	23 (77)
H. v. (Basel)	47	5	0.1	0
H. magnipapillata	10	5	0.1	0
H. v. (Basel)	50	14	1	1 (2)
H. magnipapillata	50	14	1	1 (2)
H. v. (Basel)	100	4	2	2 (2)
H. magnipapillata	120	4	2	1 (1)
H. v. (Basel)	50	2	3	10 (20)
H. magnipapillata	50	2	3	10 (20)

TABLE 3

A HIGH CONCENTRATION OF CA^{2+} IONS ANTAGONISES BRANCH FORMATION IN *HYDRA VULGARIS* (ZÜRICH) TREATED FOR 1H 45 MIN WITH 0.5 µMOL L^{-1} CANTHARIDIN

Ca^{2+} (mmol l^{-1})	animals (n)	branched animals n (%)
10	68	9 (13)
1	79	52 (66)

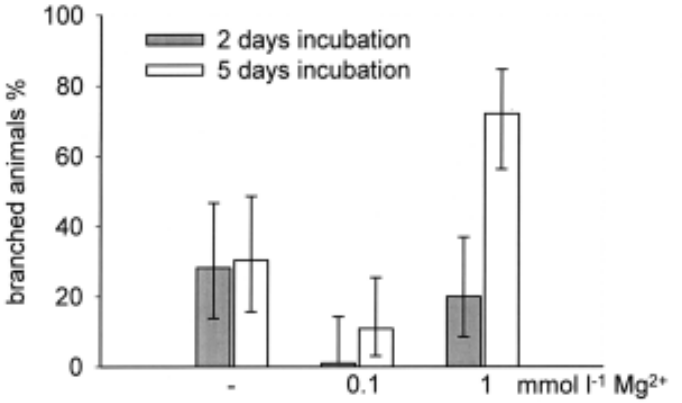

Fig. 4. The influence of Mg^{2+} ions on branching in *H. vulgaris* (Zürich). *Polyps bearing a 3-6 hour old bud were treated for 2 (grey bars) and 5 days (open bars), respectively, with 2 mmol l^{-1} Na^+/K^+-phosphate buffer enriched with different concentration of Mg^{2+} ions. Thereafter the polyps were transferred to standard culture medium. The result was scored 5 days after onset of treatment. None of the control animals in standard culture medium formed a branch (not shown). Each bar represents three independent experiments with 18 to 20 animals. The vertical bars indicate the respective 95% confidence interval.*

However, the content of Ca^{2+} ions does not reflect this difference: The amount per animal was almost identical (Table 4). Thus, the concentration of free and stored Ca^{2+} ions appears to be 2 to 3 times higher in *H. vulgaris* (Zürich) than in *H. vulgaris* (Basel). (The measured amount of K^+ ions is within the limits determined by others (Lilly 1955, Steinbach, 1963, Koblick and Yu-tu, 1967)). To our knowledge values for the Ca^{2+} ion concentration in *Hydra* are not reported.

When animals were kept in distilled water for 48 hours the amount of K^+ ions and Ca^{2+} ions decreased in members of the strain *H. vulgaris* (Zürich) (by 35 and 25%, respectively) but not in members of the strain *H. vulgaris* (Basel) (Table 4).

Discussion

In the past it was not possible to design a general scheme revealing the role of signal transduction in pattern control in *Hydra*. Rather, and for unknown reasons, such schemes had to be different for different species of *Hydra*. The data shown here indicate that one of the central processes in pattern formation is Ca^{2+} ion dependent. This was found to be true for all species studied. However, under unfavourable conditions the strains display a considerably different ability to keep their Ca^{2+} ion content above a certain threshold.

The by far most sensitive strain is *H. vulgaris* (Zürich). This could be attributed to the water quality of the Lake Zurich. The lake contains and gets water with an extremely high level of Ca^{2+} ions (about 1.2 mmol l^{-1}) which causes a high pH (in the course of a year from 7.9 to 8.8 in the surface water) and the precipitation of Ca^{2+}-minerals (Kelts and Hsü, 1978). (The culture medium used in most laboratories also contains similar high concentrations of Ca^{2+} ions ($CaCl_2$)). Obviously, the animals in the lake never have any problems to get enough Ca^{2+} ions. Rather they need -and in particular such small and thin animals like *Hydra*- very effective pumps to maintain the low intracellular level necessary for physiological processes. *Hydra* living in this lake may have adapted to these extreme conditions. Other strains may have adapted to the respective low levels of Ca^{2+} ions in their environment. It would be interesting to learn whether there is a correlation between the Ca^{2+} ion content in the water from which a certain *Hydra* strain was collected and its sensitivity against an extremely low Ca^{2+} ion concentration in laboratory experiments.

