Pheromone-Binding and Matrix-Mediated Events in Sexual Induction of *Volvox carteri*

Rainer Gilles, Claudia Gilles, and Lothar Jaenicke*

Institut für Biochemie, Universität zu Köln, An der Bottmühle 2, D-5000 Köln 1, Bundesrepublik Deutschland

Sexual differentiation of the reproductive cells (gonidia) of the green alga *Volvox carteri* f. *nagariensis* Iyengar is triggered by a glycoprotein inducer which is released into the medium by sexual male spheroids. If added, it induces the next generation cycle to become sexual. About 3000 pheromone molecules per spheroid will give full induction. Specific binding sites for the pheromone have been identified in the extracellular matrix in which characteristic events take place. Inducer binding and action are reversible for a certain time period until the start of cell division; thereafter, determination of the gonidia is final. Extent of sexual induction is proportional to the duration of inducer pulses. Inhibition of sexual induction reveals as essential extracellular components of the induction system high molecular acidic glycoproteins, cyclic nucleotides, Ca^{2+}/calmodulin, and pheromone-controlled gene products. Low matrix levels of cyclic AMP and cyclic GMP seem to stimulate induction; high levels to inhibit.

Volvox carteri, a multicellular green alga, shows two features which make this organism well suited for the study of cellular differentiation [1]: (i) dichotomy of cell-lines and (ii) inducibility of the sexual cycle.

The fundamental segregation of somatic and germ cell-lines usually gives rather complex differentiation patterns in multicellular systems because more than one type of somatic cell arises. *Volvox*, however, possesses only body cells of the same kind. At a certain stage of the embryonic development a differentiating cleavage separates the designated reproductive cells (gonidia) from those becoming — practically irreversibly — the highly specialized, flagellated somatic cells [2]. In asexual development the gonidia cleave 5 times to form an embryo with 32 cells of equal size. In the next cleavage 16 cells in the anterior part of the embryo undergo unequal division. Thus, larger gonidial stem cells are set apart from the smaller somatic precursor cells [3, 4]. This unequal cleavage is the first morphological manifestation of cellular differentiation. The cells of the 32-celled embryo continue to cleave further 5 to 6 times. They ultimately build up the globular sheath of approximately 2000 somatic cells which enclose the systematic arrangement of 16 large reproductive gonidia differentiated at the 6th cleavage. This seemingly simple morphogenetic pattern has encouraged us and other investigators [5, 6] to speculate on the biochemical mechanism of timing and spacing in embryogenesis of *Volvox*. So far, however, none of the hypotheses promulgated stood up to experimental scrutiny.

The other approach to study differentiation with *Volvox* as a paradigm is based on the second peculiarity of this system: In addition to the asexual development just described, *Volvox* is able to reproduce sexually under the influence of a molecular signal. This given, the differentiation is altered during embryogenesis. In female strains of *Volvox carteri* 32 cells of the 64-celled embryo cleave unequally*; *i.e.* differentiation is delayed one cleavage; however the same blastomeres are affected. In the male strains, on the other hand, each cell of a 128-celled embryo will undergo unequal division to form a 256-celled young spheroid with a 1:1 ratio of reproductive and somatic cells*. Obviously both parameters, timing of unequal cell division and origin of the stem cells are altered in this sexual pathway [5]. The signal for sexuality is a pheromone, the "sex-inducer" [7]. This 30 kDa glycoprotein is only produced by the sexual male strain and is released into the medium after hatching and

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* Under certain environmental conditions embryos of 64 cells in the female or 256 cells in the male strain undergo unequal cleavage, but this unequal cleavage is always the last one in embryogenesis of male spheroids.

maturing of the sperm packets [8]. It triggers the uncleaved gonidia of female and male strains to undergo sexual development. Spheroids are formed with eggs and sperm packets, respectively. The induction of the first male to start this auto-catalytic chain seems to be an inducer-independent relatively frequent event. The inducer system is impressively active: The pheromone can be diluted as low as 6×10^{-17} M (36 molecules per μl), and still shows full biological effect [9]. This calls for a very efficient signal amplification mechanism. Whereas the pheromone itself has been purified and partially characterized, the mechanism of its action is almost completely unknown. Despite some claims to the contrary [10] there is no evidence of specific binding of the inducer to any site of the spheroid, and considering the extremely small amounts required for biologically effective interaction it would seem very hard, indeed, to prove it unambiguously.

