The Microsporidian Spore Invasion Tube. IV. Discharge Activation Begins with pH-Triggered Ca^{2+} Influx

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ABSTRACT The microsporidian spore extrusion apparatus activates with a calcium influx from Spraguea Iophii spore wall/plasma membrane; this influx requires preconditioning with an extrasporular shift in medium pH to the alkaline in the presence of the polyanions mucin or polyglutamate. Undischarged S. lophii spores display calcium bound to the wall/plasma membrane with a characteristic calcium-chlorotetracycline fluorescence; this fluorescence declines significantly during spore discharge. S. lophii spores do not discharge when spore wall/plasma membrane calcium is removed with EGTA. Extrasporular mucin or polyglutamate and a pH shift to the alkaline appear to be necessary preconditions for the triggering of the influx of spore wall/plasma membrane-bound \textsuperscript{45}Ca^{2+}. Ionophore A-23187 also effectively activates spore discharge without other extrasporular polyanions. Micromolar concentrations of the calcium antagonists lanthanum or verapamil prevent spore discharge, and micromolar concentrations of calmodulin inhibitors chlorpromazine and trifluoperazine prevent spore discharge. Calmodulin, visualized with a calmodulin antibody and a peroxidase conjugate, is localized particularly on the plasma membrane and the polaroplast membranes of the extrusion apparatus.

Microsporidians are small intracellular parasitic cells with a highly specialized resistant spore stage. When activated, the spore explodes at subsecond velocity and assembles a long, fine discharge tube through which infective sporoplasm is injected into a target cell (9, 11, 12). Under natural conditions, microsporidian spores are activated while they pass along the surface of the intestinal mucosa of the appropriate host animal. Since calcium is recognized as an important second messenger signal that activates many cell events, we thought it would be of some interest to test whether a calcium influx might be in part responsible for the activation of microsporidian spore discharge. In an earlier work, a role for internal calcium was indicated for the polaroplast organelle in association with spore discharge (11). In this investigation, we observe that the spore wall/plasma membrane is equipped with calcium binding sites that can be activated externally to trigger the internal events that lead to discharge.

MATERIALS AND METHODS

Biological Materials: This work done by using Spraguea lophii spores secured from the infections found in the cranial/spinal ganglionic cells of the anglerfish, Lophius americanus. The host animals were collected by the Natural Resources Department of the Marine Biological Laboratory at Woods Hole, Massachusetts. The spores were purified by a wash cycle outlined in an earlier paper (10).

S. lophii Discharge Medium (DM): Standard 0.5 M glycylglycine or 0.5 M carbonate buffers were made up in deionized distilled water. The discharge medium (DM) was adjusted to pH 9.0 with 2.0% mucin (procine, type 1 from Sigma Chemical Co., St. Louis, MO) or 0.5 M poly-D-glutamic acid.

S. lophii Spore Extrusion-inducing Agent, Ionophore A-23187: Ionophore A-23187 (Sigma Chemical Co.) was made up in 100% dimethyl sulfoxide. A 0.5–10 ml aliquot of A-23187 was added to each milliliter of spore medium to make a final concentration of 1–20 μM. We monitored spore discharge incidence by scoring random samples of 100 spores under a phase microscope to determine whether discharge had occurred. The data presented here represent a typical experiment. Each experiment was repeated at least six times.

S. lophii Spore Discharge-Blocking Agents: The agents used are as follows: (a) EGTA. Aliquots of equal numbers of spores and media were placed into a series of tubes that contained different concentrations of EGTA to be tested. After incubations of 10 min. spores were washed (deionized water) and resuspended in DM. (b) Verapamil (Sigma Chemical Co.). Equal numbers of S. lophii spores were placed in a series of tubes that contained concentrations of this calcium antagonist in DM. (c) Lanthanum nitrate (Sigma Chemical Co.). The same protocol as that used in b. (d) The calmodulin antagonists, C-CTC, calcium-chlorotetracycline; CTC, chlorotetracycline; DM, discharge medium; ISD, incidence of spore discharge; TFP, trifluoperazine.
trifluoperazine (TFP) and chlorpromazine (Sigma Chemical Co.). Stock solutions were made up in deionized water. The spores were immersed in a range of concentrations of these antagonists in deionized water for 5 min. After this incubation, spores were immersed in DM and after 5 min, the spores were lightly fixed with glutaraldehyde, and the incidence of discharge was scored.