Short treatments with various compounds affect foot formation at the bud's base only in members of *H. vulgaris* (Zürich). The list of effective compounds includes activators of protein kinase C (PKC), such as the diacylglycerol (DAG) 1,2-dioctanoyl-*rac*-glycerol (1,2-diC_8), and the tumour promoting phorbolester 12-o-tetradecanoylphorbol-13-acetate (TPA), the tyrosine kinase inhibitors staurosporine, genistein and H-7, the phosphatase inhibitor cantharidin (Pérez and Berking, 1994, Pérez, 1996). This exclusiveness may indicate that all noted treatments funnel into one process which is (quantitatively) different in *H. vulgaris* (Zürich) compared to the other strains. We suggest this to be the pumping of Ca^{2+} ions. This suggestion prompted testing the influence of two members of this list, TPA and cantharidin, in the presence of various Ca^{2+} ion concentrations.

In *H. vulgaris* (Zürich) a treatment with TPA or 1,2-dioctanoylglycerol caused animals to branch while two related substances, which however do not stimulate PKC activity in vertebrates, 1,3-dioctanoylglycerol and MPMA (phorbol-4-o-

TABLE 4

***H. VULGARIS* (ZÜRICH) KEPT IN DISTILLED WATER REDUCE THEIR Ca^{2+} AND K^+ ION CONTENT (AAS- MEASUREMENTS)**

	Ca^{2+} (ng / animal ± SD)		K^+ (ng / animal ± SD)	
***Hydra* strain**	**std. medium**	**dist. water**	**std. medium**	**dist. water**
H. vulgaris (Zürich)	45.3 ± 7.2	33.6 ± 3.2	904 ± 8	590 ± 9
H. vulgaris (Basel)	53.8 ± 6.4	56.8 ± 6.4	2392 ± 30	2344 ± 24

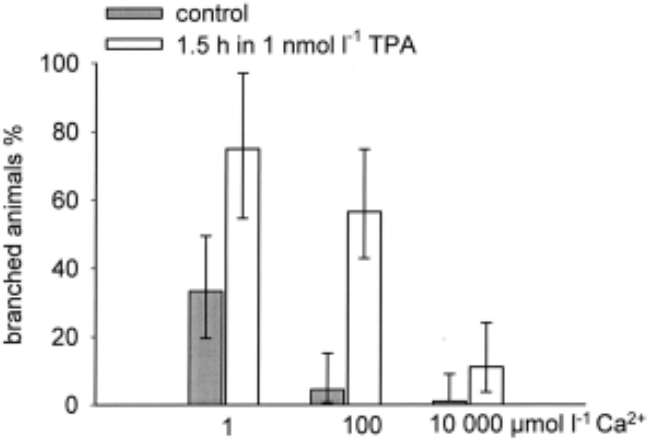

Fig. 5. Calcium ions antagonise branch formation induced by TPA. *Polyps of* H. vulgaris *(Zürich) bearing a 3 to 6 hour old bud were treated for 2 days with 2 mmol l^{-1} Na^+/K^+ phosphate buffer enriched with three different concentrations of Ca^{2+} ions (grey bars). Half of the animals were treated in addition for 1.5 hour with TPA (1 nmol l^{-1}) (open bars). This treatment starts together with the Ca^{2+} ion treatment. The result was scored 5 days after onset of treatment. Each bar represents three independent experiments with 23 to 45 animals. The vertical bars indicate the respective 95% confidence interval.*

