Rapid Communication

Laser Microsurgery of Higher Plant Cell Walls Permits Patch-Clamp Access[1]

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Plasma membranes of guard cells in epidermal peels of *Vicia faba* and *Commelina communis* can be made accessible to a patch-clamp pipet by removing a small portion (1–3 μm in diameter) of the guard cell wall using a microbeam of ultraviolet light generated by a nitrogen laser. Using this laser microsurgical technique, we have measured channel activity across plasma membranes of *V. faba* guard cells in both cell-attached and isolated patch configurations. Measurements made in the inside-out patch configuration revealed two distinct K$^+$-selective channels. Major advantages of the laser microsurgical technique include the avoidance of enzymatic protoplast isolation, the ability to study cell types that have been difficult to isolate as protoplasts or for which enzymatic isolation protocols result in protoplasts not amenable to patch-clamp studies, the maintenance of positional information in single-channel measurements, reduced disruption of cell-wall-mediated signaling pathways, and the ability to investigate intercellular signaling through studies of cells remaining situated within tissue.

It is becoming increasingly apparent that the cell wall of plants does not function solely as a structure that allows turgor development. Cell-wall components or fragments have been implicated in cellular responses as diverse as the signaling of pathogen attack (Ryan and Farmer, 1991) and the determination of cell fate (Berger et al., 1994). Cell-wall and apoplastic proteins have already been assigned fundamental roles in processes such as signal transduction in response to wounding (Pearce et al., 1991) and the control of cell expansion (McQueen-Mason and Cosgrove, 1994). Cell-type-specific labeling of plant cell walls by monoclonal antibodies implicates the presence of additional, site-specific proteins with as yet unknown functions (Knox et al., 1995). The importance of adhesions and connections between the plasma membrane of the cell and the cell wall is also being recognized (Roberts, 1990). Such connections, along with force on the plant plasma membrane exerted by

turgor, are hypothesized to play an integral role in the activation of stretch-sensitive responses, including stretch-activated ion channels (Pont-Lezica et al., 1993).

Concurrently, application of the patch-clamp technique to plant cells has begun to reveal the importance of ion channels in plant cell function (Hedrich and Schroeder, 1989; Assmann, 1993). Yet, ironically, the standard approach to patch clamping requires removal of the cell wall by enzymatic digestion, to allow the requisite physical contact between the patch electrode and the cell membrane. Although protocols have been developed (Elzenga et al., 1991; Gassman and Schroeder, 1994) for some tissues that minimize exposure to cell-wall-degrading mixtures, which are often of less than optimal purity, the fact remains that the patch clamping of the plasma membrane of higher plant cells has involved the enzymatic production of protoplasts and, thus, the unavoidable loss of biochemical and physical signaling components associated with the cell wall. In addition, once a protoplast is formed, positional information concerning the polarity of the cell, possibly including the asymmetric distribution of ion channels, is lost. It would therefore be of use to develop a technique that allowed the patch clamping of higher plant cells while leaving most of the cell wall intact. Such a technique has been developed for large algal cells, for which laser microsurgery has been used to ablate a portion of the algal cell wall, thus exposing the cell membrane and allowing patch-clamp measurements (Taylor and Brownlee, 1992; De Boer et al., 1994). Laser microsurgery has also been applied to higher plant cells (Weber and Greulich, 1992; Kurkdjian et al., 1993; De Boer et al., 1994) but has not previously been combined with patch-clamp measurements. In this article we report the successful application of laser microsurgery to access the plasma membrane of cells of a higher plant, *Vicia faba* L., for patch-clamp recording. Because of the obvious importance of the cell wall to stomatal function, our initial focus has been on development of the laser microsurgery technique for use on guard cells that remain situated in the epidermal tissue.

Abbreviations: LC, large conductance channel; P_o, ion channel open probability; SC, small conductance channel; V_m, membrane electrical potential.

MATERIALS AND METHODS

Plants

Plants of *Vicia faba* L. and *Commelina communis* L. were grown as previously described (Lee and Assmann, 1991). Leaves were harvested from 3- to 4-week-old plants in the morning, and epidermal peels were made immediately from the abaxial surface (Schwartz and Zeiger, 1984). The peels were floated cuticle side up in the dark for at least 1 h on a solution consisting of 2 mM KCl, 1 mM $CaCl_2$, and 10 mM Mes (pH 6.0, using solid Tris base).