The first biochemical effect of induction detected so far is an altered phosphorylation of distinct proteins of the extracellular matrix [11] in which also other development-dependent events occur [11a]. The observation of a causal chain between phosphorylations and induction lets us assume that it might be patterned according to the cAMP-mediated cascade in the development of animals [12] and microorganisms [13]. Considerable evidence has accumulated in recent years for the occurrence of cyclic nucleotides in lower and higher plants. But in the plant kingdom no cAMP-dependent protein kinases have been discovered till now [14], much less a complete hormonal chain working with cyclic nucleotides as second messengers.

Materials and Methods

Culture methods

HK-10 (female) and 69-1b (male) strains of *Volvox carteri* f. *nagariensis* Iyengar from the Collection of Algae, University of Texas at Austin, Austin, Texas, were kindly provided by Professor R. C. Starr. They were grown at 30 °C and in a 16 h light (12 000 lux)/8 h dark cycle as described in [7], resulting in a well synchronized generation cycle of 48 h. For bioassays 3 parental spheroids were inoculated into serial dilutions of inducer in 10 ml *Volvox*-medium [7]. After two days the percentage

of sexual individuals was determined under a dissecting microscope.

Fractionation of spheroids

100 parental spheroids were disrupted under nitrogen in a Yeda press at 3 bar. The cellular material was centrifuged off for 5 min at 10 000 × g; the supernatant contains the matrix fraction. The pellet was resuspended in *Volvox*-medium, and the gonidia were homogenized at 25 bar. The somatic cells left intact under these conditions were sedimented for 5 min at 5000 × g. For preparation of gonidia, intact spheroids were treated with 100 μg/ml pronase for 30 min at 28 °C. The gonidia were separated from the dissolved somatic sheath by filtration through a 10 μm nylon net.

Inhibition studies

Cyclic adenosine-3,5-monophosphate (cAMP); cyclic guanosine-3,5-monophosphate (cGMP); $N^6,2'$-O-dibutyryl-cAMP; $N^2,2'$-O-dibutyryl-cGMP and Concanavalin A (Con A) were obtained from Boehringer-Biochemica (Mannheim, FRG). Phosphodiesterase (3′,5′-cyclic nucleotide, E.C. 3.1.4.17, crude complex from beef heart), protease type V (pronase), poly-L-lysine (MW 15 000 − 30 000), Calcimycin (Ca-Ionophore A 23 187), Anisomycin, Monensin and Actinomycin D were obtained from Sigma (Taufkirchen, FRG). Other inhibitors were Tunicamycin (Eli Lilly, Chicago, USA), isobutylmethylxanthine (Ega-Aldrich, Steinheim, FRG) and Trifluoperazine (a kind gift from Prof. Dr. F. Hofmann, Heidelberg, FRG). Sterile stock solutions were made in *Volvox*-medium; water insoluble agents were dissolved in ethanol. Up to 5% ethanol does not harm the *in vivo*-assay. In each case the killing concentration and the effective time-length were determined for the stages of life-cycle. Interference with induction is only observed in the precleavage phase; during cleavage, however, macromolecular syntheses are most sensitive. Therefore, to avoid such effects on growth and viability, inhibitors were always diluted out prior to cell division. The following procedure was used: 10 − 25 young spheroids which had been induced previously were added immediately after release into 1 ml *Volvox*-medium (final volume) contained in the depression of a spot plate in a wet chamber (Petri dish). The inhibitors were added and the samples incubated under bright light. Shortly before cleav-

age each sample was transferred into a test-tube with 10 ml *Volvox*-medium and grown until the next generation could be scored for induction. Controls were assays with (i) inducer alone (full induction); (ii) inhibitor alone (no induction); (iii) different inducer concentrations (no effect unless indicated); (iv) various times of inhibitor addition (same effect as diluting out the inducer at that time, Fig. 2).

