A Cyclic GMP-Dependent K⁺ Channel in the Blastocladiomycete Fungus Blastocladiella emersonii

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Phototaxis in flagellated zoospores of the aquatic fungus Blastocladiella emersonii depends on a novel photosensor, Blastocladiella emersonii GC1 (BeGC1), comprising a type I (microbial) rhodopsin fused to a guanylyl cyclase catalytic domain, that produces the conserved second messenger cyclic GMP (cGMP). The rapid and transient increase in cGMP levels during the exposure of zoospores to green light was shown to be necessary for phototaxis and dependent on both rhodopsin function and guanylyl cyclase activity. It is noteworthy that BeGC1 was localized to the zoospore eyespot apparatus, in agreement with its role in the phototactic response. A putative cyclic nucleotide-gated channel (BeCNG1) was also identified in the genome of the fungus and was implicated in flagellar beating via the action of a specific inhibitor (t-cis-diltiazem) that compromised zoospore motility. Here we show that B. emersonii expresses a K⁺ channel that is activated by cGMP. The use of specific channel inhibitors confirmed the activation of the channel by cGMP and its K⁺ selectivity. These characteristics are consistent with the function of an ion channel encoded by the BeCNG1 gene. Other blastocladiomycete fungi, such as Allomyces macrogynus and Catenaria anguillulae, possess genes encoding a similar K⁺ channel and the rhodopsin–guanylyl cyclase fusion protein, while the genes encoding both these proteins are absent in nonflagellated fungi. The presence of these genes as a pair seems to be an exclusive feature of blastocladiomycete fungi. Taken together, these data demonstrate that the B. emersonii cGMP-activated K⁺ channel is involved in the control of zoospore motility, most probably participating in the cGMP-signaling pathway for the phototactic response of the fungus.

Received 20 May 2015 Accepted 30 June 2015
Accepted manuscript posted online 6 July 2015
Citation Avelar GM, Glaser T, Leonard G, Richards TA, Ulrich H, Gomes SL. 2015. A cyclic GMP-dependent K⁺ channel in the blastocladiomycete fungus Blastocladiella emersonii: Eukaryot Cell 14:958–963. doi:10.1128/EC.00087-15.
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00087-15.
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The importance of intracellular potassium ions for fungi has been extensively documented in the literature. For instance, potassium flux is involved in turgor recovery after hyperosmotic shock (12, 13) and in the regulation of membrane potential (14) in Neurospora crassa, as well as in ascospore discharge and dissemination in Gibberella zeae (15). For B. emersonii, Van Brunt and Harold (16) reported that zoospores avidly accumulate K+ ions from the medium, attaining intracellular concentrations above 50 mM and a concentration gradient ratio of 3,000; in contrast, sodium ions are expelled. These authors also showed that calcium binds to external sites in the zoospores in an exchangeable form but that zoospores transport little if any calcium across their plasma membranes. Although K+ is necessary for zoospore germination, its role is still not well understood (17). It has been suggested that membrane depolarization could trigger B. emersonii encystment and germination (16). In the case of the oomycete Achlya heterosexualis, it has been shown that the cells contain vacuolar potassium concentrations above 100 mM; most probably, potassium is the most abundant cation in this organism. In addition, potassium ions seem to be connected to the movement of Achlya zoospores, apparently stimulating the circular movement of these cells (18). Appiah and coworkers (19) also showed that changes in K+ concentration alter the speed, frequency of changes of direction, trajectory, and encystment of the zoospores of five species of oomycetes.

In the present work, by the use of specific channel inhibitors, we demonstrate changes in membrane potential induced by potassium and by cGMP in B. emersonii zoospores. Taken together, these data indicate that B. emersonii has a cGMP-activated K+ channel participating in the cGMP-signaling pathway that controls the phototactic response of the zoospore of this fungus.

**MATERIALS AND METHODS**

**Cells and growth conditions.** Cultures of B. emersonii were maintained on PYG agar (0.13% peptone, 0.13% yeast extract, 0.3% glucose, and 1% agar) plates. Large quantities of zoospores were obtained by flooding first-generation cultures grown on PYG agar for 16 to 18 h at 23°C. The zoospore suspension was filtered over nylon mesh (pore size, 30 μm) to remove vegetative-cell debris.

