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Ionic Control of Germination of *Blastocladiella emersonii* Zoospores

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Encystment and germination of *Blastocladiella emersonii* zoospores involve a rapid and radical transformation of the motile but nongrowing spore into a sessile, growing germling. Certain inorganic ions, notably 50 mM KCl, are efficient inducers of germination. By use of the carbocyanine dye DiO-C_6-(3), we found that KCl depolarizes the plasma membrane of zoospores and noted good correlation between depolarization and subsequent germination. Zoospores avidly accumulated K^+ ions from the medium, attaining an internal concentration of over 50 mM and a concentration gradient of 2,500. Sodium ions, by contrast, were expelled. Internal K^+ was required for normal germination but its function is not known. Zoospores also took up considerable amounts of calcium; most of this was associated with the external surface and appeared to be necessary for maintenance of zoospore integrity. KCl (50 mM) and other salts displaced surface calcium but this was not in itself sufficient to induce germination. The calcium ionophore A23187, in the presence of external calcium, was an effective inducer of germination, suggesting a possible role for cytosolic calcium in triggering the transformation. We propose that the first step in the induction of germination by salts is depolarization of the plasma membrane; subsequent events require the intervention of cytoplasmic signals.

Inorganic ions have been reported to induce or initiate a wide variety of developmental phenomena. A partial list of examples includes the induction of cell differentiation in embryonic frog tissue (4), crystalline lens (36), and bean cotyledons (45) by appropriate concentrations of K^+ and Na^+, as well as by the interplay of K^+ and ecdysone in inducing puffing of insect salivary gland chromosomes (24, 50). Quiescent mammalian cells in culture resume growth in response to agents that stimulate Na^+ influx and activate the Na^+, K^+-ATPase (23, 34). Certain cellular responses to virus infection may also be mediated through changes in cellular levels of K^+ or Na^+ (9). The maturation of amphibian oocytes (5, 32) and early events in the fertilization of sea urchin eggs (12, 31) provide additional examples of developmental processes that are under ionic control. On a different level of inquiry, Jaffe et al., Nuccitelli and Jaffe, and Robinson and Jaffe have shown that ion currents localize outgrowth in embryos of the brown alga *Fucus* (22, 29, 33) and in germinating pollen grains (49). Summarizing data from many sources, it appears that ion movements may affect development at two distinct levels: in the localization of growth in space, and as signals or triggers (for reviews, see references 17 and 21).

In the search for a relatively simple eucaryotic organism to serve as a vehicle for studies on the role of ion fluxes in development, we turned to the aquatic phycomycete *Blastocladiella emersonii*, whose life cycle consists of an alternation between two distinct phases, the sessile vegetative cell and the motile but nongrowing zoospore; recent reviews by Lovett (27) and by Cantino and Mills (8) summarize the available knowledge. Our point of departure is the discovery by Soll and Sonneborn (44) that zoospores can be induced to encyst and germinate with a high degree of synchrony by the addition of KCl and certain other salts. Zoospores are highly differentiated cells that swim but neither grow nor synthesize proteins (16, 27, 42, 43). Germination involves a radical architectural rearrangement which includes the de novo synthesis of a chitinous cell wall, retraction and disassembly of the flagellum, fragmentation of the single mitochondrion, and dispersal of the ribosomes from the nuclear cap throughout the cytoplasm (8, 27). Most of these changes occur within 1 min in an individual zoospore (27). A key event in germination, and one of the earliest known, is the vesiculation of the gamma particles; these organelles contain chitin synthetase in a cryptic form, and their fragmentation initiates wall synthesis

(8). The final step in the process, the emergence of the germ tube, is the only one that requires the formation of new proteins (16, 27, 39, 40, 42, 43).

The experiments described here were begun to learn how KCl and other salts initiate encystment, but grew into a broader effort to understand the role of inorganic ions, particularly K^+ and Ca^{2+}, in zoospore physiology.

MATERIALS AND METHODS

Production of zoospores. *B. emersonii* strain L17 was a gift from David Sonneborn and was maintained in the form of resistant sporangia as described by Lovett (26). Vegetative cells were grown either on the complex medium PYG (1.25 g of Difco peptone, 1.25 g of Difco yeast extract, and 3.0 g of glucose per liter, adjusted to pH 7.0 with KOH [26]); or on the defined medium DM2 (35, 40) (modified as suggested by D. L. Sonneborn).

Zoospores were produced by standard methods (26, 40), slightly modified. To produce large amounts of zoospores, organisms were grown in Roux bottles containing 150 ml of PYG agar; zoospores were released by flooding with water. The suspension was then filtered to remove vegetative cells, and the zoospores were collected by brief centrifugation at 480 × *g*. The supernatant was removed by aspiration, and the pellet was suspended by gentle swirling in water or buffer. Zoospores suspended in CaMOPS buffer (consisting of 2 mM Na^+ MOPS [morpholinopropanesulfonic acid], 0.2 mM $CaCl_2$, pH 7.0) were often nonmotile at first but generally recovered within 15 min and remained reasonably stable for 1 to 2 h. They encysted and germinated readily in response to KCl, but with poor synchrony.

