Communication

Patch Clamping Protoplasts from Vascular Plants

Method for the Quick Isolation of Protoplasts Having a High Success Rate of Gigaseal Formation

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ABSTRACT

A method is described for the isolation of protoplasts (Pisum sativum, Phaseolus vulgaris, Avena sativa, Arabidopsis thaliana) in preparation for ion flux studies using patch clamp electrophysiology. Protoplasts that have been exposed to hydrolytic, cell wall degrading, enzymes for as little as 5 minutes form gigaseals (seal resistance higher than 10 GΩ) with the patch pipette with success rates greater than 40%. Sealing of these protoplasts is fast, averaging less than 2 minutes. This method yields high rates of gigaseal formation in a variety of tissues from both monocots and dicots and will enhance data collection in ion flux studies of plasma membranes of vascular plants.

The patch clamp technique is a very powerful method for measuring ionic fluxes across membranes and for elucidating the regulatory mechanisms affecting these fluxes (2, 14). In the last decade a wealth of information on ion channel activity and regulation in animal plasma membranes has become available. Although results of studies in which the patch clamp method was used on plant plasma membranes were published as early as 1984 (11, 17), the number of studies on vascular plants, compared to that on animal cells, has remained limited. Moreover, many of the studies performed on plant material have been of the vacuolar membrane rather than the plasma membrane (6). The reason for this is the difficulty in the formation of a so-called “gigaseal” between the plasma membrane and the tip of the glass micropipette, which is necessary for high resolution, low noise recordings (5). Depending on the channels under study, seal resistances greater than 10 GΩ are considered useful (1, 12, 15). Some cell types, especially guard cells, apparently do yield protoplasts that readily form gigaseals (13). Other cell types present more difficulties. Authors have commented on the inability to obtain high resistance seals (9, 11, 15, 16); the low success rate of seal formation which can be 10% or less (4, 9, 15, 16); and the time it takes to form a seal, which is seconds with vacuolar membranes, but can be as long as 0.5 h with plasma membranes (3). Another concern regarding working with protoplasts is the possibility of introducing artifacts by the treatment with hydrolytic enzymes, especially when this treatment is prolonged for several hours (3, 15).

Although there have been efforts to improve the method of isolation and subsequent treatment of protoplasts (4), a more or less generally applicable method has not emerged. In this communication we describe a method for rapid isolation of protoplasts with a high success rate in rapidly forming gigaseals with a seal resistance greater than 10 GΩ. In our hands this method can be used without much modification, on tissues from various plant species.

MATERIALS AND METHODS

Protoplast Isolation

Solutions

The enzyme solution contained 1.7% w/v Cellulase RS (Yakult Honsha), 1.7% w/v Cellulysin (Calbiochem), 0.026% w/v Pectolyase Y-23 (Seishin), 0.2% w/v BSA (Sigma), 2.325% w/v Gamborg’s B5 (Gibco), 2 mM CaCl2, and 10 mM Mes-KOH, pH 5.5 (18). Mannitol was added to adjust the osmolarity to 610 mOsm.

The wash solution had the same composition as the enzyme solution except cellulase, cellulysin, pectolyase, and BSA were omitted.

The bath solution contained 5 mM CaCl2, 2 mM MgCl2, 10 mM K-citrate, pH 5.5, and the osmolarity was adjusted to 210 mOsm with mannitol.

Plant Material

Pea. Pisum sativum (Argenteum mutant) seeds were either imbibed on wet filter paper and cultured on vermiculite in the dark at 25°C, or were planted in soil and grown in 12 h light:12 h dark at 25°C (fluence rate 130 μE·m−2·s−1 provided by cool white fluorescent tubes).

Protoplasts from stem epidermal cells were obtained from epidermis stripped from 1 cm under the epicotyl hook of 5 to 7 d old, etiolated plants. Protoplasts from the leaf epidermis were obtained from the lower epidermis of young, unfolding,
leaves from light grown plants. Strips of epidermis were floated on the enzyme solution.

_Bean_. Seedlings of *Phaseolus vulgaris* L. were grown in moist vermiculite in continuous dim (4 μE·m⁻²·s⁻¹), red light at 25°C (20). Primary leaves of 10 d old plants were lightly abraded on the upper surface, and pieces of leaf were floated with the abraded side facing down on the enzyme solution.