Reversibility tests were done to determine whether the antagonists had a permanent effect on spore discharge. Spores were first immersed in antagonist medium (in distilled water) for 60 min, thoroughly washed free of antagonist with distilled water, and resuspended in DM. The incidence of spore discharge was then scored.

**Calcium Probe, Chlorotetracycline (CTC):** S. lophii 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 mounted on wet-mount slides and examined for fluorescence. Fluorescence was monitored with Leitz epifluorescence optics with oil at 1,200x (E. Leitz, Inc., Rockleigh, NJ). The CTC fluorescence was induced by light excitation from a mercury lamp, and emission was limited by a 520-nm barrier filter.

**Radiochromatography:** Five controlled experiments (A–E) were performed with 10⁴ S. lophii spores in each. Spores were washed in deionized water before incubations. In experiment A, spores were immersed in 0.2 M cold CaCl₂, washed in deionized water, and transferred to 0.5 ml of ⁴CaCl₂ that had a specific activity of 5.0 µCi for 10 min. In experiment B, spores were placed in 0.15 mM EGTA, washed in deionized water, and immersed with ⁴CaCl₂ that had a specific activity of 5.0 µCi for 10 min. In experiment C, spores were placed in 0.15 mM EGTA, washed in deionized water, and exposed to ⁴CaCl₂ with 0.5 µl of 15 mM ionophore A-23187, but the spores remained undischarged since the medium was at pH 7.0. In experiment D, spores were immersed into medium with polyanions but at neutral pH. Conversely, no spore discharge occurred when spores were washed in alkaline medium without the polyanions but at neutral pH. The pH of 9 induced spore discharge in the presence of ⁴CaCl₂. Experiment E was the same as D except that porcine mucin (0.2%) was used instead of A-23187. The spores from experiments A–E were washed in deionized water and transferred to Millex (Millipore Corp., Bedford, MA) 0.22-µm disposable filters. Circular cutouts (8.0 mm) of the filters were scanned with a 7230 radiochromatogram scanner (Packard Instrument Co., Inc., Downers Grove, IL).

**Immunoperoxidase:** The binding of antibody to calmodulin was detected by using the indirect immunoperoxidase method described elsewhere (4). Spores were fixed for 5 min in 0.12 M cacodylate-buffered 0.5% glutaraldehyde and washed thoroughly. The frozen pellet was cryostat-sectioned at 5-µm thickness. This sectioned spore material was washed in Ringer’s solution and immersed in sheep anti-calmodulin (provided by CAABCO, Inc., Houston, TX) diluted 1:25 in Ringer’s solution and incubated at 30°C for 30 min; cells were washed in Ringer’s solution at 4°C and exposed to rabbit anti-sheep IgG conjugated to peroxidase (provided by Cappel Laboratories, Inc., Cochranville, PA) for 20 min, washed in 0.1 M cacodylate buffer, and fixed in 1.5% glutaraldehyde-buffered cacodylate (pH 7.2) for 45 min. Cells were then washed in 0.1 M cacodylate buffer and exposed to diaminobenzidine working medium (1) for 15 min. The cells were washed in 0.1 M cacodylate buffer, postfixed in 1% osmium in distilled water, and processed for transmission electron microscopy.

**RESULTS**

Microsporidian spore anatomy is presented as a background to our observations on the role of calcium and associated factors in spore discharge activation. The major parts of the spores of S. lophii are the sporoplasm, extrusion apparatus, and spore wall (Fig. 1). The sporoplasm is the infective cell that typically discharges with subsecond velocity after activation of the extrusion apparatus. The extrusion apparatus consists of an aperture, polar filament, membrane-rich polaroplast, and a posterior vacuole (Fig. 1).

**Polyanions and pH on S. lophii Spore Extrusion**

Spores of S. lophii discharged in glycyglycine or carbonate buffer during the switch from neutral (or acid) to alkaline pH in the presence of mucin or polyglutamate (Fig. 2). The polyanions and the shift to the alkaline pH were required protocol for spore discharge. No discharge took place when spores were immersed into medium with polyanions but at neutral pH. Conversely, no spore discharge occurred when spores were placed in alkaline medium without the polyanions polyglutamate or mucin (Fig. 2).

**CTC As a Probe for Membrane-associated Ca²⁺ during Spore Discharge**

Calcium-chlorotetracycline (Ca-CTC) fluorescence was apparent at the spore boundary before extrusion (Fig. 3 a). After discharge, much of this fluorescence was extinguished (Fig. 3, a and b). Spores pretreated with EGTA showed little Ca-CTC fluorescence.