Synchronized zoospores were produced on defined medium as described by Soll and Sonneborn (40, 44). Briefly, the cells were grown in tissue culture dishes at 23 to 24°C, on medium DM2; after 17 h the growth medium was replaced with "sporulation solution" (1 mM Tris maleate, 0.2 mM $CaCl_2$, pH 6.8), and incubation was continued until most of the sporangia had released zoospores (usually 4 to 5 h). In some experiments sporulation solution was prepared with 50 μM $CaCl_2$. Zoospores were either used directly or collected by centrifugation.

Germination was routinely assayed by the dish procedure of Soll and Sonneborn (40) and scored for the three basic cell types: zoospores, round cells, and germlings.

Transport experiments. Zoospores (PYG or DM2) were suspended in buffers as indicated in the various experiments, usually CaMOPS, at densities ranging from 10^6 to 10^7 spores per ml (10^7 zoospores = 0.28 mg, dry weight, in our hands). Cell density was determined either by hemocytometer counts or turbidimetrically at 600 nm. Suspensions (3 ml) were incubated in silane-coated glass tubes immersed in a water bath at 24°C, with continuous magnetic stirring. Radioisotopes were added as indicated; cell samples were collected by filtration through membrane filters (0.45-μm pore size; Millipore Corp.) and washed immediately with water or buffer.

Experiments involving the use of ion-selective electrodes were carried out at room temperature in silane-coated beakers. A few experiments involved the use of a pH-stat.

Analytical techniques. Radioisotopes were counted by standard methods, using either a gas-flow or a scintillation counter. Ion-selective electrodes (Orion) were calibrated by the instructions of the manufacturer and were used with an Orion 801 Ionalyzer.

Internal water space was estimated with [^{3}H]H_2O and [^{14}C]inulin. The cytoplasmic pH was estimated from the distribution of [^{14}C]benzoate (3 μM) between cells and medium (28). The calculation is based on the assumption, well supported for bacteria, that benzoic acid penetrates passively across the plasma membrane whereas the benzoate anion is impermeant; benzoate distribution should then be a measure of the difference between the internal pH and that of the medium.

The membrane potential was monitored by use of the carbocyanine dye DiO-C_6-(3) (28, 48). For best results, freshly harvested zoospores were suspended in CaMOPS–0.1 M sucrose at 2 × 10^6/ml (0.05 mg/ml); dye (freshly dissolved in dimethyl sulfoxide to 0.5 mM) was added to a concentration of 0.5 μM. Fluorescence was monitored with an Aminco Bowman Spectrofluorometer (excitation 470 nm, emission 510 nm).

Materials. Radioisotopes were purchased from New England Nuclear Corp., and reagents were from standard suppliers (usually Sigma Chemical Co.). We acknowledge with thanks gifts of the following reagents: [^{3}H]triphenylmethyl phosphonium and [^{3}H]-tetraphenyl phosphonium (H. R. Kaback, Roche Institute, Nutley, N.J.); A23187 (R. J. Hosley, Lilly Research Laboratories, Indianapolis, Ind.); ionomycin (C.-M. Liu, Hoffman-LaRoche, Nutley, N.J.); phenyldicarbaundecaborane, PCB^-, *closo* form (J. Doi and M. F. Hawthorne, Dept. of Chemistry, University of California at Los Angeles); DiO-C_6-(3) (A. Waggoner, Dept. of Chemistry, Amherst College, Amherst, Mass.). All ionophores and other reagents were dissolved in dimethyl sulfoxide, which is itself innocuous at concentrations up to 1 mM.

RESULTS

Induction of zoospore germination by salts. Zoospores are delicate cells, easily damaged by centrifugation and other manipulations. However, when carefully prepared and kept in dilute, buffered $CaCl_2$ (40, 44), they remain healthy and motile for hours. Upon dilution into growth medium (DM2), virtually every zoospore germinates and grows into a vegetative cell; dilution of zoospores into solutions of KCl (25 to 50 mM) induces semisynchronous germination of the population, but growth is arrested at the germling stage.

We have repeated most of the observations made by Soll and Sonneborn (44) in their original study of salt-induced germination, with essentially the same results. Figure 1A illustrates the time course of germination in response to 50 mM KCl, divided for present purposes into encystment (conversion of zoospores into round

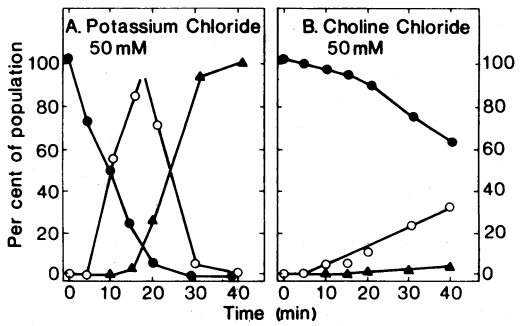

FIG. 1. *Population kinetics of zoospore germination. Zoospores produced on DM2 medium were diluted to a density of* 1.1×10^5 *cells/ml with NaMOPS-*$CaCl_2$ *(2 mM, 0.2 mM). At 0 min either KCl (50 mM; set A) or choline chloride (50 mM; set B) was added, and samples were scored at intervals thereafter. Symbols: ●, zoospores; ○, round cells; ▲, germlings.*

cells) and germination proper, which ends with emergence of the germ tube. The effects of a range of salts are summarized in Table 1. Note that even 1 mM KCl induced considerable germination, though distinctly less than 50 mM KCl. Even brief exposure (2 min) to 50 mM KCl induced complete germination (data not shown). RbCl and NaCl (50 mM) were almost as effective as KCl; CsCl and Tris-chloride were less effective. In our hands, choline chloride induced little encystment and almost no germination (Fig. 1B). Sulfates were as effective as the chloride salts, pointing to the cation as the inducing agent. It should be recalled that the percentages do not describe the rate of germination of individual zoospores but rather the fraction of the population that has been induced (40).

