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Signal transduction pathways in Volvox Carteri

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Signal Transduction Pathways in

*Volvox carteri*

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Signal Transduction Pathways in

*Volvox carteri*

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Abstract

The green alga *Volvox carteri* exhibits a virtual absence of protein synthesis in the dark, but begins synthesizing proteins necessary for growth and development almost immediately after exposure to the light. The regulation of protein synthesis in this system is known to be translational since extracts prepared in the dark have identical pools and quantities of mRNA as extracts prepared in the light (Kirk and Kirk, 1983). Exploration of the mechanism of translational regulation has lead us to consider the importance of signal transduction pathways. By using a variety of commercially available drugs that affect specific components of known signaling pathways, we have been able to propose a preliminary model for a signal transduction pathway which regulates translation in *Volvox* via a photoreceptor which responds to light and the second messenger, cyclic-guanosine monophosphate (cGMP). In addition, assays for cGMP have confirmed this model.
Introduction

The freshwater, multicellular, colonial green alga Volvox carteri has long been considered a boon to the fields of evolutionary and developmental biology. An extensive range of complexity exists within the “volvocine lineage,” which putatively defines a directed evolution of algae beginning with the unicellular, biflagellate Chlamydomonas, spanning a wide range of species with a trend of increasing numbers of uniquely organized Chlamydomonas-like cells, and reaching an apex with Volvox, which displays a distinct level of cellular differentiation and a clear division of labor between only two cell types (Schmitt et al., 1992; Kirk and Harper, 1986). The first of these cell types is the gonidium, or reproductive cell, of which there are approximately 12-16 in a typical growing and dividing organism in a favorable environment. These gonidia are surrounded by a roughly spherical shell of about 2000 somatic, or vegetative, cells in an adult Volvox. These somatic cells are biflagellate in nature, and thus morphologically resemble their evolutionary ancestor, Chlamydomonas (Schmitt et al., 1992; Kirk and Harper, 1986). The somatic cells differ sharply not only in function and biochemical composition (as shown by the differences in mRNA pools between cell types that Lai-Wa Tam and coworkers detected in 1991), but also in size and structure from the relatively large and morphologically symmetrical gonidia. This dichotomy of cell types makes Volvox an ideal system for determining and understanding the regulation of cellular differentiation and development, and a useful model for more complex systems.
Figure 1. The 48-hour synchronized life cycle of female asexual *Volvoc carteri*.

*Growth and Development.*

The point of differentiation between the somatic cells and the gonidia occurs during a stage called "cleavage" in the 48-hour light/dark dependent life cycle of asexual *Volvoc* (Figure 1). The beginning of cleavage occurs towards the end of the first 16 hour light period and is marked by the initiation of cellular division in the unicellular gonidia. The fifth division differs from the previous four, as it is asymmetric and results in 16 larger cells which do not undergo further division, but are henceforth known as the reproductive cells. The remaining 16 cells experience seven more symmetric divisions and eventually constitute the somatic shell. At the end of the cleavage stage, however, both the reproductive and vegetative cells are clustered together in a ball which is collectively known

A few hours after the *Volvox* enter the first of two eight-hour dark periods, the next stage of development, "inversion," occurs. During this stage, a hole, or phialopore, appears in one end of each embryo. The embryo loses its spherical shape as it undergoes inversion, a process where the embryo is essentially turned "inside-out" as the intact pole of the spherical embryo moves through the phialopore, and an inverted sphere eventually reforms. The somatic cells now envelope a dense, glycoprotein-rich extracellular matrix and the 16 reproductive cells which will soon begin a new cycle of cleavage. These newly inverted structures are now classified as “juveniles.” At the start of the second 24-hour period, when the *Volvox* are again exposed to light, the juveniles grow and mature until they are ready to break free from their “mom” and begin their own life cycle. This stage, appropriately termed “hatching” averages a 12-16-fold increase in the number of organisms in a culture (depending on the number of gonidia originally within each organism) and is followed by the senescence of the “mother” organism, or essentially the dissolution of the somatic shell (Kirk and Harper, 1986; Tam *et al*., 1991; Schmitt *et al*., 1992). By maintaining the *Volvox* in a laboratory setting with timed light/dark periods and a specific nutrient media, synchronous cultures can be isolated and maintained for study.

**Protein Synthesis.**

One of the most intriguing observations regarding *Volvox carteri* is its light-dependent protein synthesis (Kirk and Kirk, 1983; Girardin, unpublished observations). While protein synthesis is abundant during both light periods of the asexual life cycle, a rapid decline in the amount of proteins synthesized is observed as soon as the *Volvox* enter the dark. In general, the initiation of protein synthesis in the light and the decline of protein synthesis in the dark describes the pattern of most (if not all) proteins within *Volvox*. This pattern seems puzzling since proteins are necessary for growth, division, and
differentiation, and Volvox continue their development, in at least the first dark period, as they undergo inversion. However, because all proteins are not utilized in the same quantity nor at the same time, there must exist at least one mechanism of regulation for protein synthesis. In order to consider potential sites of the regulation of protein synthesis, we must first look at how proteins are made within eukaryotes.