**FIGURE 1** Thin section of S. lophii spore exhibiting membrane-rich polaroplast, polar filament, sporoplasm, and spore wall. x 60,000.
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then transferred for 10 min in 0.07 mM EGTA, there was

roughly in distilled water, incubated in glycylglycine buffer

was kept at 7.0. Polyglutamate or mucin effected a similar

occurred when the EGTA concentration was raised to 0.25

showed a 70% frequency of spore discharge with a pH shift

23187 (but without polyanionic mucin or polyglutamate)

EGTA, incubated in 45CaCl2, and immersed in A-23187 but

spores showed a slight amount of 4SCa2+ uptake (Fig. 6, A). The B spores, immersed

cold CaCl2 and then transferred to 45CaCl2. These spores

showed little 45Ca2+ uptake (Fig. 6, A). The B spores, immersed

in EGTA before 45CaCl2 incubation, showed slight amounts

of 45Ca2+ uptake (Fig. 6, B). The C spores were washed in

EGTA, incubated in 45CaCl2, and immersed in A-23187 but

at a pH of 7 (therefore, the spores remained undischarged).

The C spores showed a slight amount of 45Ca2+ uptake (Fig.

6, C). The D spores were washed in EGTA, incubated in

45CaCl2 and transferred to A-23187 in DM at pH 9. These

spores displayed a high level of discharge and showed a

significant increase in the 45Ca2+ uptake (Fig. 6, D). The E

spores were washed in EGTA, incubated in 45CaCl2, and

induced to discharge with mucin in the extrasporular medium

with a pH of 9. The level of incorporation of 45Ca2+ was

similar to that observed for the D spores (Fig. 6, E).

Radiochromatography of 45Ca2+ Influx into

S. Iophii Spores

Calcium influx into S. lophii spores was monitored by

radiochromatography. The A spores (which were not placed

in DM and thus remained undischarged) were immersed in

cold CaCl2 and then transferred to 45CaCl2. These spores

showed little 45Ca2+ uptake (Fig. 6, A). The B spores, immersed

in EGTA before 45CaCl2 incubation, showed slight amounts

of 45Ca2+ uptake (Fig. 6, B). The C spores were washed in

EGTA, incubated in 45CaCl2, and immersed in A-23187 but

at a pH of 7 (therefore, the spores remained undischarged).

The C spores showed a slight amount of 45Ca2+ uptake (Fig.

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spores displayed a high level of discharge and showed a

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similar to that observed for the D spores (Fig. 6, E).

Calmodulin Inhibitors, Anti-Calmodulin, and

S. lophii Spore Discharge

The calmodulin antagonists, TFP and chlorpromazine,

were used to determine whether extrusion apparatus activa-

FIGURE 2 Incidence of S. lophii spore discharge (ISD) in buffer with polyanions as a function of pH. Spores suspended in glycylglycine buffer with 0.2% mucin (O) or without polyanions (A) at different pH values. Results are reported as percent spore discharge in buffer at given pH.

EGTA and Spore Discharge

When spores were washed in 0.2 M CaCl2, rinsed thor-oughly with distilled water, and immersed in DM (pH 9.0), a

70% frequency of spore discharge took place; however, when

spores were immersed in CaCl2, washed in distilled water, and

then transferred for 10 min in 0.07 mM EGTA, there was

only a 5% incidence of spore discharge. No spore discharge

occurred when the EGTA concentration was raised to 0.25

mM. When the EGTA-treated spores were then washed thor-oughly in distilled water, incubated in glycylglycine buffer

overnight (pH 7.0), exposed to CaCl2, washed with distilled

water, and immersed in DM, there was a return to normal

spore discharge frequency. However, if the EGTA incubation

was at concentrations above 0.15 mM, there was a reduced

capability for spores to regain a discharge capacity.

