The Microsporidian Spore Invasion Tube. II. Role of Calcium in the Activation of Invasion Tube Discharge

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ABSTRACT A swelling response by the polaroplast organelle initiated microsporidian invasion tube extrusions by *Glugea hertwigi* spores. The tumescence was induced by the displacement of internal calcium. Sodium citrate, phosphate, and the calcium ionophore A23187 were effective in initiating polaroplast swelling and spore discharge; however, the addition of external $CaCl_2$ switched the expanded polaroplasts to a contracted state and blocked spore discharge. Unlike $CaCl_2$, equivalent concentrations of KCl, NaCl, $MgCl_2$, and $BaCl_2$ did not induced polaroplast contraction, and spore discharge was not blocked. $^{45}CaCl_2$ readily incorporated into spores with expanded polaroplasts; however, little calcium uptake was apparent in spores with contracted polaroplasts. Metallochromic arsenazo III yielded a color spectrum characteristic of the dye-Ca^{++} complex in the polaroplast region; furthermore, a membrane association with calcium was indicated by strong chlorotetracycline fluorescence within the polaroplast; this fluorescence was extinguished by pretreating spores with ionophore A23187. An association of the membrane with calcium was also indicated by a potassium ferrocyanide-osmium tetroxide technique. All evidence indicates that an internal calcium displacement is an important initial step in the swelling response of the polaroplast organelle.

Certain organelles are equipped to extrude (ctenophore colloblast lasso filaments, sperm acrosomes, myxosporidian polar capsules, coelenterate nematocysts, ciliate mucocysts, etc.). An explosive discharge reaction sometimes is exhibited by microsporidian spore cells (6, 8, 13). Microsporidian spores are equipped with an internal extrusion apparatus (EXA) that under certain conditions discharges a long tube with a subsecond velocity suitable for injecting cells (5, 6). The cytoplasm and nucleus of the spore (sporoplasm) pass through a slightly plastic tube into a host cell (14). Several notable studies have provided evidence that the energy produced and expended at the time of microsporidian discharge is created by an increase in osmotic pressure in the EXA (5, 6). Lubbock and Amos (7) provided evidence indicating that internal calcium displacement is the primary activator of jellyfish nematocyst discharge. This work prompted us to look for a similar phenomenon in the activation of invasion tube discharge in microsporidian spores. In this report, we provide evidence that calcium displacement is an important factor in the initiation of microsporidian spore discharge.

MATERIALS AND METHODS

Biological Materials

This work was carried out on *Glugea hertwigi* spores from cysts of the rainbow smelt *Osmerus mordax* collected by the staff of the Lake Erie Fisheries Research Station, Wheatley, Ontario, Canada. The spores were purified by a wash cycle outlined in an earlier paper (14).

Source and Method of Application of Ionophores

Valinomycin, gramicidin A, nigericin, and A23187 were supplied by Eli Lilly and Co., Indianapolis, IN. Ionophore stocks were made up in 100% dimethyl sulfoxide (DMSO). A 0.5 to 10-μl aliquot of ionophore stock was added to each milliliter of spore medium to make a final 1.0–20 μM concentration. No discharge of spores occurred in the presence of DMSO alone. At DMSO concentrations of 5–90% (vol/vol), no discharge occurred, although there was a visible discoloration of spores at higher concentrations. Random samples of 100 spores were scored using phase microscopy to determine whether spore discharge had occurred. The data presented here are representative of a typical experiment; each experiment was repeated at least six times.

Buffers

Carbonate buffer (0.1 M) was made up with sodium bicarbonate; Sorenson's phosphate buffer (0.15 M) was made up with monobasic and dibasic sodium salts. Glycylglycine (0.1 M) and other buffers were adjusted to the appropriate alkaline pH with 1.0 N NaOH.

Experiments with Chlorotetracycline (CTC)

G. hertwigi spores were placed in a medium with 1–5 μl of freshly prepared unbuffered glucose with 2 mM CTC, yielding a final concentration of 100–150 μM CTC. Spores were incubated in CTC for 20–25 min and then washed free of external CTC. Spores were mounted on wet-mount slides and examined for

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fluorescence. Fluorescence was monitored with Leitz epifluorescence optics under oil at × 1,200. CTC fluorescence was induced by broad and blue light excitation from a mercury arc lamp, and emission was limited by a 520-nm barrier filter on the microscope. The cells were photographed on Kodak Tri-X film at 800 ASA. Cells were photographed within 30 s of initial illumination with a blue excitation light (17).