The process of protein synthesis begins with transcription, the interpretation of the DNA-encoded genetic information of an organism as a complementary strand of mRNA. Transcription occurs within the nucleus of a cell, and so the new strand of mRNA must be transported to the cytosol where the ribosomes, or the functional units of protein synthesis, are located before translation can occur (Lodish et al., 1995). Translation can be subdivided into three phases: initiation, elongation, and termination (Hershey, 1991). A prerequisite for the initiation of translation is the presence of the energy source GTP and certain initiation factors, or proteins which directly bind or indirectly facilitate binding between mRNA and ribosomes and subsequent recognition of a start codon (AUG). Two initiation factors, eIF-4F and eIF-2, are known to be involved in regulating protein synthesis in many mammalian systems through a mechanism of reversible phosphorylation, which prohibits the initiation of translation (Hershey, 1991). Though many of the same initiation factors have been identified in plants, specifically wheat germ, it is unclear at this time if these initiation factors have the same importance and are controlled by the same mechanisms in plants as they are in mammals (Janaki et al., 1995; Shaikhin et al., 1992). During the second phase of translation, elongation, the ribosomes pair appropriate molecules of tRNA according to their three base sequence (anti-codon), to the complementary three base mRNA sequence, or codon. Each tRNA molecule has a specific amino acid associated with it, and in a cyclic manner, amino acids are joined one at a time by peptide bonds, forming a polypeptide. Termination of translation occurs when a stop codon is recognized, and the nascent polypeptide or protein is released (Hershey, 1991).
This multi-step description of protein synthesis provides two obvious places for regulation: transcription and translation. By comparing extracts that have been prepared from Volvox in the dark for a minimum of 12 hours with extracts prepared from Volvox that have been exposed to the light for one hour after an extended dark period (also of at least 12 hours), one can examine the issue of control. As stated previously, Volvox carteri abort protein synthesis in the dark and initiate protein synthesis in the light. Similarly, the amount of protein synthesized de novo during a certain time period (at least one hour) is much greater in the light samples than in the dark samples. It has been previously shown (Kirk and Kirk, 1985; Kirk and Kirk, 1986), however, that equivalent levels of mRNA transcripts appear in extracts of Volvox that have been exposed to either the light or to the dark. This suggests that control of protein synthesis is exerted post-transcriptionally, and that light somehow acts as a necessary trigger to turn on translation.

Signal Transduction.

The mechanism by which light acts to initiate protein synthesis is a major focal point of Volvox research. In the study described in this paper, we have hypothesized that a signal transduction pathway involving a G-protein-linked photoreceptor may mediate protein synthesis in Volvox. Though G-protein signal transduction pathways can vary extensively, there are certain components which are common to all pathways of this type. A general G-protein pathway is described as follows (Lodish et al., 1995). An external stimulus first initiates a change which affects the cell-surface-linked receptor. This receptor is associated with a G-protein that can act as a stimulatory or an inhibitory G-protein and is generally composed of multiple subunits. One of the subunits, the $G_\alpha$ cycles between an active and an inactive form by binding a diphosphate nucleotide (guanosine diphosphate (GDP) or adenosine diphosphate (ADP)) in the inactive form and a triphosphate nucleotide [guanosine triphosphate (GTP) or adenosine triphosphate] in the active form. The GTP-
bound $G_a$ is able to dissociate from the other G-protein subunits and activate an effector protein (often an adenylate or guanylate cyclase). It is the action of the effector protein which hydrolyzes the triphosphate nucleotide to its respective cyclic monophosphate form [cyclic-guanosine monophosphate (cGMP) or cyclic-adenosine monophosphate (cAMP)]. This action produces consequential changes in the concentrations of the intracellular nucleotide phosphate pool since both cGMP and cAMP can act as second messengers that permit signal amplification, trigger a cascade of intracellular changes including a series of phosphorylation/dephosphorylation reactions, and ultimately, initiate or inhibit protein synthesis.

A specific model signal transduction pathway is that of mammalian vision, which operates via the photoreceptor rhodopsin and the G-protein transducin to increase the concentration of the second messenger cGMP and activate a cGMP-dependent phosphodiesterase (which can degrade cGMP when activated by an abundance of cGMP). A cascade of events follows, including reversible phosphorylation, ultimately enabling the state of hyperpolarization which is necessary for sight to occur (Khorana, 1992). It is possible that the light-dependent signal transduction pathway responsible for translational control in *Volvox carteri* may have similar stimuli, photoreceptors, G-proteins, and second messengers as this vision pathway, though the pathways have notable differences in their kinetics: vision results in a rapid, short-term response, while the regulation of protein synthesis in *Volvox* is a slow, long-term response.