Two salts are plainly anomalous. Ammonium chloride (25 or 50 mM) induced considerable encystment but no germination; its effectiveness was greatly enhanced by KCl, but not by NaCl. LiCl (25 mM) blocked the induction of germination by KCl and by all other inducers as well.

Salts depolarize the plasma membrane. In an early experiment we compared the capacity of various salts to stabilize cyanide-inhibited zoospores against osmotic lysis. We found sucrose and choline chloride to be most effective; KCl and NaCl were much less effective. Evidently the cations that best induce germination are relatively permeant, suggesting that their primary action may be to depolarize the plasma membrane. Our observations are qualitatively consistent with this proposal, but all efforts to determine the potential quantitatively were thwarted by the delicate constitution of the zoospores, which swell or lyse upon exposure to hydrophobic probes and ionophores.

Zoospores accumulate the lipid-soluble cations dibenzyldimethylammonium (DDA^+) and triphenylmethyl phosphonium ($TPMP^+$), but only in the presence of relatively large amounts of the hydrophobic anion phenyldicarbaundecarborane (PCB^-): For example, 1 μM [^{3}H]-DDA^+ and 10 μM PCB^-. The results suggest a membrane potential of the order of −100 mV, interior negative (see reference 28 for discussion of the method), but nonspecific binding and poor reproducibility made us abandon this approach.

More satisfactory evidence that zoospores generate an electrical potential was obtained by use of the fluorescent carbocyanine dye DiO-C_6-(3). Zoospores swell and eventually lyse upon exposure to the dye, even in the dark, but could be temporarily stabilized with 0.1 M sucrose. Zoospores quench dye fluorescence (Fig. 2A), as expected if the interior were electrically negative to the external surface. Addition of salts at 50 mM reversed the quenching, and there was a clear correlation between the reversal of quenching and the capacity to induce germination: K^+, Rb^+, $Cs^+ > Na^+ > Tris^+ >$ choline. Ammonium ion was exceptional, reversing quenching even better than did K^+. Lithium (not shown) was somewhat less effective than sodium. Figure 2B shows the effect of graded amounts of KCl upon the intensity of fluorescence; the potassium ionophore valinomycin had little effect upon the

TABLE 1. *Induction of zoospore germination by salts*[a]

Salt	Concn	% of population at 40 min		
		Zoospores	Round cells	Germlings
None		97	3	0
KCl	1 mM	40	20	40
KCl	50 mM	0	0	100
RbCl	50 mM	0	0	100
CsCl	50 mM	2	25	73
NaCl	50 mM	3	17	80
Tris-chloride	50 mM	25	60	15
Choline chloride	50 mM	63	32	5
NH_4Cl	25 mM	71	28	1
NH_4Cl, KCl	25 mM each	0	23	77
NH_4Cl, NaCl	25 mM each	83	17	0
LiCl	50 mM	100	0	0
LiCl + KCl	25 mM each	100	0	0
K_2SO_4	25 mM	14	7	71
Na_2SO_4	25 mM	12	31	57
$MgCl_2$	10 mM	25	9	66
Polylysine	0.2 mg/ml	84	16	0
Sucrose	0.1 M	31	67	2

[a] Zoospores were produced on defined medium DM2, and germination assays were performed as described in the text. Salts were added at 0 min to the final concentration listed, and the population was scored at 40 min.

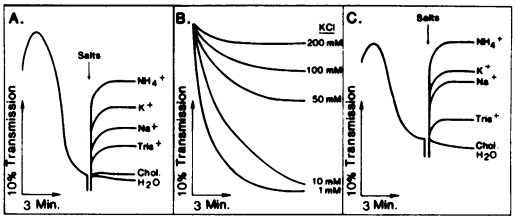

FIG. 2. *Quenching of DiO-C_6-(3) fluorescence by zoospores and its reversal by salts. Zoospores harvested from PYG agar were suspended in CaMOPS buffer plus 0.1 M sucrose, pH 7.0, at 2 × 10^6 cells/ml (60 μg, dry weight, per ml). (A) At 0 min, DiO-C_6-(3) added to 0.5 μM; at 6 min (arrow), chloride salts were injected into the cuvette to a final concentration of 50 mM. Chol., Choline. (B) KCl to the concentrations stated was added 30 s before the dye. (C) Choline chloride (50 mM) was added 90 s before the dye; chloride salts to 50 mM were added at 6 min (arrow).*

pattern, suggesting that the membrane was quite K^+ permeable to begin with. Attempts to quantitate the potential by use of a null method (19) suggest that it exceeds −60 mV but did not give reproducible values.