_Oat_. *Avena sativa* cv Victory seeds were imbibed for 1 h in aerated distilled water and sown on wet vermiculite and grown in the dark at 25°C. After 4 or 5 d the epidermis of the coleoptiles was stripped and the peeled coleoptiles were floated on the enzyme solution.

_Arabidopsis_. Seeds of *Arabidopsis thaliana* ecotype No were imbibed for 12 h in distilled water at 4°C in the dark and planted on agar supplemented with germination medium according to (19). The cotyledons and the primary leaves were cut into small (1 mm²) pieces and submerged in the enzyme solution.

**Viability Test**

The viability of the protoplasts was determined with FDA² staining (7). Two milliliters of acetone saturated with FDA was mixed with 200 μL of the protoplast suspension. Fluorescence was observed with a Zeiss fluorescence microscope after 2 min.

**Electrophysiology**

Standard patch clamp techniques (5) were used. Patch pipettes were pulled from 50 μL micropipettes (VWR), on a Narashige PB-7 pipette puller. The shank and the tip were dipped in Q-dope (poly styrene, GC Electronics, Rockford IL), which was allowed to harden for at least 2 h before fire polishing of the tip, to reduce the pipette capacitance. The tip resistance of pipettes thus obtained was typically between 5 and 12 MOhm (9.6 ± 3.7 MOhm, mean ± sd, n = 129) with the bath and pipette solutions used.

The glass bottom of the patch clamp chamber was first washed thoroughly in 5% chromic acid and then in methanol and rinsed with distilled water to facilitate the attachment of protoplasts to the glass.

The reference electrode and patch pipette solution contained 2 mM MgCl₂, 4 mM KCl, 1 mM EGTA, 100 mM K-citrate, pH 7.2. CaCl₂ was added to obtain the desired pCa (which was varied between 7 and 5) and mannitol was added to adjust the osmolarity to 210 mOsm. The tip of the electrode was filled by suction and the rest of the pipette was backfilled with a hypodermic needle. Both bath and pipette solution were filtered through a 0.2 μm filter (Supor 200 Gelman).

The establishment of the seal was monitored by measuring the current induced by a 10 ms 1 mV square pulse from a function generator (Topward type 8102). The signal was filtered (low pass cut off frequency 3500 Hz) with an 8-pole Bessel filter (type 902, Frequency Devices). The currents were monitored with a current voltage converter (List EPC-7), digitized by an A/D converter (p-box, custom made, based on a Labmaster ADC board [Scientific Solutions Inc.]), and stored on a PC hard drive.

² Abbreviation: FDA: fluorescein diacetate.
Table I. The Success Rate of Sealing Attempts Made on Various Protoplast Types

<table>
<thead>
<tr>
<th>Source of Protoplasts</th>
<th>Total</th>
<th>R &gt; 10 GΩ</th>
<th>1 GΩ &lt; R &lt; 10 GΩ</th>
<th>Fail/WC</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisum sativum leaf epidermis</td>
<td>123</td>
<td>67</td>
<td>32</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>P. sativum stem epidermis</td>
<td>98</td>
<td>42</td>
<td>31</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Phaseolus vulgaris leaf mesophyll</td>
<td>15</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Avena sativa coleoptyle cortex</td>
<td>15</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Arabidopsis thaliana cotyledon mesophyll</td>
<td>15</td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

this rupturing, making it possible to monitor whole cell currents on these cells.

The time it took for the seal to form from the first contact of the pipette tip with the protoplast was short ranging from 30 s to 5 min (in *P. sativum* leaf epidermis protoplast, 1 min 40 s ± 50 s, mean ± SD, n = 39). It is unclear why protoplasts prepared by the described method form gigaseals with such ease. The rigorous wash steps are one essential element. In one experiment we added 0.2% w/v BSA in both the wash and the bath solutions and were not able to form any gigaseals in 10 trials. Another possibility is that the cytoskeleton might be influenced by the osmotic swelling such that the O-shaped “bleb” of membrane sucked into the pipette tip, found to be involved in the seal formation in animal tissue (10), forms more readily than in protoplasts not osmotically shocked. Until now, efforts to understand the function and regulation of the plasma membrane have been hampered by the difficulty of using patch clamp techniques on this organelle. This method presented here will bring studies of the plasma membrane forward and allow useful comparisons, between it and the tonoplast, between tissues, and among plants.

**LITERATURE CITED**


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