**Photoreception.**

One specific characteristic of each somatic cell of *Volvox* is the presence of an eyespot that appears red due to the pigment rhodopsin (Van Den Hoek *et al.*, 1995). The eyespot can be easily distinguished from the green, chlorophyll-laden body of the vegetative cell (Bold and Wynne, 1985). Rhodopsin is a retinal-containing protein.
photoreceptor (Hegemann and Harz, 1993) and is known as the "universal visual pigment of animals" (Van Den Hoek et al., 1995). However, rhodopsin has also been found to exist and function as a photoreceptor in some members of the Kingdom Bacteria, as well as other members of the volvocine algae (Khorana, 1992; Van Den Hoek et al., 1995).

Multiple spectroscopic studies in Chlamydomonas have shown that the phototactic behavior of Chlamydomonas may be directed by the photoreceptor character of rhodopsin (Hegemann and Harz, 1993). For this reason, rhodopsin is also a good candidate for the photoreceptor in Volvox which intercepts the external stimulus light and results in the regulation of protein synthesis.

The studies described in the remainder of this paper were instituted in an effort to investigate potential signal transduction pathways controlling light-dependent translational control in Volvox carteri. An effective way to study signal transduction pathways is by inhibiting a pathway at different points and observing changes in the final outcome of the pathway (in this case, protein synthesis). This method of in vivo manipulation with drugs has previously been proven effective in Volvox (Gilles et al., 1985; Jaenicke, 1991) during the investigation of a cAMP-dependent pheromone induction of sexual Volvox. In addition, in Chlamydomonas, a blue-light-dependent signal transduction pathway was studied by similar manipulations with various drugs that were known to activate or inhibit essential components of many signal transduction pathways (Pan et al., 1996). Likewise, in the present study, various drugs which affect components of signal transduction pathways were administered to Volvox exposed to the light or the dark, in an attempt to elucidate the specific pathway controlling protein synthesis. In addition, we looked at the effects of some of these drugs during the two transition periods between light and dark. The results obtained allow us to propose a signal transduction pathway regulating protein synthesis that involves a cGMP intermediate. Assays of cGMP concentrations at various times during the Volvox life cycle provide evidence supporting this model.
Materials and Methods

Drugs.

All drugs were purchased from CALBIOCHEM®. 3-Isobutyl-1-methylxanthine (IBMX), K-252a, 8-methoxymethyl-3- isobutyl-1-methylxanthine (MM-IBMX), MY-5445 [1-(3-Chlorophenylamino)-4-phenylphthalozone], Quazinone, RO-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], SQ 22536 [9-(tetrahydro-2'-furyl)adenine], and Zaprinast [1,4-dihydro-5-(2-propoxyphenyl)-7H-1,23-triazolo[4,5-d]pyrimidine-7-one] were all prepared in DMSO in 1 mM stock concentrations. Dipyridamole and KT5823 were also prepared in DMSO, but at 100 mM and 0.1 mM concentrations, respectively. 8-Bromo-cyclic-adenosine monophosphate, 8-bromo-cyclic-guanosine monophosphate, dibutyryl-cyclic-guanosine monophosphate, and isoproterenol were all prepared in distilled water at concentrations of 1 mM. The sodium salt of okadaic acid was also prepared in distilled water, but at a 0.1 mM concentration. LY 83583 [6-anilino-5,8-quinolinequinone] was prepared at a concentration of 1 mM in ethanol.

Isolation of Gonidia.

Volvox were filtered in a laminar flow hood using a sterile Gelman Sciences 300-ml filtration device, and a 30 μm nylon mesh filter. Depending on the number of flasks being used, either a 15-ml or a 40-ml Dounce homogenizer was used and filled with 10 ml or 35 ml of the essential nutrient media, Standard Volvox Media (SVM), respectively. Volvox collected on the filter were rinsed into the homogenizer and physically homogenized with seven strokes of the loose (“A”) Dounce pestle. The mixture was then transferred to either a 15-ml or 50-ml sterile Fisher centrifuge tube and 0.7 ml of Percoll per 10 ml Volvox mixture was added. After inverting the tube several times to mix, the sample was centrifuged for 5 minutes at room temperature at about 1500 rpm in a Fisher
Scientific Centrifug™ Centrifuge, and the supernatant was discarded, leaving the pellet for resuspension.

Toxicity Tests.