The Laser System

The system used to laser ablate guard cell walls was similar to that described previously by Taylor and Brownlee (1992). The beam of a nitrogen pulse laser (model No. VSL-337; Laser Systems, Cambridge, MA) was directed through a Galilean-type beam expander (Spindler and Hoyer, 1990) and into the rear epifluorescence port of a Diaphot 300 (Nikon) inverted microscope. A dichroic mirror (No. 400DCLPO2; Omega Optical, Brattleboro, VT) in the microscope filter cassette reflected the beam through an X40 oil immersion objective (Fluor 40/1.3; Nikon) onto the tissue. The focal point of the laser was matched with the focal plane of the microscope by adjusting the distance between the plano-concave and plano-convex lenses of the beam expander. Consequently, as the focal plane of the microscope was changed, the focal point of the laser moved with it.

Laser Ablation of the Cell Wall and Plasma Membrane Access

Epidermal peels were transferred from the incubation solution and immobilized cuticle side down on the surface of a No. 0 coverslip, which formed the bottom surface of a Plexiglas dish. After the epidermis was placed on the coverslip, excess solution was wicked away with a Kimwipe (Kimberly-Clark Co., Atlanta, GA) so that the peel lay flat. Immobilization was achieved by application of a thin bead of vacuum grease around the perimeter of the peel. Immediately following immobilization, a perfusion system was used to introduce the following solution into the dish to plasmolyze the guard cells: 1 mM $CaCl_2$, 10 mM Hepes (pH 7.0 using KOH), and mannitol to adjust the osmolality to between 460 and 480 mmol kg^{-1}. Calcofluor white M2R (Sigma catalog No. F-6259) was added to the solution at 0.01% (w/v). This compound binds to cell-wall components and improves absorption of the laser energy (De Boer et al., 1994). Once the cells had plasmolyzed sufficiently, the laser beam was directed toward a spot on the upper guard cell wall but as far laterally from the protoplast as possible. The tissue was perfused during the ablation procedure with the same solution used for plasmolysis except with Calcofluor omitted. Following the laser ablation of several different cells, a solution was allowed to flow by gravity into the Plexiglas dish from a 100-mL linear gradient maker, one chamber of which contained 5 mM KCl, 5 mM K^+ gluconate, 0.5 mM $CaCl_2$, 10 mM Hepes (pH 7.0 using KOH), and mannitol added for a final osmolality of 100 ± 2

mmol kg^{-1}. The other chamber of the gradient maker contained an identical solution with mannitol to give an osmolality of 450 ± 5 mmol kg^{-1}. In this way, the guard cells could be deplasmolyzed slowly (De Boer et al., 1994) and observed for the first appearance of a protoplast bleb (that portion of the protoplast that protrudes through the laser hole). Blebs for subsequent patch-clamp experiments were selected on the basis of the presence of organelles and a somewhat darker coloration. Occasionally, blebs were produced that appeared transparent and contained no organelles; these were assumed to be vacuolar in origin.

Patch Clamping

Patch clamping was performed as previously described (Wu and Assmann, 1994) except that filamented patch pipet glass (No. 5968; A-M Systems, Everett, WA) was used to pull patch electrodes, and the patch-clamp amplifier used was an Axopatch 200 (Axon Instruments, Foster City, CA). The electrode solution had an osmolality of 450 ± 5 mmol kg^{-1} and otherwise consisted of the same solution as present in the bath following deplasmolysis. After an inside-out membrane patch was produced, the bath solution was exchanged for one containing 10 mM KCl, 40 mM K^+ glutamate, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.8), 4.2 mM KOH, and mannitol to give an osmolality of 243 mmol kg^{-1}. The free Ca^{2+} concentration in this solution was 2 μM, as measured with a Ca^{2+} electrode (model 93–20; Orion Research, Boston, MA). Membrane patches were voltage clamped for 15 s per pulse in 20-mV increments from +126 to −54 mV (applied potentials after correction for liquid junction potentials).

RESULTS AND DISCUSSION

A new experimental strategy was necessary to obtain useful exposure of the plasma membrane of a cell remaining situated in tissue. If the guard cell in situ is modeled as a rectangular box, it is apparent that three sides face adjacent epidermal cells, a fourth side faces the other guard cell, and a fifth side faces the coverslip upon which the tissue is fastened. The only possible surface for laser ablation followed by patch-clamp access was therefore the "upper" cell wall, facing the bath solution. In contrast to previous demonstrations of laser microsurgical removal of portions of the cell wall, it was not possible to release patch-clamp-accessible membrane by ablating the easily visualized side walls of the cells. Although ablation of side walls has been effective with single algal cells or protrusions of higher plant cells such as pollen tubes or root hairs that result from tip growth (Taylor and Brownlee, 1992; Kurkdjian et al., 1993; De Boer et al., 1994), this strategy resulted in protoplast blebs that simply protruded into the cell-wall compartment of the adjacent cell in the tissue. To release guard cell protoplast blebs that could be accessed from above with a patch electrode, it was necessary to ablate the upper cell wall, which is virtually transparent. To ensure that a hole was cut through the upper cell wall, the focal plane of the microscope, in concert with the focal

point of the laser, was swept over a small area of the cell and up and down through the cell wall.