Fluorescence microscopy

The fluorescein isothiocyanate (FITC)-coupling procedure followed that described in [8]. For fluorescence microscopy, spheroids were suspended with 100 µg/ml FITC-Con A or FITC-antibodies in *Volvox*-medium for 1 h, filtered through a 50 µm nylon net and washed. Pictures were taken with a Leitz Variolux microscope equipped with an epifluorescence illumination (Ploemopak L 2 or L 2.1 filter block).
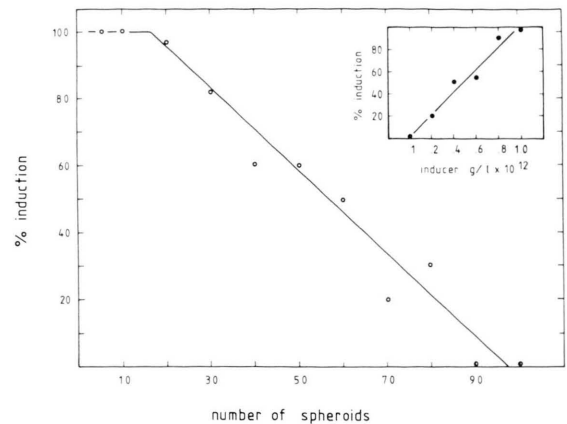

Fig. 1. Competitive binding assay. Different numbers of parental spheroids containing on the average 15 daughters each are incubated with a just sufficient concentration of pheromone $(6 \times 10^{-17} \text{ M})$ in test tubes containing 10 ml *Volvox*-medium. Percentage of sexual induction in the next generation is plotted against the number of parental spheroids. The inset shows a fine-titration of inducer. 10^{-12} g/l $(6 \times 10^{-17} \text{ M})$ inducer are diluted up to 10 fold, and the biological response is determined by bioassay [7].

Results

The *Volvox* gonidia are the obvious targets of the pheromone, and in analogy to other hormone systems, the receptor should be located on their outer membrane. We looked very carefully for specific inducer-binding membrane-receptors by means of fluorescent antibodies. The specific antisera were the same that were previously used for our sandwich-radioimmunoassay of the inducer [15]. No particular binding site could be identified on the gonidial membrane at physiological inducer level. At higher concentrations non-specific binding to the spheroidal hull was seen. The number of pheromone molecules bound to the gonidia seems to be below detection limit of this method. Attempts to enhance its sensitivity by using secondary antibodies failed.

However, evidence for actual pheromone binding in the spheroids resulted from a different strategy: If we incubate a varying number of spheroids with the same limiting concentration of sex inducer we find induction decreased proportional to the increase of the number of spheroids, indicating that the algae compete stoichiometrically for the pheromone. From the linear dependency and the slope of this titration shown in Fig. 1 we can calculate the number of molecules bound per spheroid, if we

know the range of pheromone concentration between full and no induction. This is covered by almost exactly one order of magnitude (inset Fig. 1). From the details of the experiment * we deduce that 300 pheromone molecules bound per spheroid will not suffice for induction, but 3000 molecules give complete sexual induction.

In addition, the competitive binding-assay shows not only the existence of high-affinity binding sites, but also the ability of *Volvox*-spheroids to concentrate the inducer: The pheromone is extracted from the medium (10 ml) into the matrix volume of the algae $(1350 \times 0.022 \, \mu l = 30 \, \mu l)$ which corresponds to a 300-fold concentration.

In order to localize the binding phase we fractionated the algae into:

(i) matrix − this is the mucilaginous material filling the spheroid. After gentle disruption of the somatic sheath it will partially flow out and can be separated from the cellular fraction by centrifugation.

