Cationic Interactions Regulate the Initiation and Termination of Zoospore Activity in the Water Mould *Achlya heterosexalis*

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The zoosporic phase of the water mould *Achlya heterosexalis*, initiated when zoospores emerge from cysts, is strongly influenced by cations. The concentrations of Ca^{2+} and K^{+} present in the medium can determine whether or not zoospores will be released from cysts, whether zoospore motion will be approximately linear or circular, rapid or slow, and when zoospore activity will be terminated by encystment. Ionic effects on zoospore motility are rapid and reversible over short time spans; within a 2 min period motility can be strongly suppressed by increasing the $[\text{K}^{+}]$ and then restored by increasing the $[\text{Ca}^{2+}]$. Trifluoperazine, considered to be an anti-calmodulin drug, both inhibits zoospore release, and arrests zoospore motility.

INTRODUCTION

The genus *Achlya* is characterized by immediate encystment of the asexual spores after emergence from the sporangium (Johnson, 1956). Alternative developmental paths for these spores are germination, by means of a germ tube, or zoospore production. The zoosporic phase starts with excystment; the cyst functions as a single-spored sporangium, from which the secondary zoospore emerges. While excystment in Oomycetes has received little attention, the availability of synchronously produced cysts of *Achlya heterosexalis* (Thomas, 1989) facilitates examination of this process. The zoospore phase is normally terminated by encystment, with the resultant cyst considered to have the same developmental alternatives, germination or zoospore production, as the original cyst. In the genus *Achlya* polyplanetism is common; as many as five repetitions of zoospore emergence followed by encystment have been observed (Salvin, 1940). Environmental factors affecting zoospore release and motility in Oomycetes include temperature, mechanical disturbance, nutrition (Hickman & Ho, 1966) and ions (Fischer & Werner, 1958; Mitchell & Yang, 1966; Svensson & Unestam, 1975; Grant *et al.*, 1986; Soderhall & Cerenius, 1987). Grant *et al.* (1986), working with *Phytophthora* zoospores, reported that Na^{+} induced encystment, and Ca^{2+} counteracted the effect of the Na^{+} . This ionic interaction was incorporated into a model in which Na^{+} uptake is regulated by a Ca^{2+} -gated channel for monovalent ions. In *Achlya*, Ca^{2+} is required for asexual sporulation (Griffin, 1966), amino acid transport and growth (Cameron & LeJohn, 1972); K^{+} , with a vacuolar concentration assumed to be of the order of 100 mM (Thiel *et al.*, 1988), is probably the most abundant cation present (Money & Brownlee, 1987). Our work examines cation interactions, particularly between Ca^{2+} and K^{+} , which regulate the motile phase of the *Achlya* life cycle by controlling its initiation and termination. Calmodulin has been implicated in spore release from both sporangia (Suryanarayana & Thomas, 1986) and cysts (Suryanarayana, 1985). The possible role of calmodulin in the initiation and termination of the zoospore phase was examined using the calmodulin-binding drug trifluoperazine.

METHODS

Cyst preparations. Cysts of *Achlya heterosexalis*, ATCC 52875, were prepared as described by Thomas (1989); such cysts tend to release zoospores rather than to germinate by means of a germ tube. For most experiments, cysts were induced in the presence of 5×10^{-4} M- CaCl_2 ; those induced in deionized water are termed DW cysts. Cysts

were used on the day of release or stored at 5 °C for not more than 2 d. In order to remove the spent induction medium and to control the pH, the suspension was centrifuged at 2250 r.p.m. for 4 min. Cysts were washed and resuspended in 10^{-3} M-MOPS [3-(*N*-morpholino) propanesulphonate] buffer, pH 6.6.

Zoospore emergence. Each treatment usually involved 2.4 ml MOPS suspension of cysts in a 35×10 mm Petri dish. The suspension was made up to a total volume of 3 ml using MOPS buffer with or without additives, and maintained at room temperature without shaking. Cysts were scored by microscopic observations at a magnification of $100\times$. Initial cyst numbers ranged from 3 to 4×10^4 spores ml^{-1} . Cysts were counted after they had settled to the bottom of the Petri dish; only full cysts, which are readily distinguished from empty ones, were counted. The diameter of the microscope field, 1064 μm , was established using a calibrated ocular micrometer. Each count represents the mean of five randomly selected fields. Treatments were in duplicate and experiments were repeated at least once. In experiments examining the effects of trifluoperazine on zoospore emergence from MOPS-washed cysts, concentrations tested were 0, 1.25, 2.5, 5 and 10 μM . To test for reversibility of inhibition of emergence by trifluoperazine, following a 3 h treatment, the cysts were washed and resuspended in fresh MOPS, while parallel unwashed treatments served as controls.