With practice it was possible to obtain protoplast blebs routinely from *C. communis* and *V. faba* epidermal peels (Fig. 1, A and B). Some blebs were too small to be successfully approached with a patch electrode. However, given blebs of the appropriate size (Fig. 1C), patch electrodes sealed easily onto the blebs with little or no application of suction. This is in contrast to previous reports in which the achievement of $G\Omega$ seals from higher plant plasma membranes was difficult (De Boer et al., 1994). This may be due to tissue- or species-specific differences in the plant material used.

Ion channel activity was observed in 14 of 15 cases in which $G\Omega$ seals were obtained. In several cell-attached recordings, LCs were observed (data not shown). Patch excision was used so that ionic conditions and the membrane potential would be explicitly defined. The recording shown in Figure 2A from an inside-out patch reveals two distinct channels at an applied potential of +86 mV ($V_m = -86$ mV). An amplitude histogram constructed from the raw data shows three discrete peaks (Fig. 2B), corresponding to the closed state (-2.4 pA), the open state of the SC (-3.5 pA), and the combined open states of both the SC and LC (-6.5 pA). Current/voltage relationships for each channel are shown in Figure 2C. Linear regressions of the data from each channel were used to calculate conductances of 47.4 and 23.5 picosiemens for the LC and SC, respectively. The LC reversed at -28 mV and the SC reversed at -35 mV (Fig. 2C), close to the theoretical reversal potential for K^+ (-34.5 mV), suggesting that both channels are primarily selective for K^+. The conductances of both channels appear to be larger than those reported previously, taking into account the differences in experimental solutions used by different experimenters (Schroeder et al., 1987; Wu and Assmann, 1994; Ilan et al., 1995). Consequently, a correlation cannot be made between these channels and other K^+-selective channels of guard cells already reported in the literature. The SC also shows an atypically high (Slayman and Bertl, 1994) P_o at this voltage (Fig. 2, A and B); the P_o of both channels decreases at more positive V_ms (data not shown). The observed channels may be previously reported K^+ channels behaving differently because of the new isolation method or channels not detectable in conventionally isolated protoplasts now evident because of the unique method of plasma membrane access. Additional experimentation beyond the scope of this communication will be required to further address this issue; the purpose of this report is the demonstration that nonenzymatic patch-clamp access to the plasma membrane of a higher plant cell is possible while the cell remains situated in tissue.

CONCLUSIONS

Results reported here demonstrate that it is feasible to use laser microsurgery to access the plasma membrane of higher plant cells for patch-clamp recording. Laser-assisted patch clamping may prove particularly useful in studies of guard cell function. For example, it has been suggested that
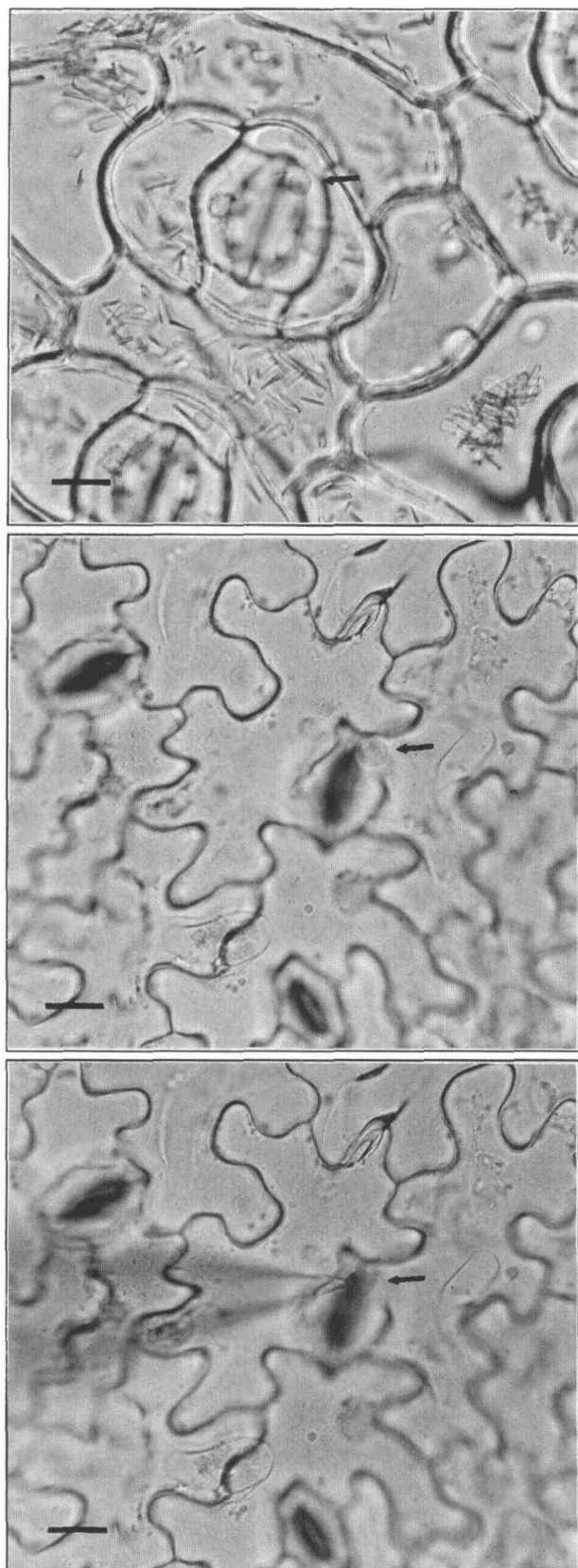