**Microfluorometric measurements of alterations in the membrane potential.** Changes in membrane potential were determined by microfluorimetry using the FlexStation III microplate reader and the FLIPR potassium membrane potential assay kit (both from Molecular Devices Corp., Sunnyvale, CA) according to the instructions of the manufacturer. This kit is 10 times faster and more stable than those with traditional dyes and provides good correlation with manual patch clamp assays. The FLIPR potassium membrane potential assay kit detects ion channel modulation by increasing or decreasing the fluorescent signal as the cellular membrane potential changes. The signal increases in intensity during membrane depolarization as dye follows the positively charged ions inside the cell. During hyperpolarization, the fluorescent signal decreases in intensity as dye follows the positively charged ions out of the cell. Briefly, B. emersonii zoospores were collected and were seeded into 96-well black microplates with clear bottoms at a concentration of 5 × 10^6/well in 100 μl of sporulation solution (1 mM Tris-maleate buffer [pH 6.8], 1 mM CaCl2, 1 mM MgCl2) plus a 10-fold dilution of the red dye of the FlexStation membrane potential assay kit (Molecular Devices Corp.) containing 1.8 mM probenecid, in a final volume of 200 μl. The cells were incubated for 60 min at 37°C. The fluorescence of samples was excited at 488 nm, and fluorescence emission was detected by the FLIPR 565 ± 25 nm band-pass emission filter. Samples were measured at 1-s intervals for 120 s, after 30 s of monitoring of the basal fluorescence intensity, as a measure of the membrane potential level of resting cells. A depolarizing agent (60 mM KCl and either 3 mM cGMP or 3 mM CAMP) was added to the cells in the presence or absence of the potassium channel inhibitor tetraethyl ammonium (TEA) chloride at 10 μM or the CNG channel inhibitor L-cis-diltiazem at 10 μM. The responses to agent addition were calculated as the peak fluorescence minus the basal level. Fluorescence intensity was determined using SoftMaxPro software (Molecular Devices Corp.). Data were expressed as mean values ± standard errors (SE).

**Measurement of potassium currents.** A total of 5 × 10^6 zoospores were loaded with 10 μM potassium-binding benzofuran isophthalate acetoxymethyl ester (PBFI-AM; Molecular Probes) for 60 min at 37°C in sporulation solution (1 mM Tris-maleate buffer [pH 6.8], 1 mM CaCl2, 1 mM MgCl2) containing 1.8 mM probenecid and 0.06% Pluronic F-127 (Sigma-Aldrich), a nonionic surfactant. After loading with PBFI-AM, the cells were washed with sporulation solution and were incubated with 0.1% low-melting-point agarose in sporulation solution. K+ imaging was performed by using the Eclipse Ti-S inverted research microscope (Nikon, Melville, NY) equipped with a 14-bit high-resolution charge-coupled device (CCD) camera (CoolSNAP HQ2; Photometrics, Tucson, AZ), and the images were analyzed with NIS-Elements software (Nikon) using image acquisition rates of two frames per second. Dye fluorescence was excited with a xenon lamp at 340 nm or 380 nm, and the light emitted was detected using a band-pass filter at 515 to 530 nm. Intracellular potassium influx was monitored in cells stimulated with either 60 mM KCl–3 mM cGMP or 60 mM KCl–3 mM CAMP in the presence or absence of the potassium channel inhibitor TEA at 10 μM. About 20 cells were analyzed for each independent experiment. The mean variation between the ratios of the fluorescence intensities obtained by exciting PBFI at these wavelengths (ratio of fluorescence at 340 nm to fluorescence at 380 nm) was then used to determine the concentrations of K+ during the stimulus (F) and the resting state (Fo), normalized to its basal fluorescence.

**Phylogenetic analysis of the BeCNG1 amino acid sequence.** To investigate the evolutionary ancestry of the BeCNG1 protein, we calculated a phylogeny. Primarily we used the BeCNG1 amino acid sequence (GenBank accession number KF309500 for the mRNA and AIC07008 for the protein) as a seed sequence for a custom bioinformatic pipeline for generating phylogenies (20). This pipeline initially uses a BLASTp search to recover a set of amino acid sequences (with the gathering threshold set to 1e−10) from a local database of published and publicly available genome databases (see Table S1 in the supplemental material) (21). The sequences retrieved were aligned using MAFiT, version 7.03b, and were masked using trimAl (22). Using this output, a preliminary phylogeny was
calculated with FastTree (23). The output tree and alignment were then used as a guide for manual improvement of taxon sampling, sequence alignment, and alignment masking. Taxon sampling was improved by performing additional BLASTp searches of the NCBI nonredundant (nr) database, the Broad Institute Origins of Multicellularity database and Fungal Genome Initiative database, and the JGI Genome Portal. Long branch sequences and closely related sequences from the same genus groups were removed. The alignments were then analyzed using ProtTest 3 (24) to predict the “best-fitting” sequence substitution model for phylogenetic analysis (see the legend to Fig. S1 in the supplemental material). The ProtTest-predicted parameters were then used—where possible—with the RAxML program (with 100 best-known likelihood [BKL] and 1,000 bootstrap [BS] analyses) under the CAT model and with MrBayes, version 3.2 (until the log likelihood reached a plateau for samples from a minimum of 500,000 generations [sampled every 1,000 generations], with burn-in calculated using Tracer, version 1.5) (http://beast.bio.ed.ac.uk/Tracer).