It may be argued that salts reverse fluorescence quenching by displacing the dye from surface sites, rather than by collapsing a transmembrane electrical potential. In particular, it has recently been found (6) that carbocyanine dyes react with sites that bear calcium, with concomitant quenching of fluorescence that is reversed by KCl. This interpretation is rendered unlikely by the finding (Fig. 2A) that K^+ is more effective in reversing quenching than Na^+ and much more so than choline, because interaction with surface binding sites would not be expected to show specificity for particular ions. This argument is strengthened by the observation (Fig. 2C) that NH_4^+, K^+, and Na^+ showed differential effects even in the presence of 50 mM choline chloride. By contrast, all the salts displaced surface calcium equally well (see Fig. 5).

We interpret our data to indicate that zoospores generate an electrical potential, interior negative, which is depolarized by the addition of KCl and other salts. However, we have not been able to determine what ions generate the potential. Proton conducting uncouplers react with the dye and could therefore not be used. Gramicidin induced rapid K^+ efflux, and presumably rendered the membrane permeable to protons as well, yet did not reverse quenching (data not shown). The origin of the membrane potential, if that is indeed what the dye reports, remains to be discovered.

Potassium accumulation by zoospores. Early in this study we tried to measure the membrane potential by the use of [$^{204}Tl^+$]thallous ion (2). The procedure proved unsuitable because zoospores accept Tl^+ as an analog of K^+, but drew attention to the remarkable capacity of zoospores to accumulate potassium.

Fresh zoospores harvested from either DM2 or PYG medium contain both K^+ and Cl^- corresponding to an intracellular concentration of 25 to 50 mM. To our surprise, zoospores avidly accumulate further K^+ from the medium (Fig. 3A). Zoospores that had been harvested from PYG agar, centrifuged gently, and suspended in CaMOPS buffer first lost part of their K^+ complement but then reaccumulated it, reducing the external K^+ level to below 20 μM; two successive additions of KCl were also absorbed, and then the entire K^+ complement was released by addition of the channel-forming ionophore monazomycin. By use of [^{3}H]H_2O and [^{14}C]inulin, we estimate the water space of zoospores at about 3 μl per mg (dry weight). These zoospores thus attained an internal K^+ concentration of some 65 mM, and a distribution gradient [K^+]i/[K^+]o of 2,600.

Figure 3B shows the accumulation of $^{42}K^+$ by zoospores grown on defined medium; note the initial lag, which reflects the time required for the zoospores to recover from the harvesting procedure. The apparent K_m for $^{42}K^+$ uptake was

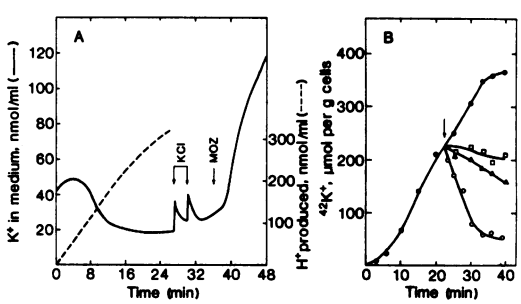

FIG. 3. *Potassium accumulation by zoospores. (A) Zoospores harvested from PYG agar were centrifuged and suspended in Tris maleate-$CaCl_2$ (at 0.25 and 0.20 mM, respectively, pH 7.0) at a density of 0.45 mg (dry weight) per ml. The pH of the suspension was kept constant by automatic addition of Tris base with the aid of a Radiometer pH-stat. Potassium levels in the medium were followed with a K^+ electrode. Potassium leaking out of the zoospores was reaccumulated. Additions of KCl were made at the arrows (20 μM each), followed by monazomycin (MOZ; 2 μg/ml). (B) Zoospores from DM2 medium were centrifuged and suspended in 0.2 mM $CaCl_2$-2 mM MOPS-0.1 M sucrose buffer, pH 7.0, at 2 × 10^6 cells/ml (60 μg, dry weight, per ml). At 0 min, ^{42}KCl was added (54 μM, 380 nCi/ml). After 22 min (arrow), the culture was dispensed into several tubes and additions were made as follows: none (●); 5 mM KCl (○); valinomycin, 0.5 μg/ml (□); tetrachlorosalicylanilide, 0.5 μM (△).*

about 15 μM (data not shown). Addition of nonradioactive KCl (100-fold excess) caused efflux of $^{42}K^+$ by exchange for K^+. The ionophores valinomycin and tetrachlorosalicylanilide blocked $^{42}K^+$ uptake and induced net efflux (in this experiment, the zoospores were protected against gross lysis by 0.1 M sucrose). The finding that K^+ undergoes rapid exchange, and that treatments which subject the zoospores to osmotic, mechanical or metabolic stress induce efflux, suggests that the pool size in the steady state is the result of pump and leak; the point is important because K^+ leakage down its steep concentration gradient may make a substantial contribution to the membrane potential.