Figure 1. Protoplast blebs (arrows) above the plane of focus of *C. communis* guard cells (A) and *V. faba* guard cells (B and C; note approaching patch-clamp pipet). Scale bar = 20 μm.
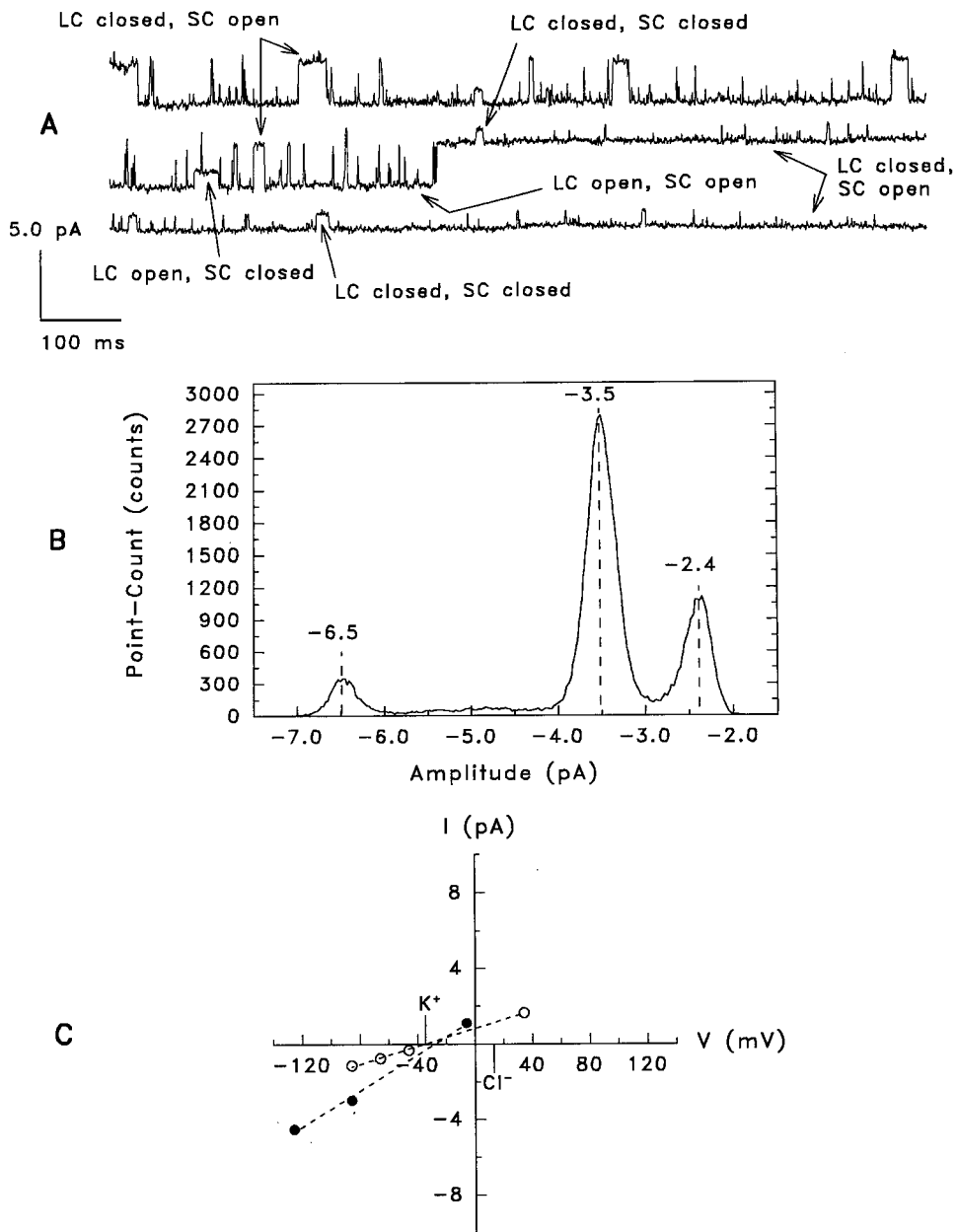
Figure 2. A, Single-channel recordings from a laser-accessed *V. faba* guard cell plasma membrane. The data were recorded from an inside-out patch to which a +86-mV potential was applied (V_m = −86 mV). Seal resistance was 25 GΩ. Each line of data presented represents approximately 1 s of channel activity. Two distinct channels are evident: an LC and an SC. Note the unusually high P_o of the SC. B, All-points amplitude histogram of single-channel data, a portion of which was presented in A. The peak at −2.4 pA corresponds to the state in which both channels are closed. The peak at −3.5 pA represents the current when the SC is open but the LC remains closed. Finally, the peak at −6.5 pA corresponds to the state in which both channels are open. Because of the high P_o of the SC, the situation in which only the LC was open was too rare to generate a distinguishable peak. In keeping with convention (Bertl et al., 1992), inward (downward) current is indicated as negative. C, Current/voltage relationships for the LC (●) and the SC (○) presented in A. Channel conductances were determined from the coefficients of linear regression equations fitted to the data. The reversal potentials for K^+ and Cl^- (−34.5 and +13.0 mV, respectively, as determined for the experimental solutions after calculating ionic activities) are indicated on the x axis. Channel activity was also monitored at +54 mV, as well as at +34 mV for the LC, but channel events were too infrequent to analyze.