RESULTS

To investigate BeCNG1 function, changes in membrane potential in zoospores in response to the addition of cGMP were determined. For this purpose, we used a membrane potential assay (see Materials and Methods), which detects a fluorescence signal according to changes in membrane potential. We observed that incubation of zoospores with cGMP, but not cAMP, increased the fluorescence detected, indicating significant membrane depolarization (Fig. 1). The results suggest that external cGMP is taken up into the zoospores, since the cGMP-responsive domain of CNG is predicted to be internal. The resting potential of most cells is determined by the relative potassium levels inside and outside the cell (high potassium levels inside and 4 mM in medium or physiological salt solution). Usually, the addition of high quantities of KCl induces some currents by depolarization. The addition of KCl (positive control) to zoospores also resulted in membrane depolarization, but to a lesser extent (Fig. 1). These results indicate that high levels of cGMP and KCl that induce membrane depolarization also inhibit zoospore motility.

To check the hypothesis that BeCNG1 is a potassium-selective channel, we performed the same experiment with the classic potassium channel inhibitor tetraethyl ammonium (TEA) (Fig. 2). Again, as in the experiment for which results are shown in Fig. 1, both KCl (positive control) and cGMP were able to depolarize the membrane potential. However, in the presence of 10 μM TEA, changes in membrane potential were significantly inhibited. Taken together, the results point to the existence of a cGMP-activated potassium channel in B. emersonii zoospores.

To confirm that the channel we are monitoring is indeed a potassium channel, the flux of this ion was analyzed in the presence of cGMP by using a fluorescent dye selective for potassium (PBFI-AM; Molecular Probes). The addition of cGMP caused a high influx of potassium (Fig. 3), which was also observed when only KCl was added as a positive control. Nevertheless, the addition of cAMP did not lead to potassium influx, indicating the Zoospore suspension causes the cells to stop swimming, whereas addition of 3 mM cAMP does not affect zoospore motility (data not shown). These results indicate that high levels of cGMP and KCl that induce membrane depolarization also inhibit zoospore motility.

To confirm that the CNG channel we are monitoring is indeed a potassium channel, the flux of this ion was analyzed in the presence of cGMP by using a fluorescent dye selective for potassium (PBFI-AM; Molecular Probes). The addition of cGMP caused a high influx of potassium (Fig. 3), which was also observed when only KCl was added as a positive control. Nevertheless, the addition of cAMP did not lead to potassium influx, indicating the Zoospore suspension causes the cells to stop swimming, whereas addition of 3 mM cAMP does not affect zoospore motility (data not shown). These results indicate that high levels of cGMP and KCl that induce membrane depolarization also inhibit zoospore motility.
specificity of the potassium channel for cGMP (Fig. 3). This experiment was also performed in the presence or absence of the potassium channel inhibitor TEA to confirm that the potassium flux observed by the addition of KCl or cGMP was caused by a potassium channel and not by nonspecific flux or diffusion. Again, KCl and cGMP produced increases in potassium influx in all recorded cells (Fig. 4A), but in different ways. KCl induced a rapid increase in potassium flux followed by a slow decrease, whereas cGMP induced a sustained and prolonged increase in potassium influx (Fig. 4B). The addition of cAMP, as expected, did not produce any significant change in potassium flux. In the presence of TEA, the increases in K⁺ concentrations produced by cGMP and KCl were inhibited, as expected for a potassium channel (Fig. 4B and C). Taken together, our results point to the presence of a potassium-selective channel in B. emersonii zoospores that is specifically activated by cyclic GMP; in the presence of this cyclic nucleotide, the channel opens, creating a potassium influx.