How is electroneutrality preserved during K^+ accumulation? Most probably by expulsion of protons generated by metabolism (Fig. 3A). Zoospores respiring in CaMOPS buffer, pH 7.0, maintained an internal pH of about 7.4; addition of KCl (50 mM) raised this to 7.7. If zoospores maintain a pH gradient (interior alkaline) as well as a membrane potential (interior negative), some means for moving protons outward against the electrochemical potential gradient must be present.

Rubidium and thallium were accumulated as avidly as K^+, but the characteristics of sodium uptake were quite different. Zoospores incubated with various concentrations of $^{22}NaCl$ (from 0.5 mM to 10 mM) did accumulate $^{22}Na^+$ (Fig. 4), but in no case did the concentration gradient $[Na^+]i/[Na^+]o$ exceed 20. The initial rate of $^{22}Na^+$ entry was approximately proportional to the external concentration over this range. Upon addition of K^+, the $^{22}Na^+$ was quickly discharged. Sodium uptake, unlike that of K^+, probably serves no specific function but may reflect electrophoretic accumulation in response to the membrane potential.

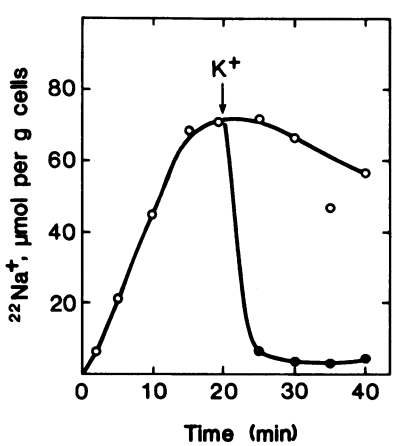

FIG. 4. *Sodium accumulation by zoospores. Zoospores harvested from PYG agar were suspended in CaMOPS buffer (pH 7.0; 2 mM NaMOPS, 0.2 mM $CaCl_2$, corresponding to 1 mM Na^+) at 0.2 μg (dry weight) per ml. At 0 min, $^{22}NaCl$ (○) was added to 600 nCi/ml; KCl at 0.5 mM (●) was added to part of the suspension at the arrow.*

Since chloride is a major ionic constituent of zoospores, its role in K^+ accumulation was examined. Zoospores do accumulate chloride against a concentration gradient, particularly in the presence of potassium. In one experiment, zoospores incubated with 400 μM $^{36}Cl^-$ and 50 μM $^{42}K^+$ attained an internal chloride concentration of about 7 mM (the internal K^+ concentration reached 63 mM). The chloride concentration gradient of 17-fold is consistent with the finding that zoospores from PYG medium (3 mM Cl^-) contain 25 to 50 mM internal chloride. Zoospores may possess some kind of chloride pump, but the gradient it can achieve is obviously too small to account for the accumulation of K^+. Indeed, chloride is not required for K^+ accumulation, since zoospores produced by sporulation in buffered calcium nitrate accumulated potassium (K_2SO_4) perfectly well.

Potassium is required for germination. *B. emersonii* grew well in medium DM2 prepared without sodium salts, and the vegetative cells then sporulated and produced normal zoospores; apparently the organism does not require Na^+, but no attempt was made to exclude sodium ions contributed by the reagents or glassware (estimated to be 10 to 50 μM). By contrast, K^+ must be added to media both for vegetative growth and for zoospore germination.

B. emersonii was grown on a series of media of reduced K^+ content, derived from DM2 by substitution of Na^+ for K^+. At 50 μM K^+ growth was limited, and upon replacement of the medium with sporulation solution, sporulation was delayed. Half of the cells did, however, sporulate within 7 h. The zoospores, albeit motile and apparently normal, were deficient in germination: when challenged with 50 mM NaCl they encysted, but even after 3 h only 10% had produced germ tubes. When challenged with 50 mM KCl instead, the K^+-limited zoospores underwent slow but nearly complete germination. Cells grown in the presence of 10 μM K^+ or less were severely inhibited but many did sporulate when kept in sporulation solution overnight. The zoospores failed to germinate normally with either NaCl or KCl, producing structures with grossly abnormal morphology. The results make it clear that K^+ is required for germination, and perhaps for normal encystment as well.

Calcium and germination. It has long been known that calcium is both necessary and sufficient for sporulation of *B. emersonii* (41) and that low levels of Ca^{2+} enhance the stability of

zoospores (8, 40, 41, 47). In fact, a minimal level of external calcium appears to be indispensable for their integrity, as zoospores became disorganized and lysed within a few minutes when exposed to amounts of ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) sufficient to reduce free calcium to 0.1 μM. Soll and Sonneborn (44) noted in passing that KCl induced "efflux" of $^{45}Ca^{2+}$ from zoospores; this caused us to carry out a series of experiments on the calcium complement of zoospores and its role in germination.