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* If 90 parental spheroids containing 15 daughters each = 1350 young spheroids exhaust 10 ml of a 6×10^{-17} M inducer solution (*i.e.* 4×10^5 molecules of inducer) the steady-state concentration during incubation should be one tenth, because no spheroid will be induced. Consequently 3.6×10^5 molecules are bound, *i.e.* 300 per spheroid.

(ii) gonidia – the reproductive cells are much larger and physically less stable than somatic cells. Both properties are used for fractionation (see Materials and Methods).

(iii) sheath – the somatic cells forming a mono-layer – not a syncytium! – called the somatic sheath, are obtained as a unit.

The cellular fractions (ii) and (iii) are always contaminated with matrix which, for functional reasons, adheres tightly. Matrix may be obtained free of cell material if care is taken on breaking the spheroids. With these fractions the above binding experiments were repeated. Each fraction was added to a test tube containing a limiting concentration of the sex inducer in 1 ml *Volvox*-medium and three parental algae (ca. 45 gonidia). As seen from Table I the main binding capacity is found in the matrix. Binding in the other fractions is due to the unavoidable matrix contamination. The strongly acidic matrix which contains polymer-bound sugar acids as well as sulfated and phosphorylated sugars seems to be predestined for interaction with the strongly basic (pI = 10.5) sex inducer.

Reversible action and binding of pheromone

Callahan [16] and Kochert [17] state that induction can be prevented by diluting out the inducer. Considering the extremely low threshold concentration this apparent reversibility sets a perplexing physiochemical problem. For, if we assume that reversibility of action reflects reversibility of binding and that for 50% sex induction 50% of the pheromone receptors have to be occupied, we come to the following paradoxical situation: The resulting dissociation constant K_D of the inducer/receptor complex is 3×10^{-17} M which would mean prac-
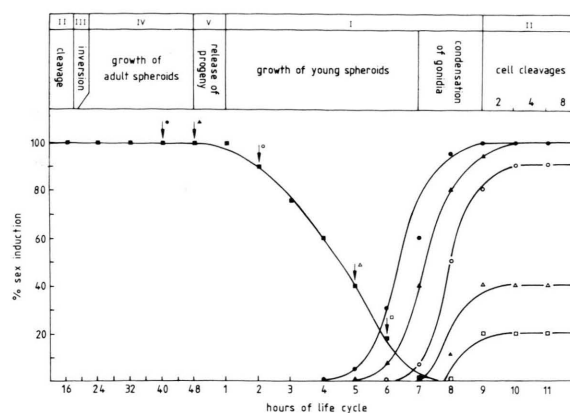

Fig. 2. Inducible and reversible phases in life cycle. Reversibility is analysed by "pulse-chase"-experiments (●, ▲, ○, △ and □). A sufficient amount of inducer to give 100% induction is added to 5 spheroids in test tubes with 10 ml *Volvox*-medium at times indicated by arrows. Every hour the inducer is diluted out in one tube by serial transfer of the algae through three test tubes with 10 ml fresh *Volvox*-medium. For control the last washing is inoculated with new algae. The washed spheroids are further grown until the next generation is formed and sexual induction can be estimated. Inducibility (■) is analysed by addition of inducer at different times before cell cleavages. Note, percent induction refers to the next following generation; after one further generation all spheroids will be induced in each case. Exactly one 48 h life cycle (from cleavage to cleavage) is shown. The developmental stages (I–V) are explained and numbered at the top.

tically complete irreversible binding ($\Delta G^0 = 97$ kJ/mol). Biological reversibility could only be achieved at a K_D at least five orders of magnitude higher. The results of the preceding section offer a possible explanation to resolve this dilemma. As indicated, the pheromone is concentrated into the matrix by a factor of 300, to an effective concentration within the spheroid of 10^{-15} M. If the whole matrix contains binding molecules it is reasonable to assume a large excess of them over the 3000 molecules of pheromone required for induction. Assuming a 1000 fold excess of anionic binding sites, the K_D would calculate to 10^{-12} M, a value found *e. g.* in high affinity antigen-antibody complexes which are known to be reversible [18].