Zoospore motion and encystment. Zoospores were obtained by allowing cysts, unwashed, or washed and resuspended in MOPS, to remain at room temperature for 1–2 h. Motility tests were conducted in 35×10 mm Petri dishes, with sequential counts of zoospores at the depth of the suspension which showed maximal zoospore activity. The effects of trifluoperazine on zoospores were tested at the concentrations mentioned previously. In fields with high zoospore activity, counts were made on one or two quadrants of the field. Spore preparations stained with 0.01% Calcofluor White M2R (Haigler *et al.*, 1980) were examined for cyst wall morphology using an Olympus BH fluorescent microscope with a BG12 barrier filter. Encystment was confirmed by testing the smooth, rounded cells for resistance to hydrolysis in 0.3 M-NaOH, based on the method of Tokunaga & Bartnicki-Garcia (1971). For video analysis a 3 ml zoospore suspension obtained from DW cysts in a 35×10 mm Petri dish was gently decanted into a fresh Petri dish in order to minimize the number of residual cysts, which tended to settle to the bottom of the dish. A pipette was used to reduce the volume to 2 ml. Motility was recorded using a $10\times$ objective of a Kyowa Medilux 12 trinocular microscope, a Panasonic WV-1500 television camera and a Panasonic AG-6050 time-lapse video cassette recorder. Randomly selected zoospores were tracked using tracings from the monitor onto acetate transparencies. Distances on the monitor field were calibrated using a stage micrometer. Each zoospore was tracked for at least 1 s, represented by 60 frames, and its positions plotted at 5- or 15-frame intervals. Zoospore speed is the mean obtained from five or more tracks.

RESULTS

Zoospore emergence from cysts

The concentrations of the salts required to obtain at least 90% inhibition of emergence were 10^{-6} M- LaCl_3 , 10^{-3} M- KCl , and 10^{-2} M- NaCl , MgCl_2 or CaCl_2 . Ca^{2+} at 10^{-3} M counteracted the inhibitory effects of K^+ and Na^+ but not that of La^{3+} at the concentrations tested. Mg^{2+} resembled Ca^{2+} in its ability to counteract the inhibitory effects of K^+ on zoospore emergence. The interaction between K^+ and Ca^{2+} , with the latter counteracting K^+ inhibition of zoospore emergence, is shown in Fig. 1. Complete inhibition of emergence by the anti-calmodulin drug trifluoperazine was obtained at 5 μM , a concentration much lower than the 50 μM required in the earlier study by Suryanarayana (1985). Differences from the present study were the use of an *Achlya ambisexualis* isolate, and unwashed cysts in the original CaCl_2 induction medium. As in the earlier study, the complete inhibition of zoospore release was partially reversed after removal of the trifluoperazine; after 3 h in trifluoperazine (at 10 and 5 μM) and a subsequent 6 h with or without a single wash in MOPS, the wash treatments contained 5.4 ± 0.9 (mean $\pm$ SD) and 4.9 ± 0.9 zoospores per field, and the unwashed treatments, no zoospores. Untreated controls had 6.1 ± 0.4 zoospores per field.

Zoospore motility and encystment

The effects on zoospore motility of a number of cations, all applied as chloride salts, were comparable to those described above for the zoospore emergence studies. Treatments which had inhibited emergence also inhibited zoospore motility, usually by inducing encystment. Concentrations of the salts yielding at least 90% encystment were 10^{-6} M- LaCl_3 , 10^{-3} M- KCl , and 10^{-2} M- NaCl or CaCl_2 . At 10^{-3} M, CaCl_2 counteracted the encystment-inducing effects of K^+ but not that of La^{3+} at the concentrations tested. The duration of motility in zoospore preparations was enhanced if the cysts had been washed and resuspended in MOPS buffer. For

Fig. 1. Effects of $\text{Ca}^{2+}/\text{K}^{+}$ interactions on zoospore emergence from cysts: (a) effect of [KCl] on excystment in the absence (O) and presence (●) of 10^{-3} M- CaCl_2 ; (b) effect of [CaCl₂] on excystment in the presence of 10^{-3} M-KCl. ▲, Effect of 6×10^{-4} M- CaCl_2 alone. Each point represents the mean of duplicates.

Fig. 2. Effects of $\text{Ca}^{2+}/\text{K}^{+}$ interactions, both at 10^{-3} M, on zoospore motion. Initial speed (—), speed after adding CaCl_2 alone (▲), after adding KCl (O) and after adding CaCl_2 subsequent to KCl (●). Each speed represents the mean of five measurements. The vertical bars, representing $2 \times$ the standard deviation of the means, are omitted when they fall within the marker symbols.

example, unwashed and MOPS-resuspended preparations after 21 h showed 27% and 81%, respectively, of their initial motility. In the original sporulation induction medium containing CaCl_2 at 5×10^{-4} M, added K^{+} at 10^{-3} M extended the duration of zoospore motility. Without K^{+} , zoospore activity was lost by 24 h; with K^{+} , zoospores remained active at 48 h. When MOPS-washed cyst preparations were used, no sustaining effect of K^{+} on zoospore motility in the presence of Ca^{2+} was observed. Analysis of video recordings revealed short-term responses of zoospores to K^{+} and Ca^{2+} . K^{+} rapidly decreased zoospore speed; subsequent addition of Ca^{2+} within 2 min reversed the inhibition, but delayed application of Ca^{2+} was ineffective (Fig. 2).