Briefly, zoospores released by cells sporulating in Tris maleate plus 50 μM $^{45}CaCl_2$ contain between 10 and 30 μmol of $^{45}Ca^{2+}$ per g (dry weight). As much as two-thirds of this could be removed by washing zoospores on a membrane filter (0.45-μm pore size; Millipore Corp.) three times with sucrose-$MgCl_2$-$CaCl_2$ (at 0.1 M, 2 mM, and 0.2 mM, respectively). Since this washing procedure did not extract $^{42}K^+$, we conclude that it does not impair the integrity of the zoospores, and therefore, that much of the calcium is bound to the external surface. This inference is confirmed by the observation that low levels of EGTA which did not cause lysis (in this instance, 75 μM EGTA and 50 μM $^{45}CaCl_2$) removed over 80% of the $^{45}Ca^{2+}$ complement from the same zoospores within 2 min. A fraction of the total $^{45}Ca^{2+}$, some 4 to 6 μmol/g (dry weight), was tenaciously retained unless the zoospores lysed. Some of this may well be internal, and it presumably includes the calcium detected in gamma particles by X-ray microanalysis (20).

Having found that zoospores accumulate a variety of univalent cations, we wondered whether calcium is also accumulated. To minimize the contribution of external binding, unlabeled zoospores were suspended in NaMOPS-$MgCl_2$-$CaCl_2$ (at 2, 2, and 0.05 mM, respectively) and allowed to recover motility. At 0 min, $^{45}Ca^{2+}$ (carrier-free) was added, and at intervals thereafter samples were collected by filtration through Unipore filters (Bio-Rad Laboratories), which trap very little external fluid. The zoospores were not washed; trapped external fluid was monitored by inclusion of [^{3}H]sorbitol, and its contribution to the $^{45}Ca^{2+}$ content was subtracted. Zoospores under these conditions quickly took up some 15 μmol of $^{45}Ca^{2+}$ per g (dry weight), but uptake then ceased (Fig. 5A). The radioactive calcium was largely exchanged upon the addition of excess unlabeled calcium, and was quickly removed by 75 μM EGTA (no lysis). Uptake of $^{45}Ca^{2+}$ by zoospores was not diminished when the experiment was performed at 0°C. We conclude that zoospores bind calcium to external sites in an exchangeable form but transport little if any across the plasma membrane.

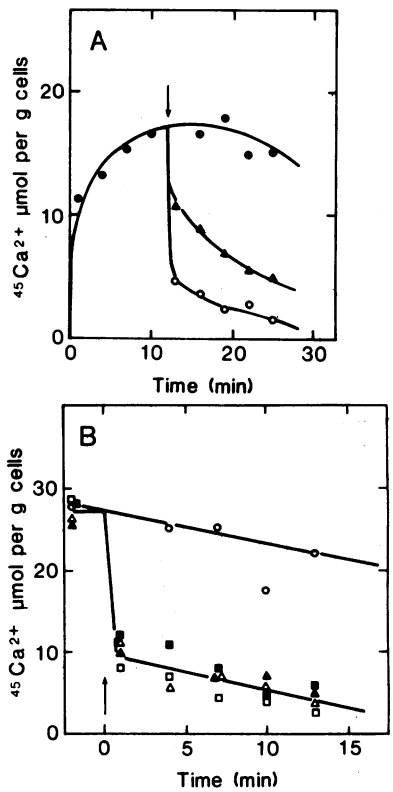

FIG. 5. *Binding and displacement of calcium. (A) Zoospores produced on DM2 were centrifuged and suspended in NaMOPS-$MgCl_2$-$CaCl_2$ (at 2, 2, and 0.05 mM, respectively), pH 7.0, supplemented with 10 μM [^{3}H]sorbitol (50 nCi/ml). Cell density was 1.5 × 10^6/ml (40 μg, dry weight, per ml). Carrier-free $^{45}Ca^{2+}$ (168 nCi/ml) was added at 0 min; at intervals samples were filtered (Unipore polycarbonate filters, 0.4 μm pore size; no wash) and assayed for both $^{45}Ca^{2+}$ and ^{3}H. The $^{45}Ca^{2+}$ associated with the cells was obtained by subtracting the amount trapped in the filter. At 12 min, additions were made to portions of the suspension, as follows: none (●); 2 mM $CaCl_2$ (▲); EGTA, 75 μM (○). (B) Vegetative cells grown in DM2 were allowed to sporulate in Tris maleate-$^{45}CaCl_2$ (at 1 mM and 50 μM, respectively), centrifuged, and suspended at 2 × 10^6 cells/ml in NaMOPS buffer (2 mM, pH 7.0). At 0 min, salts were added to 50 mM as follows: NaCl (□); KCl (△); LiCl (■); choline chloride (▲); and water only (○). Samples were taken, filtered, washed, and counted.*

Addition of 50 mM KCl released considerable $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-labeled zoospores, and so did all the other salts (Fig. 5B); we found no consistent differences between the cations, suggesting that we are observing ionic displacement of calcium from surface sites. Since the cations

differ markedly in their capacity to induce germination (Table 1), it appears that displacement of the bulk of the external calcium is not sufficient to cause germination. Careful titration of zoospores with small amounts of EGTA also induced no more than minimal germination. Nonetheless, we cannot exclude the possibility that KCl and other inducers act by displacing a special, small calcium pool that was not detected by our experiments.

Novel inducers of germination. That calcium may indeed play some special role in germination is suggested by the observation that the calcium ionophore A23187 induced encystment as well as germination of zoospores; the effect was enhanced by external Ca^{2+} (Fig. 6). KCl-induced germination was neither affected by Ca^{2+} nor inhibited by addition of EGTA (data not shown). The new calcium ionophore, ionomycin (25), also worked but was less effective and more toxic than A23187 (not shown). These effects suggest that an increase in the cytosolic calcium level mediated by the ionophores induces germination.

