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Communication

Localized Patch Clamping of Plasma Membrane of a Polarized Plant Cell[1]

Laser Microsurgery of the *Fucus spiralis* Rhizoid Cell Wall

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ABSTRACT

We used an ultraviolet laser to rupture a small region of cell wall of a polarized *Fucus spiralis* rhizoid cell and gained localized access to the plasma membrane at the growing apex. Careful control of cell turgor enabled a small portion of plasma membrane-bound cytoplasm to be exposed. Gigaohm seals allowing single-channel recordings were obtained with a high success rate using this method with conventional patch clamp techniques.

A fundamental problem in plant cell biology concerns the role of plasma membrane ion channels in the development and maintenance of polarity (see ref. 9 for recent review). However, direct recordings of single channels from localized regions of polarizing or polarized cells are still lacking. The patch clamp technique is a powerful tool enabling the study of function and regulation of single-channel currents and kinetics associated with the gating of the ion channel. Since the introduction of the improved GΩ seal patch clamp by Hamill and coworkers (8), a mass of information concerning ion channels and their behavior and regulation in the animal plasma membrane has become available. Single-channel studies of the plant plasma membrane, although increasing, have been much slower to develop. Voltage-gated channels in the plasma membrane of plant protoplasts have been described in several cell types (1, 11, 17, 19, 21), and more recently, the technique has been used to investigate their regulation (7, 10, 15, 16, 18, 20).

Removal of the cell wall to expose plasma membrane suitable for patch recording was predicted to be a major problem in the advancement of single-channel studies in plant cells by Takeda and coworkers (22). In all but a few cases, removal of the cell wall is achieved by enzymic treatment to produce a spherical protoplast. The purity of the enzymes is crucial to avoid degradation of the plasma membrane lipids and proteins. Reported success rates for obtaining high resistance (>5 GΩ) seals on enzymically isolated protoplasts vary but are generally low (5, 19). Attempts to improve the low success rates have often proved unsuccessful (6), although recent improvements to the procedure of enzymically isolating protoplasts may increase the number of successful GΩ seals (4).

Plant cell protoplasts do not retain the structural order or polarity of the parent cell. This problem is of particular importance in the study of ion channel activity, distribution, and regulation in polarizing and polarized plant cells. In *Fucus* zygotes, the plant cell wall is required for the axis of polarity to develop (13); protoplasts from young *Fucus* zygotes are, therefore, of limited use in a single-channel study of polarity because localized cell wall removal and exposure of the underlying plasmalemma is required. Furthermore, the extraction and purification of the enzymes required for cell wall removal in *Fucus* are complex and time consuming (12). Although mechanical or surgical techniques have been used to gain access to the plasmalemma of *Chara* (3, 14), these are not suitable for smaller and less robust cells.

In this report, we describe a novel technique for localized removal of the cell wall of *Fucus* rhizoid cells using a UV laser. Under appropriate conditions of turgor, the tip of the rhizoid cell is exposed, enabling high resistance GΩ seals to be obtained on the plasmalemma.

MATERIALS AND METHODS

Growth of Zygotes

Thallus tips bearing mature conceptacles of the dioecious marine alga *Fucus spiralis* were collected, washed, and stored at 4°C in the dark for 1 to 3 d before they were placed in FSW[2] at 18°C to release zygotes. Zygotes were washed and transferred into small plastic chambers with a glass coverslip base. Dishes were placed in unilateral white fluorescent light (50 $\mu Em^{-2} s^{-1}$) at 18°C to promote polarization, and zygotes were used 2 to 3 d after release.

Preparation of Zygotes

Dishes were placed on the stage of an inverted microscope (Nikon Diaphot, Nikon, Tokyo, Japan) and treated with

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[2] Abbreviation: FSW, filtered sea water.

0.001% calcofluor (Sigma, Poole, UK) in FSW for 5 min. The calcofluor FSW was washed and replaced with 10% (w/v) PEG (M_r 6000; Sigma) and 0.6 M mannitol in FSW to achieve plasmolysis.

Cell Wall Removal and Exposure of Plasmalemma

A pulsed nitrogen laser (VSL 337; Laser Science Inc., Cambridge, MA) was used to produce a high-intensity UV beam that was introduced into the microscope objective (Nikon UV-Fluor ×40, numerical aperture 0.8–1.3) via the UV port and condenser of a Nikon Diaphot microscope. Alignment was adjusted until the beam was focused to a spot (about 1 μm observed as a fluorescence on the coverslip). The laser was triggered remotely using 12-V pulses from a stimulator.

Plasmolyzed zygotes were placed such that the tip of the cell wall was aligned with the focused laser beam. After 1 to 5 pulses, the cell wall was ruptured. Extrusion of cytoplasm from the tip of the rhizoid was finely controlled via a small glass micropipette (tip diameter 5–20 μm), filled with variable concentrations of mannitol and PEG made up in FSW depending on the degree of extrusion required. The pipette was mounted on a micromanipulator (Narishige MW-3; Narishige, Tokyo, Japan) and positioned 20 to 100 μm from the cell.