^{45}Ca Incorporation Experiments

Four controlled experiments (A–D) were performed with 10^8 *G. hertwigi* spores each. Spores were thoroughly washed in deionized water before incubation. In experiment A the spores were immersed in 0.2 M cold $CaCl_2$ for 10 min, washed three times in deionized water, and transferred to 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 μCi for 10 min. In experiment B spores were placed in 0.5 M sodium citrate for 10 min, washed three times in deionized water, transferred to cold 0.2 M $CaCl_2$ for 10 min, washed three times in deionized water, and placed in 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 μCi for 10 min. Spores in experiment C were placed in 0.5 M sodium citrate for 10 min, washed three times in deionized water, and transferred to 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 μCi for 10 min. Spores in experiment D were immersed in 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 μCi for 10 min. Spores in experiments A–D were washed thoroughly in deionized water before transferr each pool was transferred onto Millex (Millipore Corp., Bedford Mass.) 0.22-μm disposable filter units. Circular cutouts (4 mm in diameter) of the filters were attached to a cardboard holder, and the spores were counted with a 7230 series radiochromatogram scanner (Packard Instrument Co., Inc., Downers Grove, IL).

Osmium Tetroxide–Potassium Ferrocyanide (OsFeCN) Postfixation

Spores to be treated with ferrocyanide reagent (OsFeCN) were fixed in 1.5% glutaraldehyde in 0.2 M cacodylate containing 5 mM $CaCl_2$ at pH 7.2 (4). Spores were washed in buffer with 5 mM $CaCl_2$ and postfixed in a buffered mix of 1.0% osmium tetroxide and 0.8% potassium ferrocyanide for 2 h. After this treatment, the spores were further stained with 2% aqueous uranyl acetate for 2 h. Control spores were prepared in the same manner but without $CaCl_2$. After fixation, spores were prepared using the standard protocol for transmission electron microscopy described previously (14).

Arsenazo III

Arsenazo III (2,7*bis*(2-arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid) was obtained as a sodium salt from Sigma Chemical Co., St. Louis, MO. Aqueous solutions of arsenazo III (AIII) were made up to 30 mM and adjusted to pH 7.5. AIII was a maroon-red in the absence of calcium and a striking blue color in the presence of calcium (16). Substitution of Mg^{++} or Ba^{++} for Ca^{++} did not produce this blue color shift. *G. hertwigi* spores were immersed in AIII at pH 7–7.2 for 15 min to 24 h. After removal of exogenous AIII, spores were examined with × 40 and × 100 objective lenses with bright-field light optics.

RESULTS

Microsporidian spore morphology is presented as a background to our observations on the role of calcium as an effector of spore discharge. *G. hertwigi* spores are equipped with two noteworthy components: an elaborate SDS-resistant wall and an extrusion apparatus (14). The extrusion apparatus (EXA) has four elements: (*a*) a polar cap, (*b*) a membrane-rich polaroplast, (*c*) a polar tube protein coil, and (*d*) a posterior vacuole (Fig. 1). The polar tube protein (PTP) coli consists of densely packed subunits at the resolution limits of our detection system (0.2–0.3 nm) (Fig. 2). The polaroplast component of the EXA consists of extensive folds of membrane that envelop layers of matrix material (Figs. 1 and 2). The final component of the EXA is the posterior vacuole. This vacuole has accumulations of osmiophilic, flocculent material that resist conventional embedding procedures (13).

Effects of pH, Salts, and Calcium-Affinity Molecules on G. hertwigi Spore Discharge

PH: *G. hertwigi* spore discharge required a 1–2 pH unit shift from neutral to alkaline. Spore discharge was mediated by the buffer present. For example, Sorenson's phosphate buffer (150 mM) effected 80–85% *G. hertwigi* spore discharge when the medium was adjusted from pH 7.0 to 9.5; on the other hand, the spores did not discharge at any pH (test range: 6–11) in 100 mM glycylglycine or 100 mM carbonate buffer.

SALTS: Sodium citrate (50 mM) and sodium phosphate buffer (150 mM) induced *G. hertwigi* spore discharge when added to spores in 100 mM glycylglycine buffer at pH 9.5. Spore discharge was blocked when 50 mM $CaCl_2$ was introduced to the buffer medium with citrate or phosphate. Other salts, such as NaCl, $MgCl_2$, KCl, and $BaCl_2$ (all 50 mM) did not block spore discharge in glycylglycine buffer with citrate or phosphate.