Truesdell and Cantino found some years ago that certain sulfonic acid dyes induce germination, especially when combined with chilling of the zoospore suspension (47). The roster of inducers grew with the addition of caffeine, theophylline, and certain cytokinins (37; P. M. Silverman, personal communication). We have used caffeine (10 mM), triacanthine (6-amino-3-dimethylallyl purine; 10 μM), and 2-methyladenine hemisulfate (10 μM) with good response. However, it appears that these three agents exert their effects in a manner different from that of either the salts or the calcium ionophores. None depolarized the plasma membrane as judged by fluorescence quenching, nor did they displace surface Ca^{2+}. Repeated attempts to detect influx of Ca^{2+} in response to substituted purines (or to 50 mM KCl), either by use of $^{45}Ca^{2+}$ or with a calcium microelectrode, were unsuccessful. We also observed no changes in membrane-associated calcium by the shift in chlorotetracycline fluorescence (10). Lithium chloride, previously found to block salt-induced germination, also blocked the effects of A23187 and of the substituted purines (reference 37, and present results), suggesting that it acts at a relatively late stage of the sequence.

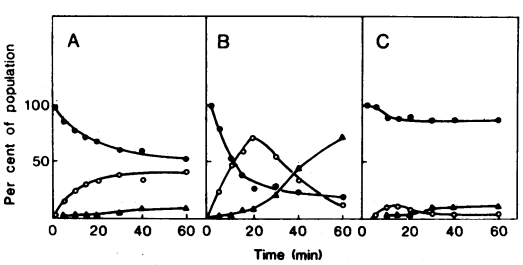

FIG. 6. *Ionophore A23187 plus calcium induce zoospore germination. Vegetative cells grown in DM2 were allowed to sporulate in Tris maleate (1 mM) + 10 μM $CaCl_2$, pH 6.8. They were then diluted 10-fold with 2 mM NaMOPS buffer, pH 7.0, to a density of 1 × 10^5 cells/ml. Three portions were incubated in parallel: (A) A23187 (0.5 μg/ml) added at 0 min; (B) A23187 and 1 mM $CaCl_2$; (C) dimethyl sulfoxide only plus 1 mM $CaCl_2$. Symbols: ●, zoospores; ○, round cells; ▲, germlings.*

DISCUSSION

The zoospore, to paraphrase Cantino, is an ephemeral cell precariously poised between lysis and germination. Physical and mechanical stress, cooling, and inhibitors of energy metabolism make the cells swell and may lead to outright lysis. Another set of physical and chemical perturbations, including dilution, chilling, sulfonic acid dyes, inorganic salts, and a number of purine derivatives (8, 37, 44, 47) trigger zoospores to encyst and germinate. Our objective is eventually to understand both the forces that maintain the exquisite architecture of a zoospore and the cascade of events that transform it into a germling.

The present study was often hampered by the tendency of zoospores to lyse in response to inhibitors of respiration, uncouplers, and ionophores. Lysis is primarily osmotic, because the inhibited zoospores can be stabilized temporarily by impermeant solutes such as sucrose or choline. Evidently, zoospores expend metabolic energy in their continuous battle against osmotic swelling, presumably in the form of ATP produced by oxidative phosphorylation. But it is not at all clear just how water is excluded: unlike zoospores of *Phytophthora* (7), those of *Blastocladiella* have no visible contractile vacuole. Calcium binding presumably helps to stabilize the plasma membrane but it does not appear that this is the process for which metabolic energy is expended. The general question, how does a cell devoid of wall and living in water utilize metabolic energy to evade lysis in the face of a large osmotic gradient, remains unresolved.

The major small osmolite of zoospore cytoplasm is probably K^+, balanced by chloride or metabolic anions or both. We have seen concentrations as high as 0.1 N K^+, although 0.05 to 0.07 N was more usual. Zoospores avidly accumulate K^+ by a process requiring concurrent metabolism, generating concentration gradients as high as 3,000. Neither expulsion of Na^+ nor uptake of Cl^- is obligatory, suggesting that over-

all electroneutrality may be maintained by efflux of protons. In fact, zoospores maintain a cytoplasmic pH equal to, or more alkaline than, that of the medium. The potassium transport system has an apparent K_m of the order of 15 μM but is of fairly broad specificity, accepting Rb^+ and even Tl^+. By contrast, movements of Na^+ are primarily directed outward; only in the absence of K^+ do zoospores accumulate Na^+, and then it is to a small degree (concentration gradient about 20). Thus, the patterns of K^+ and Na^+ movement are quite unlike those of animal cells which possess a tightly coupled Na^+, K^+-ATPase but resemble those of *Neurospora*, yeast, and bacteria (11, 17, 18). Zoospores also accumulate chloride to a limited extent (concentration gradient of 20 or less); the nature of that transport system, like the others, remains to be defined.