Electrophysiology

Patch pipettes were manufactured from thick-walled borosilicate glass (GC150F; Clark, Pangbourne, UK) on a Narishige puller (P-833). Ultrafiltered bath solution (0.22-μm Millipore millex; Millipore, Walford, UK) was used to fill the pipette. Pipettes were connected to the headstage of a patch clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA) via a pipette holder. The headstage was mounted on a micromanipulator (Leitz, Oberkochen, Germany). On contact with plasma membrane, gentle suction was applied to the pipette, and seal resistance was monitored by applying a 30-ms 20-mV square pulse at low headstage gain. After a seal was achieved, headstage gain was increased to resolve single-channel currents. The current output was low pass filtered (−3 dB 3 KHz, 4-pole Bessel). Command potentials were applied to the patch either by manual control of the voltage clamp settings or via computer software (Pclamp; Axon Instruments). Data acquisition and analysis were achieved using an analog to digital converter (Labmaster; Axon Instruments) linked to an IBM-compatible computer.

RESULTS AND DISCUSSION

Zygotes of *F. spiralis* (2–3 d old) were readily plasmolyzed by the PEG and mannitol mixture in FSW. The extent of plasmolysis varied such that a 10- to 30-μm space between the cytoplasm and tip wall was achieved, permitting rupture of the cell wall without damaging the cytoplasm. The calcofluor-stained cell wall fluoresced brightly during excitation, allowing precise alignment of the cell wall with the laser. Cell turgor was easily manipulated by microperfusion of the FSW and PEG/mannitol mixtures. The extent of cytoplasmic extrusion depended on external osmolarity. The membrane-bound cytoplasm rounded up on emerging through the cell wall incision (Fig. 1, A–C). The ability of the cytoplasmic extrusions to regulate volume in response to changes in osmolarity shows that they were bound by an intact functional plasma membrane. Preliminary experiments have shown that it is also possible to ablate regions of more mature zygotes where the cell wall is thicker.

Seal resistance increased spontaneously on contact with freshly exposed plasma membrane (up to 300 MΩ within 30 s), and gentle suction promoted the formation of seals >5 GΩ within 1 to 2 min (Fig. 1D). Seals up to 5 GΩ were obtained

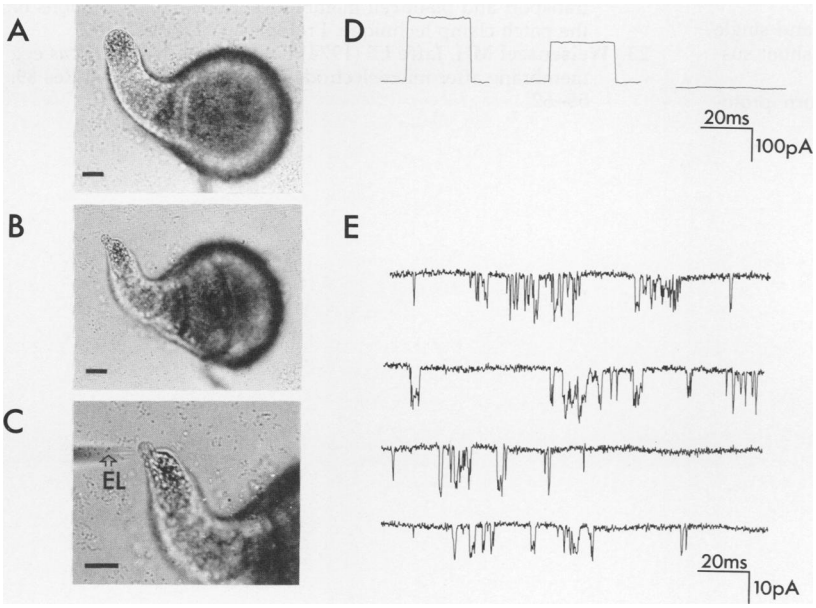

Figure 1. A, A 60-h zygote of *F. spiralis* with developing rhizoid. B, The same rhizoid after plasmolysis and treatment with UV laser to ablate the cell wall at the tip region followed by osmotic reflation. A small plasma membrane bound extrusion can be seen on which a seal was obtained with a patch electrode, EL. C, The electrode appears out of focus because of the 45° angle of approach; scale bar represents 20 μm. D, Development of a seal resistance >10 GΩ. Current through the patch was recorded in response to a 20-mV stimulus to monitor the final stages of seal formation. The decrease in patch current in response to the voltage stimulus indicates the increase in seal resistance (Ohm's law). The spontaneous outward single-channel activity was recorded in the cell attached configuration with the electrode clamped to 0 mV. E, Data were filtered at 3 KHz. The downward deflections represent currents flowing into the pipette.

35% of the time, and seals >5 GΩ were obtained at a rate of 20%. Stable recordings were maintained for up to 30 min. Improvements to the electrode fabrication protocol should further improve the rate and stability of giga-seals because the electrodes in this present study were unpolished and uncoated.

Spontaneous channel activity was recorded in 50% of patches with a seal resistance of >5 GΩ. Figure 1E illustrates channel activity recorded on plasma membrane exposed at the tip region of a rhizoid cell. With the electrode clamped to 0 mV, the only major permeant ion that would carry the outward currents at the assumed transmembrane potential of −80 mV, based on recordings from intact zygotes (2, 23), is K^+. The outward currents represented by downward deflections in the cell-attached configuration are likely to be due to K^+ channel activity.

The relatively simple technique described here should, for the first time, enable detailed studies of the types of ionic channels, their distribution, regulation, and role in the initiation and maintenance of polarity and polarized growth in a range of plant cell types, including higher plants. We are currently developing the technique to further study axis formation, fixation, and rhizoid growth in *Fucus*. The method should also be applicable to other important systems, including fungal hyphae, protonemata, root hairs, and pollen tubes, in addition to those systems in which enzymic removal of the cell wall for patch clamping is difficult.

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