CALCIUM-AFFINITY MOLECULES (CAM): CAM probes were tested on *G. hertwigi* spores in carbonate or glycylglycine buffers. These buffers were used, because spore discharge does not take place in these media at any pH. 10–50 mM CTC induced 5–10% spore discharge at pH 9.5 in 0.1 M carbonate buffer; this rate of discharge was increased to 30% with the addition of 1% procaine. Ionophore A23187 (5–10 μM) induced 95–100% spore discharge at pH 9.5 in 0.1 M carbonate buffer; this discharge rate was diminished to 5% at pH 7.0 (Fig. 3). To test the affects of external calcium on A23187 activity, we used 100 mM glycylglycine buffer as the test medium, because $CaCl_2$ precipitates in carbonate buffer. *G. hertwigi* spores averaged a 45–50% discharge rate in glycylglycine buffer with ionophore A23187 (5–10 μM) at pH 9.5. Little or no spore discharge occurred at pH 7. $CaCl_2$ blocked spore discharge in glycylglycine with 5–10 μM A23187 at pH 9.5 (Fig. 4). The CAM probe, EGTA, had little or no affect on spore discharge. This lack of affect on spore discharge was anticipated, because EGTA does not readily penetrate cells (12). Ionophores, nonactin, gramicidin S, valinomycin, and nigericin, had no affect on *G. hertwigi* spore discharge in carbonate buffer at pH 7 or 9.5.

Action of Ionophore A23187 and Certain Salts on Polaroplast Size

G. hertwigi polaroplasts were condensed in the unprimed spores; however, ocular micrometer readings indicated a 15–25% increase in volume at the time of spore discharge. Ionophore A23187 (5 μM) and 0.5 mM sodium citrate induced polaroplast swelling at pH 7 in glycylglycine buffer (100 mM); the addition of 50–100 mM $CaCl_2$ reversed the expanded polaroplast to a condensed state; conversely, equivalent concentrations of $MgCl_2$, KCl, NaCl, and $BaCl_2$ had no affect on polaroplast size.

^{45}Ca Incorporation into G. hertwigi spores

^{45}Ca uptake was tested, because it was the only cation to reverse the expanded state of the polaroplast to the normal condensed state. Spores were incubated in sodium citrate, because this salt was effective in inducing polaroplast swelling. *G. hertwigi* spores were washed in deionized water before the experiments. Experiment A consisted of 10^8 spores washed in 0.2 M $CaCl_2$ for 10 min, washed in deionized water, and transferred to 0.5 ml of $CaCl_2$ with a specific activity of 12.5 μCi for 10 min. A low level of $^{45}CaCl_2$ incorporation was observed in these spores (Fig. 5). The 10^8 spores in experiment B were incubated in 0.5 M sodium citrate, washed, and transferred to 0.2 M cold $CaCl_2$, washed, and immersed in 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 μCi for 10 min. The results showed a similar low incorporation of $^{45}CaCl_2$ into the