Fig. 3. Examples of zoospore tracks over a 1 s period: (a) initial, (b) after adding KCl to 10^{-3} M and (c) after subsequent addition of CaCl_2 to 10^{-3} M.

The tracks of the zoospores were strongly influenced by the ions (Fig. 3). Untreated zoospores maintained a constant course, their tracks approximately linear; K^+ -treated spores moved slowly with frequent changes in direction, typically moving in circles. The relationship between encystment-promoting treatments and germination was not examined in this study. Trifluoperazine at $5 \mu\text{M}$ caused an almost immediate loss of motility in zoospores. Cell shape as observed using conventional light microscopy or Calcofluor fluorescence appeared irregular; however the cells appeared to be resistant to hydrolysis by NaOH, suggesting that encystment had occurred.

DISCUSSION

Ionic conditions inhibiting emergence and those inhibiting zoospore motility (by promoting encystment) are similar, consistent with a common mechanism. If the fresh cyst can be considered to contain a zoospore primordium, then similarities between treatments that inhibit zoospore emergence and those that induce zoospore encystment may be expected on the grounds that both types of treatment may affect similar aspects of zoospore biology. One possibility is that ions which inhibit emergence may induce an encystment-like response of a zoospore initial within the cyst itself. Although the present results are restricted to the zoospore phase, and do not deal with the development of spores subsequent to encystment, we observed that K^+ -induced cysts tend to produce germ tubes rather than zoospores.

Although Ca^{2+} was a relatively weak inhibitor of both zoospore emergence and zoospore mobility, the inhibitory effects of low concentrations of the anti-calmodulin agent, trifluoperazine, and of La^{3+} on emergence and on mobility, and the antagonism between Ca^{2+} and other ions during both processes are consistent with crucial (Thiel *et al.*, 1988) roles of Ca^{2+} in the development of *Achlya*. Apparently distinct swimming modes, 'linear' promoted by Ca^{2+} and circular induced by K^+ , suggest effects of these ions on a zoospore steering mechanism.

Ca^{2+} has been implicated in the regulation of flagellar and ciliary motility of several organisms (Schmidt & Eckert, 1976). Interestingly, the circular swimming mode of secondary zoospores about to encyst resembles the motion described as characteristic of primary zoospores of Saprolegniales (Hickman & Ho, 1966).

The interaction Ca^{2+} and K^{+} in *Achlya* bears some resemblance to the $\text{Ca}^{2+}/\text{Na}^{+}$ interaction in *Phytophthora*, which has been interpreted by Grant *et al.* (1986) in terms of a Ca^{2+} -gated Na^{+} channel. While their work, consistent with that of Tsien *et al.* (1987), shows a strong Ca^{2+} response at 1/100th the Na^{+} concentration, the interaction in *Achlya* occurs at approximately equal osmolarities. The critical events in the life of a zoospore emphasize the importance of osmotic regulation. Excystment, like spore release from sporangia (Money & Webster, 1988) is inhibited by the non-permeant osmotic agent polyethylene glycol-4000 (D. L. Butler & D. Laviolette, unpublished). Zoospore motility is terminated by swelling and encystment, in which osmoregulation plays a critical role (Grant *et al.*, 1986).

The zoosporic phase is a vulnerable one, during which growth is suspended; while chemotaxis may increase proximity to a suitable substrate, prolonged motility will deplete energy reserves. Successful termination of this phase in nature would probably involve adhesion to, and encystment on a potential nutrient substrate (Nicholson, 1984). Bartnicki-Garcia & Sing (1987) have demonstrated the critical importance of timing in determining whether encystment will occur with or without adhesion. Termination of the motile phase may be induced by ions diffusing from a potential host (Svensson & Unestam, 1975) or substrate. The colonization of wounded fish by certain Oomycetes including *Achlya* sp. (Scott & O'Bier, 1962) may be initiated by encystment induced by blood plasma ions, which can be present at concentrations exceeding 200 mM (Forster & Berglund, 1953). Evidence for a distinction between chemotactic and encystment-inducing signals (Fischer & Werner, 1958; Royle & Hickman, 1964) suggests a means of decreasing premature encystment, and thus increasing success in substrate colonization.

A convenient method (Thomas, 1989) for raising synchronous populations of zoospores capable of sustained motility shows that oomycetous zoospore development although less studied (Soderhall & Cerenius, 1987) is not inherently less tractable (Grant *et al.*, 1984) than that of the chytrid *Blastocladiella emersonii* which has been the subject of intensive analysis (Soll & Sonneborn, 1969; Lovett, 1975; Caldwell *et al.*, 1986).

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