Prior to use in assays for protein synthesis determination, each drug and solvent was tested for its general toxicity to Volvox in order to determine appropriate concentrations of drugs to be used during each assay. One milliliter of isolated gonidia in SVM was added to each well of a 24-well tissue culture plate, and various amounts of each of the aforementioned drugs, and DMSO and ethanol alone, were added to separate wells until a concentration could be found that did not seriously alter the organisms' color, size, motility, morphology, or development in a period of two hours or less as compared to controls. Concentrations that severely disturbed one or more of these properties (i.e. whole organisms became non-motile and brown, white, shrunken, dissociated, and/or had shrunken or darkened reproductive cells) were considered "toxic" for reasons other than their potential ability to inhibit protein synthesis, and were decreased until the majority of Volvox in a well were found to be unaffected, but were severely affected at the next highest concentration of the same drug. This "unaffected concentration" was considered appropriate for use in further assays.

Measurements of Protein Synthesis:

Preparation of Volvox. Two flasks of Volvox carteri were prepared one day prior to assaying for protein synthesis, one for exposure to light and one to remain in the dark until after sonication of the organisms. Preparation included isolation of the gonidia from asexual Volvox in the first 24-hours of their life cycle (preferably during cleavage, but never before), resuspension in a 500-ml flask of fresh, sterile SVM and covering each flask with heavy duty aluminum foil after entering the dark period, and finally returning the
flasks to a 32°C bath. The next day, both flasks of juveniles were harvested by filtration in
a Gelman Sciences 300-ml filter device using a 30 μm filter and rinsed and resuspended in
an appropriate amount of sulfur-free SVM (SF-SVM) depending on the number of assay
tubes to be used (appropriate volume equals 1.0 ml/tube). The Volvox were resuspended
in a Petri dish, and 1 ml of the Volvox/SF-SVM mixture was aliquoted to each Fisherbrand
12 x 75 mm polypropylene test tube. Manipulations of one flask of Volvox (the “dark”
sample) were performed in the dark, without exposure to light, while the other sample (the
“light” sample) was prepared in the presence of normal fluorescent lighting. The dark
samples were covered with aluminum foil and left in the dark for approximately one hour,
while the light samples remained uncovered and were exposed to the light. Both sets of
samples were returned to the water bath during this time. After one hour in this sulfur-
starved state, approximately 2 μCi of 35S[H2SO4] (ICN) and an appropriate drug were
added to experimental samples, while only 2 μCi 35S[H2SO4] per tube was added to control
samples.

*Measurements of protein synthesis inhibition.* As controls, at least one dark sample and no
drugs were added to at least one light sample per assay. Experimental samples were
prepared as “light” samples, and after drug and label addition, they remained in a water
bath exposed to light for one additional hour before reactions were stopped by putting tubes
on ice and adding 4 ml of ice cold SVM to each tube. Other studies of drugs’ inhibitory
abilities were done by using “dark” samples, but then exposing these to the light after drug
and label addition.

*Measurements of protein synthesis activation.* Initial assays of this type were done using
“dark” samples in addition to dark and light controls. Samples were kept in the dark for an
additional hour after drug and label addition before reactions were stopped as described
above. Later assays of this type were done by using “light” samples, but after drug and
label addition, these samples were exposed to the dark for one hour. Assays of this later type also yielded information about the transition state as the organisms undergo the light-dark transition.

Concentration Curves.

In an attempt to characterize the nature of the drugs that successfully inhibited translation, and also to provide information to aid future studies to discover mutants resistant to these drugs, drug concentration dependence was examined. A range of concentrations between 0 and 200 μM for 8-bromo-cGMP, and between 0 and 20 μM for MY-5445 and Zaprinast were added along with the radioactive label to “light” samples, and reactions were stopped after one hour in the light. Immediately after stopping the reactions, samples from each concentration tube were taken for microscopic analysis of the morphology and motility of the organisms.

Time Effect of 8-bromo-cGMP.

In order to confirm the effectiveness of the drugs over the experimental time (one hour), the level of protein synthesis was measured over time. A constant concentration of 100 μM 8-bromo-cGMP was added with the radioisotope to “light” Volvox samples. The reactions were stopped as described above 5, 15, 30, 45, 60, and 90 minutes after time of drug addition.

Quantification of Protein Synthesis:

Labeled extract preparation. After reactions were stopped, samples were uniformly suspended by inversion and vacuum filtered using 0.45 μM nylon MAGNA-R membranes (Micron Separations Inc.) in a Fisherbrand 15-ml glass filtration device. Samples were rinsed with SVM, resuspended in 1 ml of SVM in microcentrifuge tubes, and put on ice in the dark until all samples were filtered. Tubes were shaken to free as many Volvox as possible from the nitrocellulose membranes, and then centrifuged briefly in order to pellet
the Volvox. Membranes were removed at this time, and Volvox were sonicated 3 times for 12-15 seconds each time, keeping tubes on ice between sonications. After removing 50 μl each for TCA precipitation, samples were either frozen at -20°C or kept on ice (briefly) until protein determination using the BioRad Standard, Microassay, and/or Standard procedure for microtiter 96-well plates depending on the amount of protein per sample (Bio-Rad Laboratories, LIT-33).