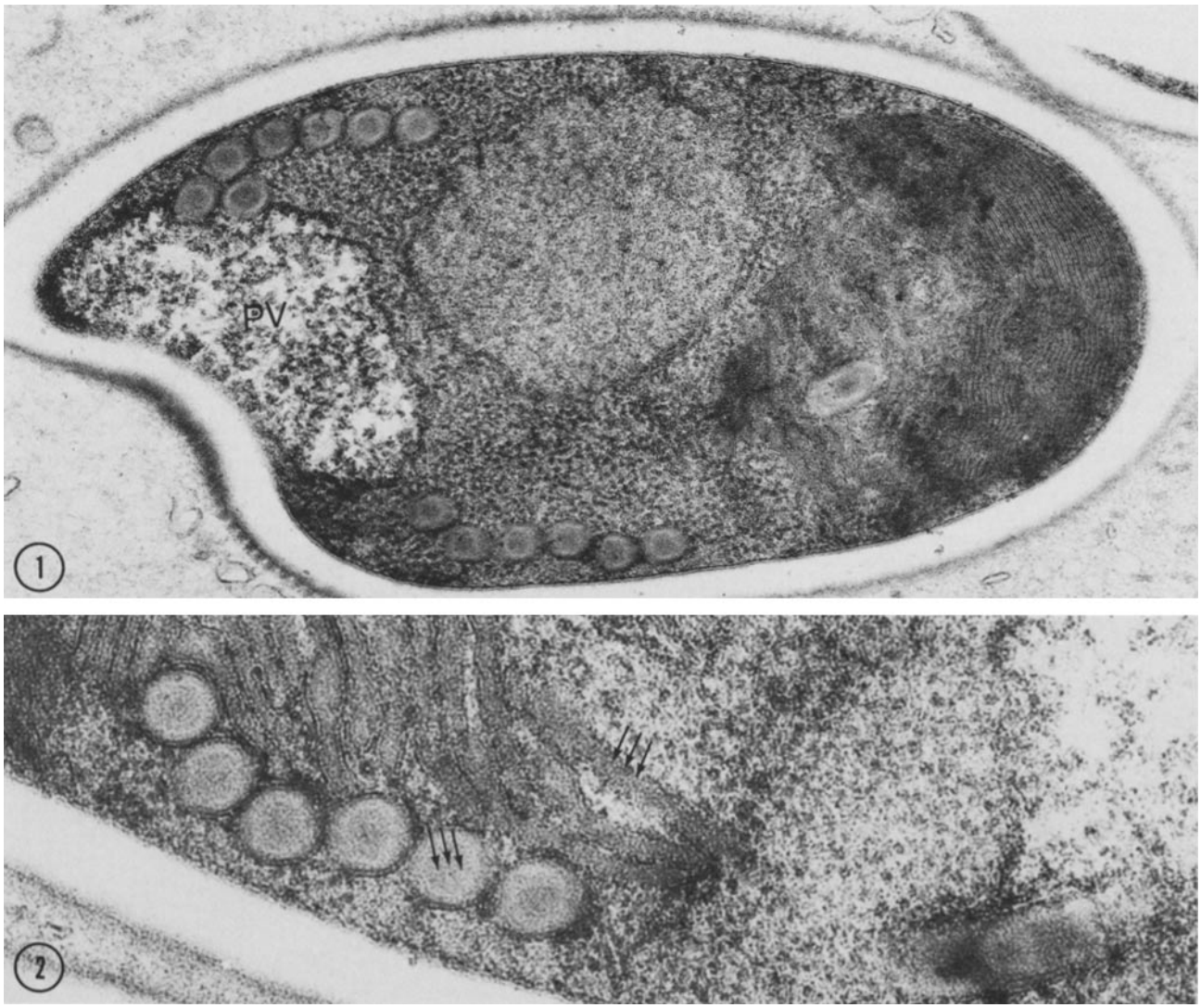

FIGURE 1 Thin section of microsporidian spore exhibiting extrusion apparatus: polar filament and posterior vacuole (*PV*). Note extensive membrane profiles of polaroplast at right end of spore. × 60,000.

FIGURE 2 Higher magnification of polar filament and associated polaroplast in filament (arrows) shows an electron density and particle pattern similar to those of matrix in polaroplast. Posterior vacuole indicated (*PV*). × 130,000.

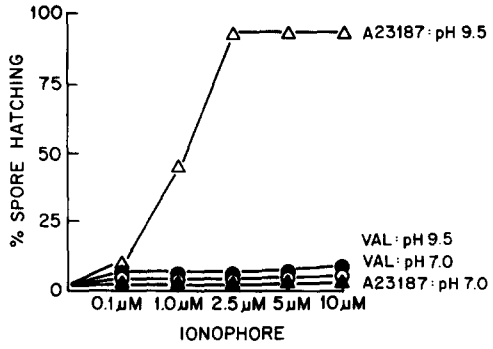

FIGURE 3 Level of *G. hertwigi* spore discharge (hatching) in ionophores A23187 and valinomycin in 100 mM carbonate buffer at pH 7 and 9.5. Note affect of alkaline pH and A23187 on spore discharge.