Activation of S. lophii Spores with

Ionophore A-23187

S. lophii spores in glycylglycine buffer (0.1 M) with A-

23187 (but without polyanionic mucin or polyglutamate)

showed a 70% frequency of spore discharge with a pH shift

of the extrasporular medium to 9.0; however, the incidence

of spore discharge remained near zero if the pH of the medium

was kept at 7.0. Polyglutamate or mucin effected a similar

70% frequency of spore discharge; this discharge frequency

did not increase when A-23187 was added to the medium

with polyglutamate or mucin. S. lophii spores pretreated with

CaCl2, washed in distilled water, and then incubated in 0.15

mM EGTA would not discharge when placed in DM with A-

23187. The calcium channel antagonist verapamil prevented

S. lophii spore extrusion in DM at concentrations as low as

20 μM (Fig. 4); this apparent inhibition was reversed with

verapamil removal (six washes in distilled water), spore

incubation overnight in glycylglycine buffer at pH 7, and the

spore immersion in DM (Fig. 4). Lanthanum, a noted Ca2+

antagonist, prevented S. lophii spore discharge at concentra-

tions of 50 and 100 μM (Fig. 5); this inhibition did not occur

when lanthanum was removed from the extrasporular me-

dium and the spores were transferred to the standard DM

(Fig. 5).

Radiochromatography of 45Ca2+ Influx into

S. lophii Spores

Calcium influx into S. lophii spores was monitored by

radiochromatography. The A spores (which were not placed

in DM and thus remained undischarged) were immersed in

cold CaCl2 and then transferred to 45CaCl2. These spores

showed little 45Ca2+ uptake (Fig. 6, A). The B spores, immersed

in EGTA before 45CaCl2 incubation, showed slight amounts

of 45Ca2+ uptake (Fig. 6, B). The C spores were washed in

EGTA, incubated in 45CaCl2, and immersed in A-23187 but

at a pH of 7 (therefore, the spores remained undischarged).

The C spores showed a slight amount of 45Ca2+ uptake (Fig.

6, C). The D spores were washed in EGTA, incubated in

45CaCl2 and transferred to A-23187 in DM at pH 9. These

spores displayed a high level of discharge and showed a

significant increase in the 45Ca2+ uptake (Fig. 6, D). The E

spores were washed in EGTA, incubated in 45CaCl2, and

induced to discharge with mucin in the extrasporular medium

with a pH of 9. The level of incorporation of 45Ca2+ was

similar to that observed for the D spores (Fig. 6, E).

Calmodulin Inhibitors, Anti-Calmodulin, and

S. lophii Spore Discharge

The calmodulin antagonists, TFP and chlorpromazine,

were used to determine whether extrusion apparatus activa-

FIGURE 3 Views of undischarged and discharged S. lophii spores exposed to 100 μM CTC. (a) Arrow on left indicates undischarged spore with CTC fluorescence confined to internal polaroplast region and spore wall/plasma membrane. Arrow on right shows significantly reduced CTC fluorescence in discharged spore. (b) Arrow on left indicates discharged spore CTC fluorescence. Arrow on right shows extruded sporoplasm. × 1,500.

FIGURE 4 Effects of verapamil on S. lophii ISD. Spores activated to discharge with 0.2% mucin in glycylglycine buffer at pH 9.0 in different verapamil concentrations. Results displayed as percentage of discharged spores compared with control without verapamil (+). G, ISD after ver-

apamil removal from spore me-
dium and subsequent exposure to fresh DM (reversibility test).

FIGURE 5 Effects of lanthanum nitrate on S. lophii ISD. Spores activated to discharge with 0.2% mucin in glycylglycine buffer at pH 9.0 in different verapamil concentrations. Results displayed as percentage of discharged spores compared with controls without verapamil (-). G, ISD after vera-

pamil removal from spore me-
dium and subsequent exposure to fresh DM (reversibility test).
tion was calmodulin-associated. These phenothiazine derivatives significantly reduced the discharge capability of *S. lophii* spores at concentrations of 5–40 μM (Figs. 7 and 8). The phenothiazine-treated spores regained the capacity to discharge after the drug was removed from the extrasporular medium (by thorough washing in distilled water) and the spores were resuspended in fresh DM. However, incubations of the spores in 80 μM chlorpromazine had an obvious permanent negative effect on spore discharge; these spores showed little capacity to regain a discharge capability when the spores were washed and placed in DM (Fig. 8). Anti-calmodulin and a peroxidase conjugate were used to visualize

**FIGURES 6** Radiochromatogram scan of 4SCaCl$_2$ uptake into *S. lophii* spores. The A spores were exposed to CaCl$_2$, washed in deionized distilled water, and incubated in 4SCaCl$_2$; the B spores were treated with EGTA, washed, and incubated in 4SCaCl$_2$; the C spores were treated with EGTA, washed, and incubated in 4SCaCl$_2$ with A-23187 at pH 7; the D spores were pretreated with EGTA, washed, incubated in 4SCaCl$_2$ with glycylglycine buffer with A-23187 at pH 9; and the E spores were pretreated with EGTA, washed, and incubated in 4SCaCl$_2$ with glycylglycine buffer with 0.2% mucin at pH 9. Counts of final wash supernatant for each sample are depicted in a–e.

