The Effect of Ecdysterone on the Cyanobacterium *Nostoc* 6720

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The insect hormone ecdysterone stimulated dinitrogen fixation, heterocyst formation, cell size and protein synthesis in suspension cultures of the cyanobacterium *Nostoc* 6720 within 48 h when added to the culture at concentrations between 10^{-9} and 10^{-7} M. In the presence of the hormone the volume of vegetative cells increased by 6-fold and that of heterocysts by nearly twice as compared to untreated controls. All the observed effects were inhibited by the calcium-calmodulin inhibitor, trifluoperazine. Cholesterol used as a null control had no significant effect.

Introduction

Ecdysone, a steroid hormone required for insect development, is present in insect larvae in two forms, α and β, where the active β form is known as 20-hydroxy ecdysone or ecdysterone. Insects are unable to synthesize ecdysone *de novo,* and it is probably derived from cholesterol or some related steroid obtained from their diet. This hormone is able in insects to increase mRNA and protein synthesis [1, 2], promote differential gene expression [3], and influence both phosphodiesterase activity and protein phosphorylation [4]. It has been found for example to induce cell differentiation in *Drosophila* at a concentration of 10^{-7} M [5] and enhance cell differentiation and acetylcholine esterase activity in *Chironomus tentans* [6]. The ecdysteroids have also been found by GC-MS in higher plants [7–9], where they affect various metabolic activities and the levels of endogenous phytohormones [10].

Such findings imply that ecdysteroids, as biologically functional molecules, are more widespread in nature than was previously thought. This is also true of the plant regulatory substance, abscisic acid, which has been shown to be present in and have similar effects in organisms as diverse as cyanobacteria and mammals [11]. We report here on the effects of ecdysterone in filamentous heterocystous cyanobacteria.

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Materials and Methods

The cyanobacterium *Nostoc* 6720 (ATCC 27895, PCC 6720) once known as *Anabaenopsis circularis* (G. S. West) Wolosz. et Miller, was grown in suspension cultures of nitrate supplemented AA/8 medium [12] in an orbital incubator at 28 °C and 180 rpm under a continuous illumination of 6.8 $\mu E \cdot m^{-2} \cdot s^{-1}$. The position of flasks in the incubator was rotated in order to ensure all cultures received equal illumination. Parental cultures were maintained on AA/8 medium until the $OD_{680\,nm}$ reached a value of 0.8 at which time the cultures were resuspended to a final optical density of 0.15 in nitrate-free AA/8 medium so as to induce heterocyst differentiation. Aliquots (10 ml) of the resuspended cultures were immediately placed in 50 ml conical flasks and incubated as above. After a further 24 h, ecdysterone, cholesterol and/or trifluoperazine (TFP) were added to the flasks as solutions in AA/8. Untreated controls received an equal volume of AA/8 medium.

Dinitrogen fixation was measured by the acetylene reduction assay [13] using 3 ml samples of cultures in 30 ml bottles. After the addition of 10% v/v of acetylene, samples were incubated for 2 h under identical conditions. Care was taken to ensure that each sample received identical illumination. After incubation ethylene formation was determined by gas chromatography. Heterocyst

frequency was routinely estimated by counting interheterocyst distances according to [14], but also included absolute estimates in which the total number of cells were counted using the criterion that a functional heterocyst was one attached to vegetative cells. Heterocyst frequency is recorded as a percentage of the total cell count (vegetative cells plus heterocysts in the filaments). The protein content was measured as described by Bradford [15] using bovine serum albumin as a standard. Cells were collected by centrifugation at 4 °C and 4000 rpm for 6 min and extracted in buffer (50 mM Hepes, 0.1 mM $CaCl_2$, pH 7.0) by twice freezing in liquid nitrogen and thawing followed by ultrasonic disintegration (3 times for 20 sec). The chlorophyll *a* content was estimated as per Strickland and Parsons [16]. Ecdysterone, cholesterol and trifluoperazine were obtained from the Sigma Chemical Company (U.S.A.).

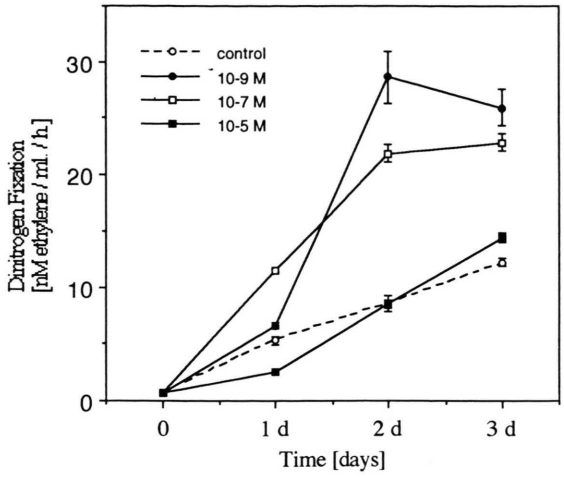

Fig. 1. Dinitrogen fixation (NF) in *Nostoc* 6720 treated with 10^{-9}, 10^{-7} and 10^{-5} M ecdysterone in comparison with that in untreated control cultures during 3 days after application. Means of 4 replicates, bars represent standard deviations.