We determined the period in the life cycle during which induction is reversible by incubating the algae for a certain time interval with a just sufficing concentration of inducer, then washing the spheroids free of inducer. The results of such a "pulse"-experiment is given in Fig. 2. Induction is com-

Table I. Binding of inducer to spheroid fractions.

Fraction	% Induction [a]
control (3 spheroids)	90
spheroids (100 algae)	0
matrix from 100 algae	0
sheath from 100 algae	47
gonidia from 100 algae [b]	7
gonidia from 100 algae [c]	30

[a] Mean of 3 experiments.
[b] Mechanically isolated.
[c] Pronase-treated

pletely reversible up to 4 h before onset of cleav-
ages of the gonidia. Then, together with the conden-
sation phase of the gonidia (see Fig. 2), an all-or-
none process sets in by which the gonidia are
determined to differentiate sexually. We tested
whether presence of the inducer is required at all
during the reversible phase by adding the inducer at
different times before onset of cell division and
found that induction is maximal only when the
pheromone is added 8 to 9 h prior to the beginning
of cell division (Fig. 2). This means that induction
takes place not as sudden switch but as a slow and
dynamic reaction in which the interval of the pre-
incubation with the inducer reflects the kinetic
course of the inducer-dependent process forming a
secondary substance or a different state. In this case
induction should be proportional to that interval.
We tested for this hypothesis by an additional
"pulse"-experiment in which inducer was added at
certain points in the descending part of the in-
ducibility-curve as shown in Fig. 2. True to ex-
pectation maximal response corresponds to the
percent inducibility at the time of addition of the
pheromone. To correlate between the duration of
the pulse and the percentage of sexual induction we
have to take into account that intermediate inducer-
dependent substance(s) are formed which neces-
sarily have to be unstable to be reasonable re-
gulatory agents. Assuming for simplicity that their
formation and degradation proceeds with the same

velocity (steady-state-concentration established) we
obtain net synthesis of the intermediate signal by
subtracting the time between end of pulse and first
cleavage from the interval of inducing pulse. In-
deed, a plot of this corrected pulse time against
induction as in Fig. 3 gives a straight line.

The evidence presented lets us postulate that the
inducer after combining with certain components of
the spheroid starts a process by which a substance is
accumulated or the state of the spheroid is altered
which finally leads to the expression of sexuality.
Upon dissociation of the pheromone the process is
completely reversible until just before cleavage the
inducer-dependent substance (state) has reached the
critical concentration to shift development quickly
and irreversibly to the sexual cleavages.

Inhibition of sexual induction

1) Reactions of the matrix

Binding studies had revealed the importance
of the matrix for induction. Free resting gonidia
obtained by disrupting young spheroids by suction
through a drawn-out pipet will undergo asexual
development almost unperturbed but cannot be
sexually induced no matter whether inducer is
added before or after isolation. Only gonidia just
before or during cleavage can be isolated from
spheroids without effect on inducibility or induc-
tion. Thus, it seems essential for induction or main-
tenance of induction that the gonidia are mixed
with matrix. Reconstitution of the system by adding
cell-free matrix isolated from a 100 fold excess of
mature, non-induced spheroids is partially success-
ful as may be read from Table II, but higher matrix
concentrations are lethal. This may be due to ir-
reversible loss of matrix-ultrastructure, indication of
which is seen in Fig. 4.

Polycations may act as intermediate messengers
[19] or may interact with the negatively charged
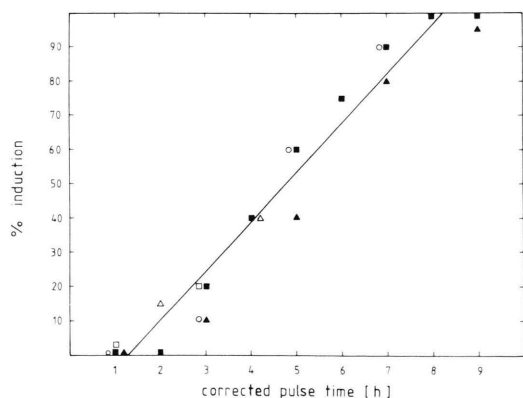groups of the matrix [20] involved in induction such

Fig. 3. Dependency of sexual induction on net inducer
pulse duration. The data from the "pulse-chase"-ex-
periments in Fig. 2 are corrected: The time between
washing and beginning of cell division is subtracted from
the inducer pulse duration. This gives the net inducer-
pulse time. The 5 sets of data which are labelled according
to the 5 pulse-chase experiments in Fig. 2 are plotted
together.