TCA precipitation. Fifty μl of each of the above extracts was added to 1 ml 5% trichloroacetic acid (TCA) in microcentrifuge tubes. Samples were boiled 5 minutes and put on ice for approximately one hour. After incubation, samples were centrifuged briefly to remove condensation from cap and pipetted onto Fisherbrand Glass Fiber Filter Circles G6 in a Stainless Steel Filtration Device for vacuum filtration. Samples were rinsed with 5% TCA, and filters were dried and put in 7-ml scintillation vials with 5-ml Econosafe Scintillation Fluid. Radioactivity was counted by liquid scintillation using the Beckman LS3801.

cGMP Assay. cGMP was assayed using an ELISA kit (Neogen Corporation, Lexington, KY). Initial assays utilized Volvox extracts prepared from Volvox exposed to the light for one hour or remaining in the dark for 18 - 24 hours. Additional experiments monitored changes in cGMP concentrations that occurred as freshly isolated, cleaving gonidia went from being exposed to light in their normal light/dark cycle to remaining in the dark for an extended period of time. Extracts were prepared at the end of the light period, immediately after the dark period began, and 1, 2, 4, 6, 12, and 20 hours after the dark period began. Extracts were prepared in this case by harvesting the gonidia either in the light or in the dark using a 30 μm filter in a Gelman Sciences filtration device. Gonidia were resuspended in PBS pH 7.5 and pelleted by centrifugation. Supernatant was removed.
until the remaining volume totaled 1 ml, and samples were sonicated 5 times for 10 seconds each. Samples were centrifuged at 14,000 rpm in a table top Eppendorf™ Centrifuge for one minute to remove any remaining whole gonidia. 100-400 μL were frozen at -20°C for protein determination at a later time, and the remainder of each sample was boiled for 4 minutes for deproteinization. Samples were centrifuged 10 minutes at 14,000 rpm, and supernatant was collected and used immediately in assay or frozen at -20°C for later use. Concentrated samples or dilutions in PBS pH 7.5 from 1:1 to 1:19 were used in cGMP assays.

Calculations:

Results of protein synthesis were analyzed using Microsoft Excel version 5.0 by subtracting the instrument blank from the total counts per minute (cpm) per sample, determining cpm/μL, and normalizing for the amount of protein per μl.
The results of the toxicity test are shown in Table 1. In addition to this information, *Volvox* were able to tolerate pure ethanol and dimethylsulfoxide (DMSO) in concentrations up to 5% and 10%, respectively (data not shown). These “effective concentrations” were used in subsequent assays, with the exception of the concentration studies, which were performed using wider ranges of concentrations, above and below the determined effective concentration. Concentrations higher than the effective concentrations listed in Table 1 caused immotility in addition to dissociation of somatic cell sheath, discolored (brown, white, or translucent) cells, shrunken organisms, shrunken gonidia, arrested development prior to inversion, enlarged somatic cells, and/or irregular distributions of somatic cells, while concentrations lower than the effective concentration had no visible effects on normal motility or morphology.
Table 1. A classification of the drugs used (based on their known activity in other systems) and their effective concentrations. Abbreviations: PK-A = cAMP-dependent protein kinase; PK-G = cGMP-dependent protein kinase; PDE = phosphodiesterase; CaM = Calmodulin; AC = adenylate cyclase; GC = guanylate cyclase (CALBIOCHEM®, 1996/97).

<table>
<thead>
<tr>
<th>CLASS OF DRUG</th>
<th>DRUG</th>
<th>SOLVENT</th>
<th>CONCENTRATION (μM)</th>
</tr>
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<tbody>
<tr>
<td>β-Adrenergic Agonist</td>
<td>Isoproterenol</td>
<td>Water</td>
<td>100</td>
</tr>
<tr>
<td>PK-A Activator</td>
<td>8-bromo-cAMP</td>
<td>Water</td>
<td>100</td>
</tr>
<tr>
<td>PK-G Activator</td>
<td>8-bromo-cGMP</td>
<td>Water</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Dibutyryl-cGMP</td>
<td>Water</td>
<td>100</td>
</tr>
<tr>
<td>PDE Inhibitor</td>
<td>IBMX</td>
<td>DMSO</td>
<td>5</td>
</tr>
<tr>
<td>cAMP-specific</td>
<td>RO-20-1724</td>
<td>DMSO</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Dipyridamole</td>
<td>DMSO</td>
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</tr>
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<td>cGMP-specific</td>
<td>MY-5445</td>
<td>DMSO</td>
<td>0.5</td>
</tr>
<tr>
<td>CaM-specific</td>
<td>Quazinone</td>
<td>DMSO</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Zaprinast</td>
<td>DMSO</td>
<td>1.0</td>
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<td>General PK Inhibitor</td>
<td>K-252a</td>
<td>DMSO</td>
<td>5</td>
</tr>
<tr>
<td>PK-G specific</td>
<td>KT5823</td>
<td>DMSO</td>
<td>0.05</td>
</tr>
<tr>
<td>AC Inhibitor</td>
<td>SQ 22536</td>
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<td>5</td>
</tr>
<tr>
<td>GC Inhibitor</td>
<td>LY 83583</td>
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<tr>
<td>Phosphatase Inhibitor</td>
<td>Okadaic acid,</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>Sodium Salt</td>
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</tr>
</tbody>
</table>