**DISCUSSION**

CNG and K⁺ channels share significant amino acid sequence identity, and they have been suggested to present a common ancestral 3-dimensional (3D) architecture. However, these two types of channels have quite different selectivity properties: K⁺ channels are permeable primarily to K⁺ and Rb⁺ ions, whereas CNG channels are permeable to all monovalent alkali cations (Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺), to a variety of organic cations, and to some diva-
lent cations, such as Ca$^{2+}$, Mg$^{2+}$, Sr$^{2+}$, and Mn$^{2+}$. This functional difference is ascribed to the presence of the GYGD motif in the pore of K$^+$ channels, which in CNG channels is replaced by a single glycine (25). As a consequence, the pore in CNG channels is presumably shorter than that in K$^+$ channels and possibly more flexible (25). CNG channels are ubiquitously expressed in sensory and nonsensory cells, and their role in sensory transduction in vertebrate photoreceptors and olfactory sensory neurons has been well established (26). K$^+$-selective CNG (CNGK) channels have been implicated in sea urchin sperm chemosensations and in light signaling by ciliary photoreceptors in the scallop; cGMP is the signaling molecule in both signal transduction pathways, and a rise in cGMP levels opens the K$^+$ channels, causing hyperpolarization of the cells (2, 10).

In this report, we show that the blastocladiomycete fungus B. emersonii expresses a K$^+$ channel that is activated by cGMP. The use of specific channel inhibitors confirms the activation of the channel by cGMP and its K$^+$ selectivity. Interestingly, other blastocladiomycete fungi, which also possess a flagellum during the zoospore phase of their life cycle, such as Alomyces macrogyrus and Catenaria anguillulae, possess genes that encode an orthologous putative K$^+$ channel (see Fig. S1 in the supplemental material), as well as the rhodopsin–guanylyl cyclase fusion protein (6). However, both these gene orthologues families are absent in non-flagellated fungi, such as members of the classes Ascomycetes and Basidiomycetes (see Fig. S1) (6). Furthermore, in the flagellated chytridiomycete fungus Batrachochytrium dendrobatidis, a pathogen of amphibians, orthologues of BeGC1 and BeCNG1 are also absent, even though other elements of cGMP signaling are present (27).

Current drafts of the genome of yet another chytrid, Spizellomyces punctatus, show the presence of an orthologue of BeGC1 (see Fig. S1), but no true homologue of BeGC1 was detected (6). Nevertheless, the genome contains several adenylate/guanylate cyclase domains, in contrast to the genomes of fungi that do not form flagella, which lack genes encoding components of cGMP signaling (27). Thus, these observations indicate that the presence of orthologues of both BeGC1 and BeCNG1 genes as a pair may be an exclusive feature of blastocladiomycete fungi. Additionally, these data agree with the hypothesis that cGMP signaling pathways are restricted to motile flagellated fungi (27) and that the loss of flagella within the fungi was accompanied by the loss of cGMP-signaling genes.

Taken together, our data indicate that a CNGK channel triggers the phototaxis pathway in B. emersonii zoospores. Analysis of the B. emersonii genome revealed that no other gene encoding an ion channel with these characteristics is present, suggesting that the BeCNG1 channel is a key component of this signaling pathway. The role of a cGMP-activated K$^+$ channel in the scallop phototransduction pathway (2, 3) and in the sea urchin sperm chemotactic response (9, 10) suggests that systems analogous (and possibly paralogous) to BeCNG1 in the B. emersonii phototactic response are present. In our proposed model, the increase in cGMP promoted by the exposure of zoospores to green light leads to activation of the BeCNG1 channel and to K$^+$ influx. The increase in intracellular K$^+$ concentrations in B. emersonii zoospores leads, directly or indirectly, to changes in flagellar beating, producing the phototactic response of the fungus.

It is worth mentioning that the presence in B. emersonii of a K$^+$ channel activated by light due to its association with a photoreceptive guanylyl cyclase can constitute an interesting optogenetic device to be used in eukaryotic cells. The capacity to control a K$^+$ channel by exposure to light could provide the means to manipulate the ability of K$^+$ to terminate excitatory currents within cells. Thus, the rhodopsin–guanylyl cyclase (BeGCC1) together with the CNGK channel (BeCNG1) could represent an efficient tool acting in many cellular processes, including neuronal firing and hormone release, as recently discussed by Cosentino and coworkers (28).

ACKNOWLEDGMENTS
This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Provost’s Office for Research of the University of São Paulo (grant 2011.1.9533.1.3) (NAPNA-USP), São Paulo, Brazil. G.M.A. and T.G. acknowledge fellowship support by FAPESP. S.L.G. and H.U. were partially supported by CNPq.

We thank Luci D. Navarro and Sandra M. Fernandes for expert technical assistance.

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