Table II. Induction of isolated gonidia.

Treatment	% Induction
gonidia + inducer	0
gonidia + inducer (preincubated)	0
gonidia + inducer + matrix from 100 algae	lethal
gonidia + inducer + matrix from 100 algae (1 : 2 dil.)	20

Table III. Inhibition by polycations and Con A.

| Inhibitor	Concentration [μg/ml]	Induction %	Other effects
poly-Lysine	3	0	agglutination
Spermine	2	0	agglut., hulls opened
Protamine sulfate	2	0	agglut., hulls opened
Con A + 10-fold excess inducer	2.5	0	number of gonidia reduced
Con A + 100-fold excess inducer	2.5	60	number of gonidia reduced
Con A + 1000-fold excess inducer	2.5	100	number of gonidia reduced
Con A + 10-fold excess inducer + мM α-methyl mannoside	2.5	100	
Succinyl-Con A	50	100	
Con A + 30 μg Colchicine [a]	2.5	0	
Con A + 25 μg Colcemide [a]	2.5	0	
Con A + 10 μg/ml Cytochalasin D [a]	2.5	0	

[a] Without ConA: 100% induction.
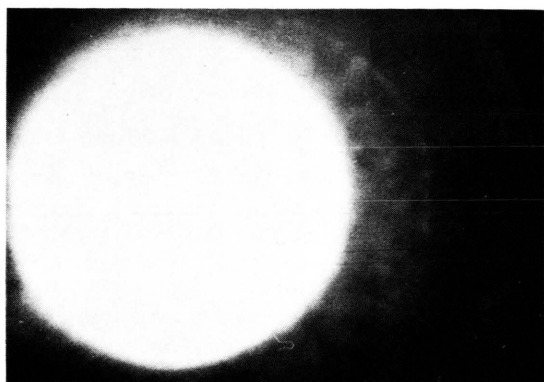
Fig. 4. FITC-fluorescence of gonidia and their envelopes. The gonidial cell wall, not their membrane, shows very strong fluorescence with FITC-Con A. Also in the matrix surrounding the gonidia distinct structures become visible which are not resolved in light microscopy. An envelope exists which has approximately twice the diameter of the gonidium. It encloses matrix material showing a higher background fluorescence than the rest of the matrix. In the region close to the gonidium filamentous structures can be discerned. Their actual form varies from preparation to preparation. We infer from this that they are formed randomly by a 3-dimensional net work of glycoprotein-Con A-complexes.

as the inducer-specific phosphoprotein which has a pI < 3. Poly-L-lysine, spermine and protamine sulfate coagulate the matrix drastically; at lower concentration they agglutinate the algae and strongly interfere with induction (Table III).

Concanavalin A was reported to be an inhibitor of induction [21]. We infer from Table III that native Con A acts competitively with the inducer, gradedly blocking induction at inducer concentrations be-

tween one and 100 times the threshold. Succinyl-Con A, however, which has a reduced ability to aggregate glycoproteins, does not affect induction [22]. Most Con A effects known from other systems are based on binding to membrane glycoproteins. Our fluorescence microscopic studies in *Volvox* revealed many sites for binding FITC-Con A in the matrix, particularly in that material filling the space around the gonidia. It is unlikely that membrane proteins are the primary targets of Con A, since FITC-Con A shows only a very low affinity to membranes of uncleaved gonidia. Simultaneous incubation of Con A with cell-poisons such as colcemide or cytochalasin cannot prevent Con A effects (Table III). Such interference is reported for other systems when redistribution of membrane proteins is involved [23].