**FIGURE 7** Effect of trifluoperazine (TFP) on *S. lophii* ISD. Spores immersed in DM with different TFP concentrations. Results report ISD as compared with control samples without TFP (+). [] results of reversibility tests of TFP-inhibited spores washed and transferred to fresh DM without TFP.

**FIGURES 8** Effect of chlorpromazine on *S. lophii* ISD. Spores immersed in DM with different chlorpromazine concentrations. Results report ISD as compared with control samples without chlorpromazine (+). [] results of reversibility tests of chlorpromazine-inhibited spores washed and transferred to fresh discharge medium without chlorpromazine. Note that spores in 80 μM chlorpromazine did not respond well to reversibility test.

the calmodulin within cryostat-sectioned spores. Presumptive anti-calmodulin peroxidase conjugates were mostly found near the membranes of the spore extrusion apparatus (Fig. 9, a and b). Control spores exposed to peroxidase but without anti-calmodulin showed no reaction (Fig. 9 c).

**DISCUSSION**

The results indicate that three extrasporular conditions are required to activate *S. lophii* spore discharge: (a) a pH shift from acid/neutral to the alkaline; (b) the presence of calcium bound to the spore wall/plasma membrane; and (c) the presence of polyanions (such as polyglutamate or mucin). The following evidence indicates that a calcium influx is a primary signal for *S. lophii* spore discharge. First, the calcium chelator EGTA blocked spore discharge;
the CTC fluorescence studies indicate that the EGTA removed the bound Ca\(^{2+}\) from the spore wall/plasma membrane. The work of Wolniak et al. indicates that Ca-CTC fluorescence is clearly distinctive when associated with hydrophobic regions (13). Secondly, A-23187 induced a Ca\(^{2+}\) influx immediately before spore discharge. The following evidence supports Hagiwara and Byerly's criteria for possible calcium channels in S. lophii plasma membranes: (a) The organic channel antagonist verapamil strongly blocked S. lophii spore discharge at concentrations typical for inhibiting calcium channels (2, 5); as expected, the verapamil-treated spores could effectively activate and discharge after the removal of the verapamil. (b) The inorganic channel antagonist lanthanum blocked S. lophii spore discharge. The lanthanum-blocked spores still could be activated and discharged after removal of the lanthanum from the extrasporelum medium. (c) Finally, the phenothiazine inhibitors calmodulin, chlorpromazine, and TFP also blocked spore discharge. These antagonists have a strong affinity for calmodulin (3). The interference caused by the phenothiazines may be due to their hydrophobic affinities; however, it is unclear whether the blocking action is due to a binding to the specific ports in the membrane, or whether the molecules complex directly with the hydrophobic regions on the calmodulin molecule (3, 6).

The anti-calmodulin peroxidase conjugates seem to indicate that calmodulin is in the vicinity of the spore extrusion apparatus membrane. The evidence indicates a good probability for a calcium–calmodulin mediated system involved in the initiation of spore discharge.

Phenothiazines are likely involved in the activation of the calcium influx into S. lophii spores. The sialic acid residues on the mucin molecule may be partly responsible for ejection overflow due to other anionic groups (such as polyglutamate) also affect the triggering of the calcium influx. Indeed, substrate-associated SiO\(_3^-\) groups on the surfaces of glass slides could activate S. lophii spores charged with calcium and shifted to an alkaline pH; this activity was reduced to zero when the slide surface was covered with polylysine. Probably mucin serves as a natural stimulator for S. lophii spore discharge along the mucosal lining of the host animal intestine. The mucin may create an ion exchange phenomenon during the shift of the extrasporelum medium from acid/neutral to alkaline pH. Such a phenomenon fits the Spray et al. model for the triggering of the opening of calcium channels in plasma membranes (8). This model indicates that under low free hydrogen conditions (alkaline), calcium channels are open; however, a shift to a free hydrogen medium (slightly acid) closes the calcium channels. Perhaps the polyanions in the extrasporelum environs create a build-up in the ion potential so that when a pH shift to the alkaline occurs, a sudden influx of calcium with the opening of the channels is permitted. Examples of cationic influxes are believed to be common phenomena along the mucosal surface of the intestine (7).

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