Results from the experiments measuring protein synthesis in variable conditions of light and dark exposure are displayed in figures 2, 3, 4, and 5. Figure 2 illustrates that though nearly all of the drugs tested revealed some inhibitory effect (30% or less), we were able to find only three drugs which consistently reduced the amount of protein synthesis by more than 60% in *Volvox* which were kept in the light throughout the experiment: MY-5445, Zaprinast, and 8-bromo-cGMP. LY83583 also showed some ability to inhibit protein synthesis when exposed to light before and after drug addition, however, the
results which we obtained using this drug varied greatly among experiments. In fact, we also observed activation of protein synthesis in *Volvox* by LY83583 in the dark (figure 3). Other than the inconsistencies observed with LY83583, we did not find evidence of significant activation of protein synthesis in *Volvox* which remained in the dark throughout the experiment (figure 3). Similarly, in figures 4 and 5, there was no indication of significant activation of protein synthesis by any drug when *Volvox* were either maintained in the dark until after drug and label addition or when *Volvox* were exposed first to the light and then the dark.
Figure 2. The effects of various drugs on protein synthesis in Volvox in the light.
Figure 3. The effects of various drugs on protein synthesis in *Volvox* in the dark.
Figure 4. Effects of various drugs on protein synthesis in *Volvox carteri* kept in the dark until the addition of drug and radioactive label, at which time, samples were exposed to the light.

It is possible, however, to see inhibition of protein synthesis by KT5823 and K-252a in figure 4 where *Volvox* were kept in the dark before drugs and label were added, but were then exposed to the light (figure 4). It should be noted however, that inhibition by these drugs could not be seen in figure 2 when *Volvox* remained in the light both before and after drug and label addition.
Figure 5. Effects of various drugs on protein synthesis in *Volvox carteri* exposed to the light until the addition of drug and radioactive label, at which time, samples were kept in the dark.

The concentration curves of both MY-5445 and 8-bromo cGMP (figures 6 and 7) show a clear correlation between the amount of drug used and the extent of the inhibition of protein synthesis. For both drugs, the trend is for an increased level of protein synthesis inhibition with an increase in drug concentration. For 8-bromo-cGMP, a maximal level of inhibition is obtained near concentrations of 150 μM, though a basal level of translation still exists above that of dark control samples (figure 6). In MY-5445 however, 10 μM concentrations show maximum inhibition and this level of inhibition is comparable to the level of protein synthesis observed in the dark control samples (figure 7). \( K_i \) values (the concentration at which inhibition is 50% of the maximum protein synthesis) are approximately 60 μM for 8-bromo-cGMP and 5μM for MY-5445.
Figure 6. The dependence of protein synthesis in *Volvox carteri* in the presence of light on the concentration of the phosphodiesterase resistant cGMP analog, 8-bromo-cGMP.
The results of the time-dependent effect of 8-bromo-cGMP (figure 8) revealed that 8-bromo-cGMP began to affect translational activity between 5 and 15 minutes after time of drug addition to *Volvox* in the light. After the initiation of the drug's activity, the level of protein synthesis in the experimental sample closely mimicked that of the dark control, though the slight increase in the amount of protein synthesis observed between 45 and 60 minutes in the two inhibited samples was greater for the experimental sample than for the dark control.

**Figure 7.** The dependence of protein synthesis in *Volvox carteri* in the presence of light on the concentration of the cGMP-specific phosphodiesterase inhibitor, MY-5445.
Figure 8. The effect of a constant concentration of 8-bromo-cGMP on translation in *Volvox carteri* in the presence of light over a period of 90 minutes after the addition of drug and radioisotope label. Dark and Light controls are included for comparison.

cGMP assays showed nearly 3 times more cGMP in "dark" *Volvox* extracts than in light *Volvox* extracts, with a mean value (n=3) of 24.6 ng cGMP/mg total protein in the dark and a mean value (n=3) of 9.32 ng cGMP/mg total protein in the light. In addition, the amount of cGMP in *Volvox* increased with the amount of time spent in the dark, and there was more cGMP after 20 hours in the dark than at any other time. The plot of cGMP concentration versus time, however, did not reveal a clear trend (figure 9).
Figure 9. Intracellular cGMP concentration in *Volvox carteri* over a 20 hour period spent in the dark.
Discussion

The drugs that were capable of inhibiting protein synthetic activity in *Volvox carteri* fall into three major categories: cGMP analog, cGMP-specific phosphodiesterase inhibitors, and protein kinase inhibitors. We will consider the implications of each of these separately.