Table IV. Inhibiton by blockers of macromolecular synthesis.

Inhibitor	Concentration [μg/ml]	% Induction	Other effects
Actinomycin D	10	0	partially abortive cleavages
Anisomycin	2.5	0	partially abortive cleavages
Tunicamycin	0.15	0	no inversion
Monensin	4	20	partially abortive cleavages

Table V. Inhibition by agents affecting Ca^{2+} and cyclic nucleotide level.

Inhibitor	Concentration [mM]	% Induction	Other effects
Trifluoperazine	5×10^{-4}	40	no motility
Trifluoperazine	8×10^{-4}	5	no motility, no inversion
CaCl$_2$	1.5×10^{-2}	20	condensed matrix
Calcimycin + 3.5 mM CaCl$_2$	1×10^{-4}	40	slow development
3,5 cAMP	1	100	
3,5 cGMP	1	100	
Dibutyryl-3,5 cAMP	1	100	
Dibutyryl-3,5 cGMP	1	100	
Dibutyryl-3,5 cAMP	1	100	
IBMX	0.4	0	no hatching
IBMX	0.1	80	
IBMX + 1 mM cAMP	0.1	0	
IBMX + 1 mM cGMP	0.1	0	

2) Inhibitors of macromolecular syntheses

SDS-polyacrylamide gel electrophoresis revealed the formation of new protein bands upon induction. Inhibition studies support this *de novo* protein as shown in Table IV. Actinomycin D and Anisomycin prevent induction. Also the inhibition of protein glycosylation in *Volvox* by Monensin [24] and Tunicamycin [25, 26] interfere with sexual induction, indicating the essential involvement of glycoproteins.

3) Inhibitors of "second messages"

In many regulatory systems the primary regulating signal leads to a second message which usually is at the cyclic nucleotide or Ca^{2+}/calmodulin level [27]. There is evidence for the occurrence of calmodulin in higher plants [28] and in *Volvox* [29]. Trifluoperazine (TFP) which inhibits calmodulin-dependent reactions [30] blocks induction and motility; the same is observed for Calcimycin (A 23187) and/or an elevated level of Ca^{2+} in the medium (Table V).

Externally added cyclic nucleotides in the presence of inducer as well as their dibutyryl derivatives have no influence on induction ([10] and Table V). On the other hand, we found that isobutylmethylxanthine (IBMX), a potent membrane-permeant inhibitor of phosphodiesterase (PDE) [31], prevents induction increasingly at concentrations between 25 and 400 μM. These contradicting results may mean that the PDE proven to be present in the matrix of *Volvox* spheroids (unpublished) cleaves added cyclic nucleotides too fast to let their level increase sufficiently for the time interval needed in the *in vivo* induction experiments. To test for this assumption we combined a just inhibitory concentration of IBMX with cyclic nucleotides. As shown in Table V, this results in a significant amplification of inhibition of induction, as would be predicted.

Sexual induction by phosphodiesterase

The inhibition of induction by IBMX alone and — still more — together with cyclic nucleotides seems to be a pivotal function of the steady-state level of cAMP and cGMP in pheromone action. This is stressed further by the finding, reported in Table VI, that phosphodiesterase added externally acts as an inducer of sexuality. Controls also shown in this table, exclude the possibility of contamination with heat-stable pheromone carried through into the assay. Phosphodiesterase induction is not blocked by Con A; it seems to bypass the Con A-sensitive step(s).

Table VI. Induction by phosphodiesterase.

Enzyme	Milliunits	% Induction
phosphodiesterase (cyclic)	12.5	100
phosphodiesterase (cyclic)	1.2	20
phosphodiesterase (cyclic) (15 min at 80 °C)	12.5	5
inducer (6×10^{-17} M) (15 min at 80 °C)	–	100
phosphodiesterase (cyclic) + 3 μg/ml Con A	12.5	95
5'-nuclease	0.04 [a]	0
alkaline phosphatase	0.007 [a]	0

[a] Higher concentrations kill the algae.