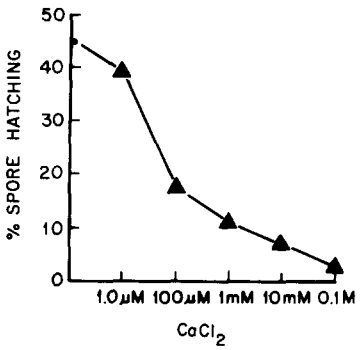

FIGURE 4 Action of $CaCl_2$ on *G. hertwigi* spore discharge in 100 mM glycylglycine buffer with 5 µM ionophore A23187 at pH 9.5.

spores. In experiment C, 10^8 spores were incubated in 0.5 M sodium citrate for 10 min, washed, and transferred to 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 µCi for 10 min. These spores showed a 20-fold increase in the level of ^{45}Ca incorporation (Fig. 5). In experiment D, the spores were placed directly into 0.5 ml of $^{45}CaCl_2$ with a specific activity of 12.5 µCi for 10 min, washed, and counted. The spores took up only a negligible amount of ^{45}Ca from the medium.

CTC Fluorescence in G. hertwigi Spores Treated or Untreated with A23187

G. hertwigi spores, incubated for 15 min in 100 µM CTC,

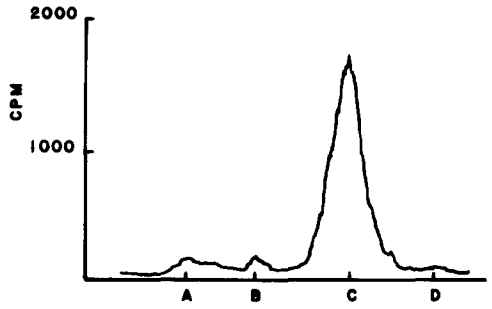

FIGURE 5 Radiochromatogram scan of $^{45}CaCl_2$ incorporation into *G. hertwigi* spores. Sample *A* spores were first pretreated with $CaCl_2$, washed, then incubated in $^{45}CaCl_2$; sample *B* spores were pretreated with sodium citrate, washed, and then incubated in $CaCl_2$ before a final wash and incubation in $^{45}CaCl_2$; sample *C* spores were pretreated with sodium citrate, washed, and then incubated with $^{45}CaCl_2$; sample *D* spores were incubated in $^{45}CaCl_2$.

were examined for calcium-bound (Ca-CTC) fluorescence. Fluorescence was confined to the polaroplast region of the spore (Fig. 6 *a*). This fluorescence was virtually eliminated by pretreating spores with 5 μM ionophore A23187 (Fig. 6 *b*). Fluorometric analysis of spores treated with CTC yielded a greater excitation peak at 530 (indicative of Ca-CTC) than at 520 nm (indicative of Mg-CTC).

Staining of Polaroplast Membrane with OsFeCN in the Presence of Calcium

OsFeCN has been used with some success in staining membranes with known calcium-dependent activities (2, 4). This method applied to *G. hertwigi* spores produced some stain accumulation in and around the membranes of the polaroplast (Fig. 7). When external calcium was omitted from the fixing medium, little or no staining was apparent within the spores.

Reaction of AIII and Ionophore A23187 on Spores

Undischarged *G. hertwigi* spores were incubated in 20 mM AIII at pH 7 for 15 min to 24 h. Incubations were carried out at this pH, because it is the pH at which the stain works best and at which the spores resisted discharge. The dye underwent a striking change from red to blue in the presence of calcium. AIII was selected because it selects Ca^{++} over Mg^{++} by about 50-fold (16) and because the dye forms stable complexes with calcium at neutral pH and the color emission produced by Ca-AIII is easily distinguishable from that of Mg-AIII (16). In experiments on *G. hertwigi* spores, a blue emission indicative of Ca-AIII was concentrated in the polaroplast region of the spore (Fig. 8 *a*); there was a similar color intensity in polaroplasts of spores pretreated with ionophore A23187 (Fig. 8 *b*).

DISCUSSION

This study provides information on the nature of the release mechanism for microsporidian invasion tube discharge. The use of CAM enabled us to analyze the activation of microsporidian spore extrusion. The main events in spore discharge are as follows: (*a*) intraspore polaroplast swelling activated by calcium displacement; (*b*) a polar cap explosion across the spore wall; (*c*) discharge and assembly of the polar tube; and (*d*) extrusion of the spore contents (sporoplasm) through an assembled discharged tube. This study was principally concerned with the initiation of the intraspore organelle swelling response and the activation of spore tube discharge. More information on step *c* is presented in the following paper (15), and step 4 was presented in an earlier work (13).