By adding the cell-permeable cGMP analog 8-bromo-cGMP (which is more resistant to cGMP phosphodiesterases than normal cGMP and also has the ability to activate protein kinase G) the cytosolic concentration of cGMP has effectively been increased. If an increase in the cytosolic concentration of cGMP affects the amount of protein synthesis in the light, but not in the dark, we can postulate that under normal (without exogenous drugs) conditions there are low levels of cGMP, and that an increase in this concentration causes protein synthesis to be inhibited. On the other hand, if the levels of cGMP are already high in the dark, then adding more in the form of an analog will have no observable effect on the protein synthetic activity. If we imagine a model in which the presence of cGMP is needed to turn off protein synthesis by binding to and activating another enzyme, perhaps a phosphatase, then only a threshold level of cGMP would be required. A model of low levels of cytosolic cGMP in *Volvox* in the presence of light that are somehow increased during the transition from light to dark would therefore agree with the observed results thus far.

When MY-5445 and Zaprinast, inhibitors of cGMP-specific phosphodiesterases, were added to *Volvox* in the presence of light, we again observed decreased protein synthesis. Phosphodiesterases act to break down cyclic nucleotides in the cytosol. By inhibiting the action of cGMP-specific phosphodiesterases, we are again causing an effective increase in the amount of cGMP in the cytosol. The results show more than 60% inhibition of protein synthesis in *Volvox* exposed to the light throughout the experiment in the presence of both MY-5445 and Zaprinast (figure 2). In addition, there was nearly
100% inhibition of protein synthesis in Volvox which were not exposed to the light until after drug addition (figure 4). We postulate a situation in which high levels of cGMP inhibit protein synthesis. These results are in complete agreement with those obtained using 8-bromo-cGMP.

Finally, two protein kinase inhibitors, KT5823 specific to protein kinase G and K-252a (a general protein kinase inhibitor) were found to inhibit protein synthesis. Because these two drugs did not inhibit protein synthesis in the same experimental conditions in which the phosphodiesterase inhibitors or the cGMP analog did (light before and after drug addition), but only inhibited translation when the Volvox were kept in the dark before drug addition and were exposed to light afterwards, these results must be interpreted differently. Protein kinases are common components of signal transduction pathways in which they are activated by cyclic nucleotides, and then go on to phosphorylate other enzymes in the cascade. Applying this to our model, we would expect that in the dark (high [cGMP]) phosphorylation would occur, which would have to inactivate protein synthesis. By inhibiting this kinase in Volvox in the dark, however, we would expect an absence of phosphorylation and an activation of protein synthesis. Since we did not observe this activation, we have looked toward a model of a cGMP-dependent protein phosphatase. Phosphatases normally function to dephosphorylate, and thus have an antagonistic role with regard to kinases. In this model, we would expect active dephosphorylation by a cGMP activated phosphatase to occur in the absence of light, which would ultimately result in the inhibition of protein synthesis. If a situation of phosphorylation persisted, then translation would be perpetuated. If a protein kinase were then inhibited in the light, the equilibrium would shift toward a state of dephosphorylation, which would result in the inhibition of protein synthesis. Because this state of dephosphorylation is not dependent upon the activity of a phosphatase, but merely a lack of activity of a kinase, we must realize that if the Volvox were exposed to light prior to the addition of a protein kinase inhibitor, phosphorylation would already be abundant, and therefore, inhibition of translation would
not be observed. If, however, the Volvox were kept in the dark until the addition of the protein kinase inhibitor, and only then were exposed to the light, phosphorylation would not occur (or only minimal phosphorylation would occur, depending on the time needed for the drug to permeate the membrane and to reach full inhibitory capacity), and the result would be inhibition of protein synthesis. Indeed, this is what we have observed in this study.

By compiling these three concurring analyses, we have come up with the model depicted in figure 10. The presence of light suppresses the cytosolic level of cGMP which thereby reduces the activity of a cGMP-dependent phosphatase and increases phosphorylation of some component required for active translation. Conversely, in the dark, abundant cGMP results in increased phosphatase activity and reduced phosphorylation. Necessary enzymes for translation cannot be activated, and protein synthesis does not occur.

**DARK:**

Active

High [cGMP] → cGMP-dependent phosphatase ↔ Translation

**LIGHT:**

Inactive

Low [cGMP] → cGMP-dependent phosphatase ↔ Translation

Figure 10. A possible model for cGMP-dependent translational control in Volvox carteri relying on a cGMP-dependent phosphatase.