Discussion

For successful sexual reproduction in *Volvox carteri* it is necessary that both sexes start developing sexual gonidia almost synchronously. This is provided by the inducing pheromone released by the male strain into the medium. In order to enhance the chance of mating, the signal has to reach many other algae simultaneously. Indeed, one male *Volvox carteri* spheroid produces enough pheromone to induce all (and only) *Volvox carteri* spheroids present in a volume of 1 m^3. The inducing system has been optimized for maximal sensitivity. The limiting concentration is 6×10^{-17} M or very close to a one-molecule event as postulated by Pall [32]. Reasoning along conventional lines one would postulate a high-affinity receptor on the surface of the gonidia which, after binding the pheromone to induce all (and only) *Volvox carteri* ultimately affecting nuclear events. On the basis of our experiments we wish to propose for discussion a different way of pheromone binding and signal processing.

Many independent lines of evidence point to the extracellular matrix − not to the gonidial membrane − as the binding site of the pheromone. By fluorescence microscopy with FITC-labelled anti-inducer antibodies we were not able to identify any specific binding on that membrane. On the other hand, to obey the observed reversibility, we have to postulate for physio-chemical reasons a great excess of binding sites in the spheroids. If these binding sites were evenly distributed in the total matrix volume, it is obvious that the local concentration would be below the detection limit of this method. Nevertheless, we demonstrated specific pheromone extraction by the competitive binding assay. Spheroids or fractions of them compete with one another for the limiting number of inducer molecules added. From the low threshold (10^{-17} M) we infer that only specific high affinity interactions have to be considered and locate them within the matrix without having yet detected their exact positioning or isolated a distinct matrix component which binds inducer. A further argument for the essential function of matrix is the non-inducibility of isolated gonidia and the inhibition by matrix directed agents.

We have to postulate augmenting second − and perhaps even further − messages before the pheromone signal from an extracellular site reaches the nucleus. Both, cyclic nucleotides and calmodulin seem to be involved. Although the evidence for cAMP is only circumstantial, the results are convincing. If inhibition of sexual induction by IBMX is caused by an increment of the level of cAMP, one would expect that lowering of this level would stimulate induction. This is exactly what was found, since added phosphodiesterase mimicks the inducer over a certain concentration range. It is unlikely that external phosphodiesterase alters the intracellular cAMP level. Thus, extracellular (namely matrix) cyclic nucleotides are a signal for induction. High levels of cyclic nucleotides block induction whereas low levels induce.

cAMP as an external biochemical differentiation signal was reported in *Dictyostelium* [33] and *Chlamydomonas* [34]. Also in *Volvox* this concept fits excellently into the observation of extracellular binding sites for the pheromone. However, we cannot answer yet the obvious question how the message is transported into the cell. A candidate may be Ca^{2+}, the extra- and intra-cellular concentration of which is regulated by calmodulin.

Volvox uses a cascade-like signal amplification in order to enhance the sensitivity of its induction system. Starting with a pheromone concentration of only 4000 molecules per ml, the inducer is first concentrated in the matrix by a factor of 300.

The inducer lowers − by a mechanism as yet unknown − the level of cyclic nucleotides in the matrix. This itself or the gradient generated may be the signal which may be further amplified during transmission into the cells. Finally new genes are activated and induction-specific proteins produced.

In addition to these low-molecular signals, we have recently found that extracellular phosphoproteins are involved in induction [11]. The missing link between the cyclic nucleotide/Ca^{2+}-message and the phosphorylated proteins would be the demonstration of a cAMP- or calmodulin-dependent protein kinase.

The occurrence of inducible and reversible phases in the sex-induced generation cycle is in complete agreement with a matrix-mediated induction. It turns out to be a reversible long-term process ruled by the time required for signal processing. During this time the level of cyclic nucleotides is lowered or phospho-proteins (which may be involved indirectly or directly in the Ca^{2+}-transport) are accumulated

in the whole matrix. The reversibility of induction upon diluting out the pheromone is easily met by a message stored in the matrix, because the matrix evidently will be in steady-state equilibrium with the outer medium.

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