What is the functional significance of massive K^+ accumulation by cells so prone to osmotic stress and even lysis? Since pond water contains some 30 μM K^+ (1), accumulation is likely to occur in nature as well as in the laboratory; zoospores produced by cells grown in K^+-deficient medium did not germinate normally unless supplemented with K^+. Several possible functions come to mind. A high level of K^+ is required for protein synthesis, which is necessary for formation of the germ tube (3, 42, 43) and subsequent growth of the germling. Positive turgor may be a factor in maintaining the zoospore configuration, or in the proper deposition of cell wall and construction of a germ tube. Finally, one should not overlook the possibility that outward leakage of K^+, down the steep concentration gradient, may make a major contribution to the membrane potential (negative inside).

Encystment and germination of zoospores can be readily triggered by particular shifts in the ionic environment of zoospores. Soll and Sonneborn (44) reported, and we have confirmed, that 50 mM KCl, RbCl, and NaCl are particularly effective; CsCl (also Tris-chloride) is less so; in our hands choline chloride induced little encystment and no germination. The series K^+, $Rb^+ > Na^+ > Cs^+ >$Tris >choline immediately suggests a relation to membrane permeability, but any mechanism must account for the anomalous position of NH_4^+ and Li^+. Ammonium ion is a fair inducer of encystment but did not elicit germination; KCl enhanced its action, but NaCl did not. By contrast, lithium inhibited induction by K^+, and by nonionic inducers as well (references 37 and 44, and present results).

Soll and Sonneborn (44) considered but rejected the hypothesis that an increase in the external osmotic pressure is a sufficient cause of encystment; we concur, albeit with the reservation that in our hands (Table 1) 0.1 M sucrose did induce considerable encystment and some (variable) germination. Displacement of calcium from the exterior surface is another possibility, particularly in view of the evident role of calcium in stabilizing zoospores (references 8 and 41, and present results). We are inclined to discount this hypothesis because all the univalent cations were equally effective in displacing calcium (Fig. 5B) yet differed markedly in their capacity to induce encystment. However, we cannot exclude the possibility that encystment results from the displacement of a small fraction of the calcium, bound to critical sites, by more specific exchange for particular univalent ions. A third possibility can be derived from the suggestion of Gingell (13) that reduction of the surface potential may trigger developmental events. Addition of univalent salts should help screen surface charges, but this hypothesis offers no explanation for the graded response to different ions; the failure of polylysine to induce germination and the pronounced capacity of Ca^{2+} to stabilize zoospores also argue against this notion.

Instead, we offer the hypothesis that univalent cations depolarize the plasma membrane and that this triggers encystment and germination. In favor of this proposal we can cite the correlation between the capacity of various ions to pass across the plasma membrane and to induce germination. Note, for example, that K^+ induced considerable germination even at 1 mM; sodium ion, less readily accumulated, was effective at higher levels; the relatively impermeant Tris cation was an indifferent inducer; and the most impermeant cation, choline, induced very little germination. More direct evidence comes from the observation (Fig. 2) that zoospores quench the fluorescence of the carbocyanine dye DiO-C_6-(3) and that quenching is reversed by salts in the sequence K^+, Rb^+, $Cs^+ > Na^+ >$Tris$^+ >$choline. Carbocyanine dyes have been widely used to report electrical potentials, interior negative, over the range of -60 to -150 mV (for a review, see reference 48). Unfortunately we were not able to obtain satisfactory quantitative data, nor were we able to determine just how the electrical potential arises. An attractive possibility is the leakage of K^+ down a steep concentration gradient, established by an ATP-linked K^+ pump; however we would not exclude a proton pump, known to occur in *Neurospora* and other fungi (for reviews, see references 11 and 17). Another unresolved question is the anomalous position of NH_4^+ and Li^+. Ammonium ion was the most effective depolarizing ion (Fig. 2B) and appears to be permeant by other criteria as well; we believe that it passes across the membrane but

that germination is aborted by interference with a function of potassium ion. Lithium ions evidently exert their inhibitory effects at a relatively late stage, unconnected with membrane events.

It is not likely that depolarization of the plasma membrane is the immediate stimulus for the release of chitin synthetase vesicles from gamma particles and other early events in encystment. Presumably this involves the intervention of one or more intracellular messengers. Nonionic germination signals, such as caffeine or triacanthine, may modulate the messenger level by a route that does not involve the membrane potential; a surface receptor with affinity for purines may be an element in the way encystment is triggered in nature. The identity of the hypothetical second messenger is, of course, entirely unknown. However, several examples are now known of developmental events being initiated by a sudden increase in the cytosolic free calcium (12, 21, 31). The observation that the calcium ionophores A23187 and (to a lesser extent) ionomycin trigger encystment is suggestive, and calmodulin has recently been detected in zoospores (15). We hasten to add that, aside from the ionophore effects, we obtained no evidence for a specific role of cytosolic calcium. We are mindful of the widespread involvement of cyclic nucleotides in the control of development, and particularly of the finding that in several fungi membrane depolarization is correlated with a burst of cAMP synthesis (30, 46). Although *Blastocladiella* zoospores lose 80% of both cAMP and cGMP during germination (38), an initial transient increase in intracellular cAMP has recently been reported (14). However, too little is presently known to warrant formulation of a specific hypothesis.

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