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Patch clamp pipette giga seal forming success on the nanosurgery-obtained filamentous fungi protoplasts

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Abstract: The success of patch-clamp giga-ohm seal formation on filamentous fungi *Phycomyces blakesleeanus* protoplasts was investigated to evaluate their usefulness in ion channel studies on filamentous fungi. Protoplasts were obtained by laser-mediated nanosurgery of the cell wall stained with Calcofluor White. To enable a successful seal formation, it is critical to prevent cell wall regeneration. Since wall integrity responses in fungi involve kinase-dependent pathways, we aimed to sufficiently reduce intracellular ATP availability using the respiratory inhibitor azide. The effect of azide on phosphate metabolites of *Phycomyces blakesleeanus*, as determined by ^{31}P NMR spectroscopy, was a reduction in intracellular ATP accompanied by a decrease in long chain polyphosphates. Subsequently, all seal formation measurements were performed in the presence of azide, and protoplast viability was confirmed by cytoplasmic streaming. The success of seal formation depends on the size of the protoplasts, as larger protoplasts are more prone to successful seal formation. It was also found that the laser power used in nanosurgery could influence the success of seal formation, as higher values were associated with a lower success rate. The protoplasts released by laser nanosurgery produced by our newly developed method are a good model system for patch clamp on filamentous fungal membranes because their plasma membrane can easily form high-quality seals with the patch pipette.

Keywords: *Phycomyces*, azide, laser surgery

1. Introduction

Filamentous fungi (FF) hyphae perform important functions in most ecosystems based on their ability to excrete, transport, and exchange signals with the environment. Although FF plasma membrane ionic currents are thought to play an essential role in their excreting/transporting/signaling functions, they remain insufficiently explored because of technical difficulties in accessing the plasma membrane of FF, which is covered by a cell wall that has a distinct regenerative capacity [1]. The patch-clamp method, the gold standard for electrophysiological measurements, can only be applied to a healthy, clean plasma membrane accessible to the patch pipette. We developed a laser nanosurgery-based method of FF cell wall removal to obtain naked fungal cells (protoplasts) with a healthy and clean plasma membrane [2]. Briefly, the principle of the method was as follows: 1. Plasmolysed hyphae were subjected to laser sectioning of the cell wall stained with Calcofluor White; 2. Hyphae were partially deplasmolyzed. Cell wall regeneration was inhibited throughout the procedure. Successful formation of a gigaohmic resistance (GΩ) seal between the patch-clamp pipette and protoplast plasma membrane is a prerequisite for the patch-clamp current recording. Here, we investigated whether azide is a good wall regeneration inhibitor and which parameters in our new method affect the formation of the GΩ-seal.

2. Material and Methods

The culture of the filamentous fungus *Phycomyces blakesleeanus* (Burgeff) wt. (NRRL 1555(−)) was grown from the spore stock according to the standard protocol [3]. The ^{31}P NMR spectra were recorded from a control culture and an age-matched culture treated acutely with 5 mM azide. The details of the procedure, experimental setup and quantification of peak intensity were as previously described [3]. For the nanosurgery patch-clamp experiments, the cultures were grown with the following modifications: Spore cultures were plated on glass coverslips coated with 50% collagen type I and grown in media with glucose reduced to 10 g/l. Cultures (19-30 hours old) were stained with 1% Calcofluor White dye (Sigma Aldrich) in nominally isosmotic solution (ISOSOL, contains in mM: 60 KCl, 65 K-glutamate, 2 $MgCl_2$, 1 $CaCl_2$, 2 mM azide, 10 HEPES pH 7, supplemented with sucrose to 495 mOsm) with the addition of 10 μM brefeldin A for 10 min at 20°C. Subsequently, the stained cultures were washed and plasmolyzed in hyperosmotic solution (HYPERSOL, composed as ISOSOL, with [Ca^{2+}] increased to 30 mM and sucrose replaced with sorbitol to bring the osmolarity to 620 mOsm). Details of the experimental setup for Nonlinear Laser Scanning Microscope for Two Photon Excitation Fluorescent (TPEF) imaging and laser nanosurgery were previously described [2, 4]. The nanosurgical pattern was positioned in the empty hyphal section (where the hyphal protoplast had retracted from the area due to plasmolysis) (Fig. 1a, left). After laser nanosurgery, the resulting cell wall incision was verified by TPEF and bright-field imaging (Fig. 1a, middle and right, respectively). The release of protoplasts through the wall incision was monitored by bright field microscopy. Subsequently, the fungal culture was transferred to the patch-clamp setup (an EPC8 amplifier connected to an

Istrutec 1600 digitizer, along with Pulse software (all from HEKA)). The ¼ of the solution was carefully replaced with an appropriate solution to bring the final osmolarity of the chamber bath solution to 595 mOsm. The pipettes for the patch clamp, pulled on a Sutter p-97 puller had a resistance of 10-20 MΩ. Statistical comparisons were performed using ANOVA with multiple comparisons and Holm-Sidac correction, and an unpaired two-tailed t-test with Welch's correction for unequal variances. Confidence levels for statistical significance were 0.05 (*), 0.01 (**), and 0.005 (***).