Four lines of evidence indicate that *G. hertwigi* spore extrusion is activated by the displacement of internal calcium. First, AIII emits a distinct calcium-positive blue emission confined to the spore polaroplast. Second, Ca-CTC fluorescence was concentrated in the membrane-rich polaroplast component of the EXA; furthermore, the substantially reduced Ca-CTC emission in the polaroplast region with A23187 pretreatment indicates a calcium displacement (3). Third, CAM probe A23187 induced polaroplast swelling and spore discharge. The internal calcium pool was likely affected, inasmuch as spore discharge was induced in the absence of external calcium. Finally, sodium citrate induced a rapid polaroplast swelling before spore discharge; the tumescent state was reversed to the native, condensed condition by the addition of calcium. $^{45}CaCl_2$ surged into spores which were pretreated with sodium citrate; however, the calcium incorporation was greatly reduced in spores with polaroplasts in the condensed state.

Polaroplast Swelling, Tube Discharge, and Calcium

The importance of calcium in spore discharge was suspected when (*a*) ionophore A23187 induced polaroplast swelling and spore discharge in the absence of external calcium, (*b*) when A23187-induced spore discharge was blocked upon the addition of external $CaCl_2$, and (*c*) when ionophores with other ionic specificities had little or no affect on polaroplast swelling or spore discharge. Lubbock and Amos (7) have reported that jellyfish nematocysts are activated to discharge by the displacement of internal capsule calcium. They observed that the addition of $CaCl_2$ induced capsule contraction and blocked discharge; however, similar concentrations of salts such as KCl, $MgCl_2$, and NaCl did not induce capsule shrinkage and failed to block capsule discharge stimulated by the addition of citrate to the medium. A perpetual shrink-swell oscillation was effected by *G. hertwigi* polaroplasts spores were transferred from a medium with $CaCl_2$ to one containing citrate and then back to the $CaCl_2$-containing medium. The contraction of the polaroplast in $CaCl_2$ probably was not due to the direct osmotic effects of the salt, because equal concentrations of $MgCl_2$, NaCl, KCl, and $BaCl_2$ did not induce polaroplast contraction. A23187-induced polaroplast swelling is likely due to calcium displacement from membrane; however, this ejection is likely not from the polaroplast since this organelle response to AIII indicator for Ca^{++} before and after A23187 exposure.

The observed difference in Ca-CTC and Ca-AIII responses after ionophore A23187 treatment may be due to differences in the activity of the two probes. CTC reacts to calcium by emitting a strong fluorescence in association with apolar environments such as membrane (3, 16). CTC fluorescence in spore polaroplasts indicated a membrane-associated calcium binding. However, a substantial reduction in the Ca-CTC fluorescence emission in the spores treated with A23187 indicates a loss of calcium from the membrane. The calcium probe AIII produces a blue emission equally well in apolar and polar environments. Therefore, if calcium were released from the polaroplast membrane but were not displaced outside this organelle, it would still be detectable by the AIII method. Our AIII results indicate that calcium was not transported from the polaroplast during the A23187 treatment of *G. hertwigi* spores, since AIII color