Results and Discussion

The effects of ecdysterone on *Nostoc* 6720 cultures were studied in samples harvested 1, 2 and 3 days after the application of 10^{-5}, 10^{-7} or 10^{-9} M of ecdysterone. Ecdysterone at a concentration of 10^{-5} M significantly (confidence limits of mean, $p = 0.01$) decreased total nitrogenase activity 24 h after treatment. Ecdysterone in concentration of 10^{-7} M caused a significant increase in N_2 fixation after 24 h, while concentrations of both 10^{-9} and 10^{-7} M ecdysterone significantly increased nitrogenase activity after 48 h and 72 h (Fig. 1). Heterocyst frequency was also increased (Table I). Up to 48 h after addition of ecdysterone the chlorophyll *a* content was not significantly changed, but the following 24 h (72 h sample) showed a significant increase in samples treated with either 10^{-7} or 10^{-5} M ecdysterone as compared to untreated controls (data not shown).

The most obvious, but unexpected effect of ecdysterone that was observed in preliminary experiments was a substantial increase in cell size. This was assessed by direct measurement of the images of cells obtained by overhead projection of photographic negatives. Both vegetative cells and heterocysts were enlarged. The former was enlarged relative to untreated control cultures by a factor of 1.6 along the axis of the filament and a factor of 2 perpendicular to the axis (Table II). Heterocysts were enlarged by factors of 1.2 and 1.3 respectively. Assuming both cell types approximate a cylinder these values indicate that vegetative cells increase in volume more than 6-fold and heterocysts, 2-fold.

Table I. Heterocyst frequency [% of total cells] in *Nostoc* 6720 treated with 10^{-9}, 10^{-7} and 10^{-5} M of ecdysterone in comparison* with that in untreated control cultures for 3 days after application. Means of 4 replicates ± standard deviations.

Treatment	24 h	48 h	72 h
Control	6.02 ± 1.06	6.20 ± 0.85	6.77 ± 1.28
Ecdysterone, 10^{-9} M	5.58 ± 0.87	8.52 ± 1.72	8.82 ± 1.73
Ecdysterone, 10^{-7} M	6.59 ± 1.72	7.53 ± 1.33	8.90 ± 0.88
Ecdysterone, 10^{-5} M	4.86 ± 0.98	6.68 ± 1.03	6.72 ± 1.02

* No significant difference (confidence limits of mean, $p = 0.05$).

Table II. The relative dimensions and calculated volumes of *Nostoc* 6720 cells in cultures treated with ecdysterone (10^{-8} M) for 48 h.

Treatment	Width	Length	Calculated volume
Vegetative cells			
Control	3.55	3.70	146
Ecdysterone	7.25	5.91	975
Heterocysts			
Control	6.62	11.68	1608
Ecdysterone	8.38	17.55	3865

Length = cell diameter along axis of filament, width = cell diameter perpendicular to the axis of the filament. Volume calculated, assuming a cylinder, as $\pi r^2 l$.

It is plausible that the effect of ecdysterone relates not to its hormonal function, but to its steroid structure. This question was assessed by comparing the effects of the hormone to those of cholesterol. This was no significant effect of cholesterol on the culture parameters studied (cell size, N_2 fixation, heterocyst frequency and protein content) in nitrate-free cultures after 48 h incubation (data not shown).

The effects of abscisic acid on heterocyst frequency and nitrogenase activity in *Nostoc* 6720 is inhibited by the calmodulin inhibitor trifluoperazine (TFP) (Smith *et al.* 1987 [14]). To assess whether the ecdysterone effects are also dependent upon a calcium-mediated response nitrate-free cultures containing combinations of TFP and ecdysterone were analyzed (Table III). TFP at 10^{-7} M effectively and significantly inhibited the enhancement of heterocyst frequency and N_2 fixation in combination with an ecdysterone concentration of 10^{-8} M. TFP also inhibited the ecdysterone promoted increase in cell size and the associated increase in protein content (Table IV). Dinitrogen fixation as qualified by either culture density or protein content was not enhanced by ecdysterone as compared to the untreated control and only slightly enhanced when expressed as nitrogenase activity with respect to chlorophyll *a* content (Table V). In all combinations of TFP and ecdysterone, excepting 10^{-9} M ecdysterone plus 10^{-7} M TFP, N_2 fixation as qualified by chlorophyll *a* content was decreased as compared both to ecdysterone and untreated control cultures.

These results demonstrate that ecdysterone is able to influence N_2 fixation and heterocyst frequency in *Nostoc* 6720 and that the effects may be inhibited by TFP. In this respect the gross effects of ecdysterone appear comparable to those of ABA and imply that the steroid hormone is able to influence Ca^{2+}-mediated regulatory pathways. Unlike ABA, ecdysterone promotes an increase in cell size resulting in vegetative cells and heterocysts

Table IV. The characterization of growth by measurement of chlorophyll *a* and protein content 48 h after application of ecdysterone and ecdysterone [E] in combination with trifluoperazine [TFP]. Means of 4 replicates ± standard deviations.