The model shown in figure 10 is not the only possibility for a signal transduction pathway that agrees with our observations. Another possibility is a model that directly involves a common regulatory component in signal transduction pathways, the G-protein (figure 11). The absence of light would cause the photoreceptor to activate a stimulatory
G-protein which would activate guanylate cyclase, resulting in high [cGMP]. Abundant cGMP, acting as a second messenger may react with another intracellular component, and cause downstream phosphorylation/dephosphorylation events that eventually lead to translation. In contrast, in the presence of light, the inhibitory G-protein would be activated, thus yielding low intracellular concentrations of cGMP, and by a similar cascade of events, inhibiting protein synthesis.

\[
\text{LIGHT} \rightarrow \text{Inhibitory G-protein} \rightarrow \text{Low cGMP} \rightarrow \text{Translation} \\
\text{DARK} \rightarrow \text{Stimulatory G-protein} \rightarrow \text{High cGMP} \rightarrow \text{Translation}
\]

Figure 11. Another possible model for cGMP-dependent translational control in *Volvox carteri*, based on G-protein regulation.

In order to address certain discrepancies which appeared in the results, it will be helpful to consider the nature of many of the drugs utilized in this study. First, all of these drugs were characterized in systems other than algae, and while it is known that certain elements such as protein kinases, phosphatases, phosphodiesterases, and cyclic nucleases, among others, do exist within *Volvox carteri*, it has not been proven that they play the same roles in algal systems as they do in the systems in which they were characterized. One must also consider the permeability of these drugs to both the cell wall and the plasma membrane of *Volvox carteri*, because if the drugs are never entering the cell, no intracellular changes would be detected. The fact that the drugs may not be acting on the *Volvox* in the expected manner (table 1) or the fact that the drugs may not be entering the cells, could both explain the lack of any changes in translation in experiments using many of the drugs. Another explanation for the lack of effectiveness of the drugs could be in the specificity of the drugs to enzymes which are not important in the relay between light and protein synthesis. For example, though we might postulate that a phosphatase inhibitor
(like okadaic acid) should have some capacity to activate protein synthesis, especially in the model in which _Volvox_ are exposed to light prior to drug addition, and then kept in the dark, we know that okadaic acid is specific for only protein phosphatases 1 and 2A, but does not affect any acid, alkaline, or tyrosine phosphatases (CALBIOCHEM®, 1996/97). [Unpublished results, however, suggest that tyrosine phosphatases are not involved in the regulation of protein synthesis in _Volvox_ (Jovanovic, 1996)]. In addition, LY83583 is described as an inhibitor of soluble guanylate cyclase (CALBIOCHEM®, 1996/97), but it is possible that if guanylate cyclase is important in this signal transduction pathway, it is a membrane bound protein which remains unaffected, or only minimally affected by LY83583. There could be other incidences of similar alterations in forms of the targeted protein that result in the ineffectiveness of a specific drug. Finally, many of these drugs have multiple effects. LY83583 is again a perfect example of this potential complication since not only is it known to inhibit the activity of soluble guanylate cyclase, but it is also a known inhibitor of certain nitric oxide effects (such as smooth muscle relaxation) and certain interleukin-1 induced effects (such as cGMP accumulation in aortic vascular cells) in rats (CALBIOCHEM®, 1996/97). SQ22536 is also known to have multiple effects including an enhancement of oxygen radical production (CALBIOCHEM®, 1996/97). We have therefore attributed the high degree of variability in the results of our experiments with LY83583 to the multiple effects of this drug.

Examining intracellular cGMP concentrations has provided evidence which supports both of the above models in which high levels of cGMP induce low levels of protein synthesis and low levels of cGMP yield high levels of protein synthesis. As expected we found increasing amounts of cGMP with time spent in the dark, however, we expected a plot of this data to produce a predictable trendline such as a rectangular
hyperbola. We are currently in the process of repeating these experiments to determine whether or not this data is reproducible.

Further studies that should be done to further examine this model include obtaining mutants to drugs which are capable of inhibiting protein synthesis. The availability of mutants would permit useful genetic and molecular analyses of the necessary components in this putative signal transduction pathway. In addition, identifying a cGMP-dependent protein phosphatase which is more active in the dark than in the light would give supportive evidence to figure 10. Studies such as these are currently underway in our laboratory. Once this portion of the pathway is understood, a more directed progression towards the targets of phosphorylation will be possible, as well as regression towards the photoreceptor end of the pathway, including identification of inhibitory and stimulatory G-proteins, as well as qualification of any light-dependent activities of these proteins which would provide support for figure 11. By obtaining and compiling this type of information, we hope to develop a complete picture of the light-dependent signal transduction pathway which controls translation in *Volvox carteri*.
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