methylether-(12-myristol-13-acetyl)) did not have this effect. It thus appears that a stimulation of a PKC is responsible for the branching (Pérez and Berking, 1994). We found the effect of TPA to be considerably reduced by an increased external concentration of Ca^{2+} ions. Protein kinases of type C are well studied in *Hydra* (Hassel, 1998, Hassel *et al.*, 1998), but a PKC which is inhibited by Ca^{2+} ions is unknown. A simple interpretation of the results obtained would be that in *H. vulgaris* (Zürich) PKC stimulation provokes the export of Ca^{2+} ions out of the cytoplasm into stores and / or out of the cells. The resultant low internal Ca^{2+} ion concentration provokes the observed effect. An increased external level of Ca^{2+} ions partly compensates the induced loss of Ca^{2+} ions from the cytoplasm. The effects observed after a treatment with cantharidin and Ca^{2+} ions can be explained similarly. Based on the observation that only in *H. vulgaris* (Zürich) the other noted compounds cause animals to branch, we suggest that all these substances decrease the internal level of Ca^{2+} ions by stimulating a Ca^{2+}-pump. In the non-responding species the compounds may decrease the Ca^{2+} ion level as well but not down to the threshold.

Some species were found to respond to certain regimes of treatment with these compounds in a quite different way (see Hassel and Berking, 1990, Müller, 1989, Müller, 1990, De Petrocellis *et al.*, 1993). These responses may be due to different dispositions of the strains tested, so that in these cases other effects of the compounds prevail. For instance, it is well known that the stimulation of a PKC can affect a large number of quite different processes. Whether or not in members of a certain species a reduced Ca^{2+} ion level contributes to or even is responsible for the effects observed, appears to be accessible by the following experiment: one may treat the animals with the compound in question in the presence of high and low levels of Ca^{2+} ions, respectively, (with or without DMSO). In addition the influence of Ba^{2+} and Sr^{2+} ions on the process in question should be tested.

Materials and Methods

Animals

Two strains of *Hydra vulgaris* were used, the Zürich strain, which was collected by Pierre Tardent (1966) (commonly referred to as "*H. vulgaris*" in other laboratories) and the Basel strain collected by Thomas Honegger (Technau and Holstein, 1996). Further, we used *Hydra magnipapillata* strain 105, the standard wild type strain, and the multiheaded mutant strain, mh-1, a sexual inbred of *Hydra magnipapillata* (Sugiyama and Fujisawa, 1977), and the green *Hydra* (*Chlorohydra viridissima*). The animals were maintained at 20°C in standard culture medium (1 mmol l^{-1} $CaCl_2$; 0.5 mmol l^{-1} $MgCl_2$; 0.1 mmol l^{-1} $KHCO_3$; 0.35 mmol l^{-1} $NaHCO_3$ and 25 µmol l^{-1} EDTA, pH = 7.4). They were fed five times a week on freshly hatched nauplii of *Artemia salina*. All animals used in the experiments were starved for 24 hours prior to the experiment.

Test Assay

If not stated otherwise animals bearing a 3 hour old bud (0 is the visible appearance) were selected and treated with the test-medium (see below) for 48 hours. Then they were washed twice with the culture medium. Three days later the results were scored the first time.

Chemicals

Test substances were applied in distilled water or 2 mmol l^{-1} Na^+/K^+-phosphate buffer, pH 7.4. All ions tested like those of barium, strontium, calcium or magnesium were applied as chlorides. A stock solution of the phorbolester TPA (12-o-tetradecanoylphorbol-13-acetate) and of cantharidin (Sigma, Deisenhofen, Germany) was prepared by dissolving the agents in dimethyl sulfoxid (DMSO).

Peroxidase Staining

For demonstrating the formation of foot specific cells we used the peroxidase stain according to Hoffmeister and Schaller (1985).

Ca^{2+} and K^+ Ion Measurements

Hundred animals were cultured for 2 days either in 1 ml standard culture medium or in 1 ml distilled water. The experiment was repeated 6 times. After 48 hours the animals were washed twice with distilled water and then homogenised. Then distilled water and trichloracetic acid (TCA) were added to reach a final concentration of 10% TCA in 800 µl. The homogenate was kept on ice over night. Then the probes were homogenised and centrifuged for 3 minutes at 10,000g. The supernatant was removed and centrifuged through a filter (Micro Spin Filter Tubes, 0,2 µm PVDF; Alltech). Of every probe 3 x 200 µl were analysed by means of Atom Absorption Spectrometry.

Statistics

In the graphs the means of at least three identically performed experiments are shown. The bars indicate the respective confidence interval (95%-level).

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