Treatment	Chlorophyll *a* [μg ml^{-1}]	Protein content [μg ml^{-1}]
Control	3.15 ± 0.07	16.43 ± 0.70
Ecdysterone, 10^{-8} M	3.42 ± 0.18	28.85 ± 0.75*
E + TFP, 10^{-9} M	3.45 ± 0.09*	16.65 ± 0.81
E + TFP, 10^{-7} M	3.24 ± 0.11	13.85 ± 0.56*
Ecdysterone, 10^{-9} M	3.12 ± 0.06	26.15 ± 0.60*
E + TFP, 10^{-9} M	2.86 ± 0.09*	14.17 ± 0.62*
E + TFP, 10^{-7} M	3.28 ± 0.14	15.45 ± 0.56

Significant differences, confidence limits for means, p = 0.01, between treated cells and controls are marked by an asterisk.

Table III. Dinitrogen fixation and heterocyst frequency in *Nostoc* 6720 cultures after treatment with ecdysterone (10^{-8} M) [E] in combination with trifluoperazine [TFP]. Means of 4 replicates ± standard deviations.

Treatment	Dinitrogen fixation [nM ethylene · μg chl $a^{-1} \cdot h^{-1}$]	Heterocyst frequency [% total cells]
Control	72.02 ± 3.36	6.4 ± 0.6
Ecdysterone	97.56 ± 7.02	11.3 ± 1.15
E + TFP, 10^{-9} M	68.24 ± 2.48	6.7 ± 0.7
E + TFP, 10^{-7} M	53.60 ± 2.84	6.4 ± 0.5

Table V. Dinitrogen fixation [NF] in *Nostoc* 6720 48 h after treatment with ecdysterone and ecdysterone [E] in combination with trifluoperazine [TFP] referred to chlorophyll *a* and protein. Means of 4 replicates ± standard deviations.

Treatment	NF [nM ethylene · μg chl $a^{-1} \cdot h^{-1}$]	NF [nM ethylene · μg $protein^{-1} \cdot h^{-1}$]
Control	22.86 ± 1.07	4.38 ± 0.20
Ecdysterone, 10^{-8} M	28.53 ± 2.05*	3.38 ± 0.24*
E + TFP, 10^{-9} M	19.78 ± 0.71	4.10 ± 0.15*
E + TFP, 10^{-7} M	16.54 ± 0.88*	3.87 ± 0.21
Ecdysterone, 10^{-9} M	27.17 ± 1.35*	3.24 ± 0.16
E + TFP, 10^{-9} M	19.83 ± 2.55	4.00 ± 0.51
E + TFP, 10^{-7} M	24.33 ± 2.39	5.17 ± 0.51

Significant differences (confident limits for means, p = 0.01) between treated cells and controls are indicated by an asterisk.

having a increased volume of approximately 6 and 2 times respectively greater than those of untreated controls. This expansion in cell volume is accompanied by increased protein synthesis which enhances the protein content of the treated culture by 60 to 70% within 48 h and by a more variable increment in chlorophyll *a* content. This increase parallels that of N_2 fixation and implies that the increase in N_2 fixation reflects an increased proportion of nitrogenase enzyme in the enlarged heterocysts. The average filament length of ecdysone-treated cultures is also reduced to a variable extent and this may account, at least in part, for the slight increase in heterocyst frequency.

The identification of endogenous ABA in cyanobacteria [17, 18] raised the question of whether it is employed as an intracellular effector of regulatory processes. ABA is present and has effects upon Ca^{2+} transport and cytosolic calcium concentrations in organisms as evolutionary diverse as cyanobacteria and higher animals [11, 22]. The finding that ecdysteroids are present and have metabolic effects in higher plants suggests that a similar diverse distribution of ecdysterone and its biological effects may occur. Whether the effects in cyanobacteria are the result of a specific activity, involving a receptor conserved across a substantial evolutionary divide, or a non-specific function such as an influence upon plasma membrane structure, remains to be determined.

The effect of ecdysterone on cell size of *Nostoc* 6720 is distinct and may provide a means to investigate the mechanism(s) that regulates cell size and cell structure. It is notable that, despite the increased protein synthesis in ecdysterone-treated cultures, the protein concentration in vegetative cells is reduced to around 25% of that in untreated controls, and that the effect upon heterocysts is limited compared to that upon vegetative cells. Ecdysterone-treated cultures also continue to grow, in the sense of cell division, apparently normally for at least 3 days and although the heterocyst frequency increases slightly, a normal pattern, lacking double heterocysts or irregular spacing for instance [19, 20], is maintained. With regard to the inhibitor theory of heterocyst pattern formation [21] this would imply that mechanisms determining or predetermining the propensity of a vegetative cell to differentiate into a heterocyst are not affected by the increase in cell volume. The effects of ecdysterone, whether representing an endogenous regulatory mechanism or not, should provide a new perspective on these questions.

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