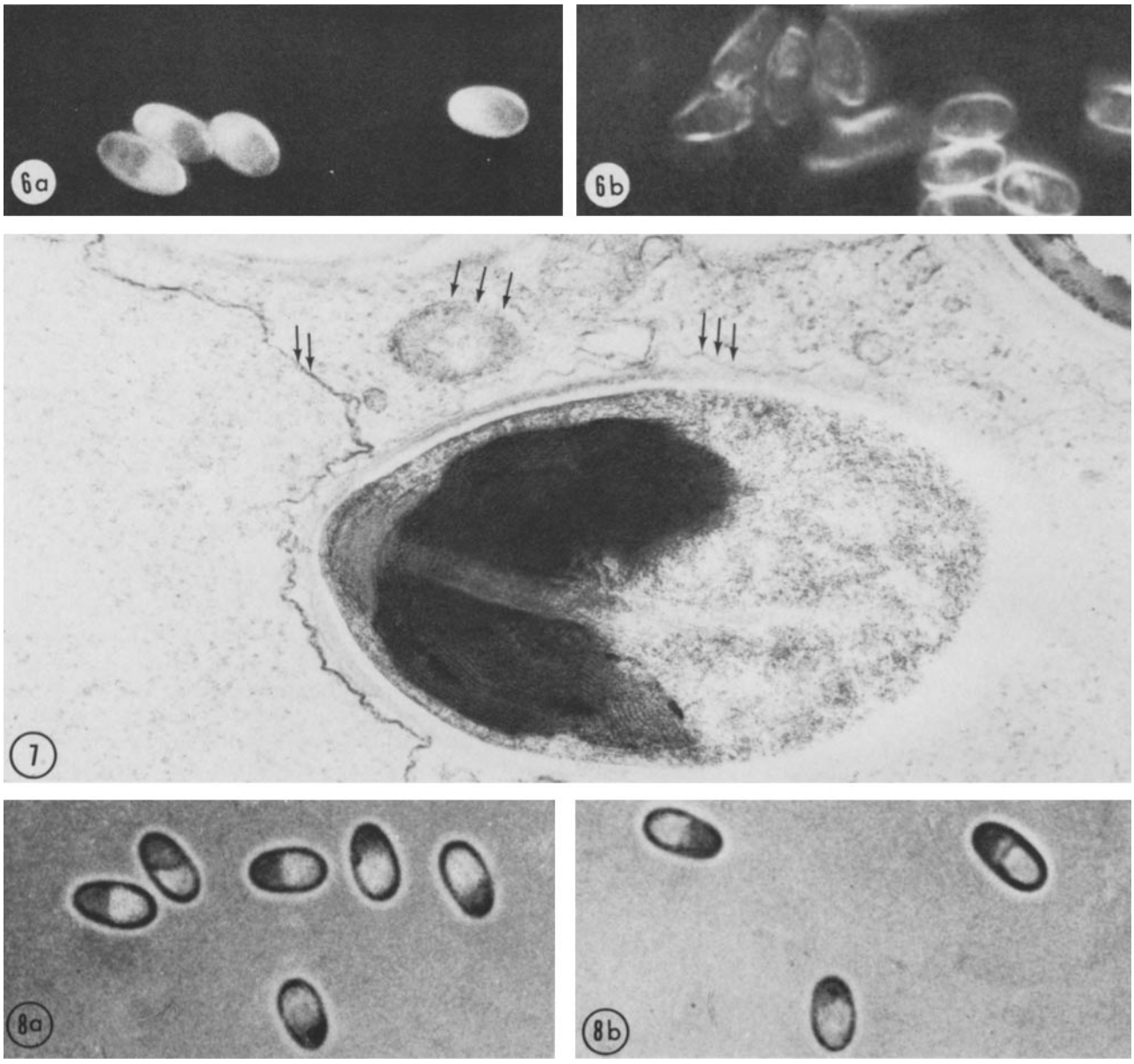

FIGURE 6 *G. hertwigi* spores treated with 100 μM CTC. (*b*) Spores pretreated with ionophore A23187 at pH 7 before CTC incubation. × 4,000.

FIGURE 7 Intracellular vegetative and spore cells of *G. hertwigi* within host tumor cell postfixed with OsFeCN. Stained membrane is apparent on vegetative cell (double arrows); intense stain within polaroplast. Note lack of stain on host cell membranes (triple arrows). × 45,000.

FIGURE 8 (*a*) *G. hertwigi* spores treated with 30 mM AlII for 15 min. AlII dye response restricted to polaroplast region. *G. hertwigi* spores pretreated with 5 μM ionophore A23187 and subsequently exposed to AlII. × 4,000.

emission remained the same in the polaroplasts with or without A23187 treatment.

How Does Calcium Work in Polaroplasts?

Whereas an assortment of different molecular probes with calcium affinity appear to provoke polaroplast expansion (sodium citrate, sodium phosphate, ionophore A23187, CTC), only calcium appears to induce polaroplast contraction. Our CTC fluorescence and OsFeCN findings indicate that there is calcium on the polaroplast membrane when the organelle is in the condensed state. There are three ways in which a calcium association in polaroplasts might induce organelle contraction. First, high calcium binding onto phospholipids might induce contraction directly; this is not a likely mechanism for polaroplast contraction, although certain investigators have demonstrated that membrane components can contract up to 13% with high calcium (9–11). A second possibility is that calcium triggers polaroplast contraction by acting on a submembranous contractile apparatus adjoining the membrane. Such an apparatus has not been identified in the polaroplast; however, such complexes in other systems are known to be particularly sensitive to calcium (1). A third possibility is that a calcium shift occurs from the polaroplast matrix to the membrane and back; when the calcium combines with the matrix, it shifts the order of this component and induces polaroplast swelling. This one

is possible because the polaroplast matrix component closely borders the membrane.

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