3. Results and Discussion

To enable successful seal formation, it was critical to prevent cell wall regeneration, both at the hypha prior to protoplast release and at the released protoplasts. Since wall integrity responses in fungi involve kinase-dependent pathways [1], we aimed to reduce intracellular ATP availability by using the respiratory inhibitor azide. The effect of azide on phosphate metabolites of *P. blakesleeanus*, as determined by ^{31}P NMR spectroscopy (Fig. 1B), was a decrease in intracellular ATP (measured as a 20 ± 8% decrease in the

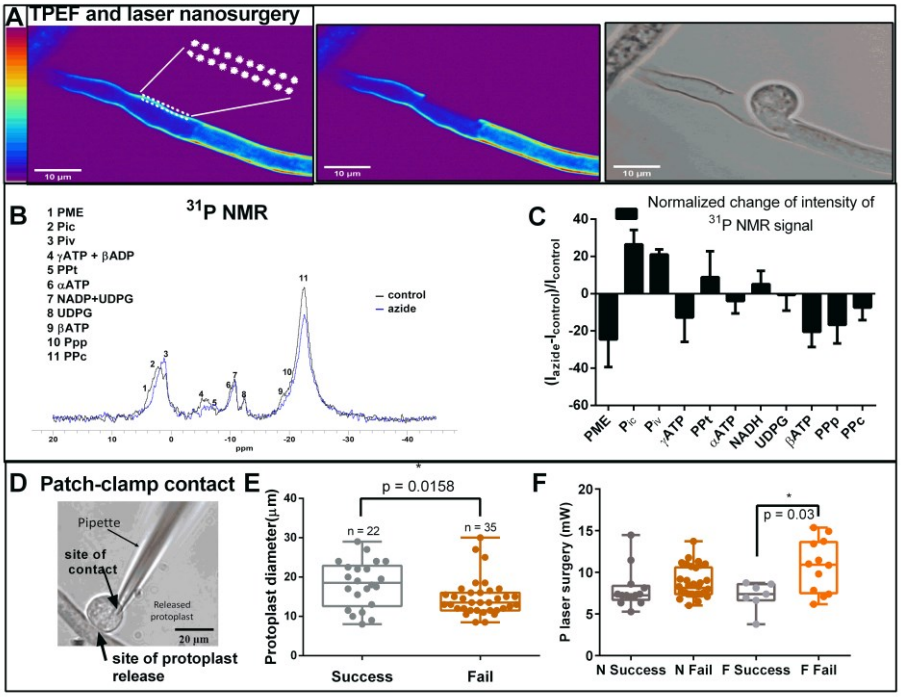

Figure 1. Succsess of patch-clamp pipette contact with the membrane of protoplasts released by laser nanosurgery. A. The procedure of obtaining protoplasts by laser nanosurgery. Left and middle: TPEF images before and after laser nanosurgery of hypha cell wall; Right: brightfield image of protoplast crawling through laser-made incision. Color bar for TPEF images is on the far left B. Overlapped ^{31}P NMR spectra of *P. blakesleeanus*, obtained in control conditions and after azide addition. Peaks (annotated by numbers) can be assigned to corresponding phosphate-containing molecules: 1. sugar phosphate (PME); 2. inorganic phosphate cytoplasmatic (Pic); 3. Inorganic phosphate vacular (Piv); 4. γ-ATP and β-ADP (γATP); 5. terminal phosphate residues of polyphosphates (PolyP) and pyrophosphate (PPt); 6. α-ATP; 7. NAD(H) and uridine diphosphoglucose (UDPG); 8. UDPG second resonance; 9. β-ATP; 10. penultimate phosphates of PolyP (PPp); 11. central PolyP residues (PPc). C. Normalized change of intensity of ^{31}P NMR signal, calculated for each peak as $(I_{azide}-I_{control})/I_{control}$. Values shown are mean ± SD (n=3). D. Brightfield image of the patch-clamp pipette on the nanosurgery-released protoplast during forming GΩ contact (seal). E. Diameters

of the nanosurgery-released protoplasts, sorted according to failure or succsess in forming the seal. T-test with Welch's correction. F. Laser surgery power at the sample, used for nanosurgeries performed in a close proximity (<3 μm) of retracted protoplast (N-"near") and for nanosurgeries at distance larger than 3 μm from the retracted protoplast in hypha (F-"far"), sorted according to failure od succsess in forming the seal. ANOVA with Holm-Sidac correction.

signal intensity of the β-phosphate group of ATP) accompanied by acidification of cytoplasm reflected through upfield shift of Pic signal and decrease of long-chain polyphosphates (Fig. 1C). The desired inhibition of wall regeneration by azide during nanosurgery and patch clamp was confirmed by 1. the released protoplasts; 2. formed GΩ-contacts at the protoplast plasma membrane (as shown in Fig. 1D). In addition, the viability of hyphae and the obtained protoplasts was confirmed by observing the presence of cytoplasmic streaming in bright field microscopy. Protoplasts obtained after nanosurgery exhibited a range of diameters, with a significant difference (p=0.016) between groups sorted by GΩ-seal formation success (in μm): d_{succ} =18±6 (n=22), d_{fail} =15±5 (n=35) (Fig. 1E). It is possible that a skilled patch-clamp operator could improve the success rate on smaller protoplasts, with further practice. Variation in the laser power applied during nanosurgery showed that it is safer to use lower values, as a significant difference in laser power was found for nanosurgery sorted by GΩ-sealing success, that was performed farther from the hyphal protoplast (Fig. 1F).

3. Conclusions

The protoplasts released by our newly developed laser nanosurgery method are a good model system for the patch-clamp method on filamentous fungal membranes because their plasma membrane can easily form high-quality seals with a patch pipette.

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