regulation of anion channels by apoplastic malate is an integral component of the guard cell response to CO_2 (Hedrich et al., 1994). This hypothesis may be better tested when the cell wall and apoplast remain largely intact, as is the case with laser-assisted patch clamping. Preservation of the intact epidermal peel also should enable the experimenter to observe the effect of a stimulus on ion channel behavior and, simultaneously, record the effect of this stimulus on apertures of adjacent stomata.

It cannot be argued that laser microsurgery allows patch clamping in the complete absence of membrane perturbation, since the cell still undergoes plasmolysis and deplasmolysis. However, the retention of the cell wall should allow an assessment of channel function under conditions that more truly approximate the in vivo situation. Although dual-barreled voltage clamping (Blatt, 1991) also has this advantage, that approach is limited in that single-channel behavior cannot be directly measured.

The ability to use laser microsurgery for patch-clamp access to the higher plant plasma membrane has several important ramifications, some of which have been enumerated previously (De Boer et al., 1994). First, it will be feasible to study signal transduction pathways involving the cell wall or cell-wall components. Second, it may be possible to patch clamp cells for which enzymatic protocols for membrane access either have not been developed or have not resulted in protoplasts amenable to patch clamping (Fairley-Grenot and Walker, 1989). Third, nonspecific effects of cell-wall-degrading enzymes on plasma membrane characteristics (Morris et al., 1981; Browse et al., 1988; Hahne and Lörz, 1988; Chen and Boss, 1990) can be avoided. Fourth, because positional information is retained, it will be possible to access defined regions of the plasma membrane of an individual cell and evaluate whether channel distribution is uniform or asymmetric. Fifth, because this technique can be used to perform patch clamping in intact tissue, it will be possible to assess locus-specific patterns of ionic fluxes and intercellular signal transduction pathways. For example, in barley epidermis, it has been discovered that even morphologically similar epidermal cells may differ in their ion content in a site-specific manner (Fricke et al., 1995). The ion fluxes responsible for this patterning can only be studied by using a technique, such as laser-assisted patch clamping, that leaves positional information intact. For these reasons, laser-assisted patch clamping should prove to be a useful tool that will complement conventional patch-clamping and voltage-clamping techniques.

ACKNOWLEDGMENTS

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