Advanced patch-clamp techniques and single-channel analysis

Philip J. White¹, Bernhard Biskup², J. Theo M. Elzenga³, Ulrike Homann², Gerhard Thiel², Frank Wissing⁴ and Frans J.M. Maathuis⁵

¹ Department of Cell Physiology, Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK
² Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Universität Göttingen, Untere Karßpüle 2, D-37073 Göttingen, Germany
³ Department of Botany, University of Groningen, PO Box 14, Haren 9750 AA, The Netherlands
⁴ Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
⁵ Department of Biology, University of York, PO Box 373, York YO10 5DD, UK

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Abstract

Much of our knowledge of ion-transport mechanisms in plant cell membranes comes from experiments using voltage-clamp. This technique allows the measurement of ionic currents across the membrane, whilst the voltage is held under experimental control. The patch-clamp technique was developed to study the ionic currents flowing through single channels, but in its whole-cell configuration it is probably the most widely used voltage-clamp technique. This article provides a basic introduction to several advanced patch-clamp techniques, and evaluates methods for single-channel analysis. The initial sections describe the fabrication and use of perfusion pipettes, which enable the experimenter to manipulate the composition of solutions on both sides of the membrane, and a molecular-genetic approach, based on cell-specific expression of green fluorescent protein (GFP), that allows the experimenter to identify protoplasts from specific cell types for patch-clamp studies. This is followed by descriptions of the perforated-patch technique, which allows ionic currents to be determined in cells whose cytoplasm is relatively undisturbed and whose endogenous signal transduction cascades are intact, and the use of ‘macropatches’ for studying ion-transport processes in vacuoles which are too large for conventional patch-clamp techniques to be effective and are unsuitable for impaling electrodes. Methods for modelling the kinetics and permeation of ion channels using single-channel recordings are presented. How the ionic currents underlying an action potential (AP) can be identified using the AP-clamp method is discussed. Finally, the use of the patch-clamp technique to investigate endocytotic and exocytotic processes through measurements of capacitance is described. The advanced patch-clamp techniques presented in this article have the potential to broaden the horizons of plant cell electrophysiology and it is expected that many will become the standard laboratory techniques of the future.

Key words: Action potential clamp, Arabidopsis thaliana, endocytosis, exocytosis, green fluorescent protein (GFP), kinetics, macropatch, permeation.

Introduction

The patch-clamp technique, developed in the 1970s by Sakmann and Neher (1983), can be regarded as a high-resolution voltage-clamp method, based on the earlier two-electrode voltage-clamp technique. Initially pioneered in animal systems, patch-clamp technology is also now well established in plant transport research with reports describing, in various tissues and membranes, the characterization of ion channels (reviewed by Tyerman, 1992;...
Schroeder et al., 1994; Maathuis et al., 1997a), primary pumps (Coyaud et al., 1987; Hedrich and Schroeder, 1989; Davies et al., 1994) and secondary transporters (Maathuis and Sanders, 1994). Especially in combination with molecular-genetic techniques, such as the cloning of transporter genes and their heterologous expression (reviewed by Logan et al., 1997), patch clamping has enabled our understanding of the underlying mechanisms of ion transport across plant cell membranes to develop rapidly.

It is now necessary to progress from the cataloguing and biophysical characterization of transport mechanisms to the elucidation of their physiological roles. This will require experimentation on specific tissues and cell types, and an ability to study, characterize and predict the activities of transport processes under physiological conditions. Several new techniques (and old techniques, hitherto applied mainly in animal systems) will facilitate this process. For example, utilizing tissue-specific expression of reporter genes will enable the experimenter to identify, and compare, transport activities in protoplasts from contrasting cell types. The so called ‘perforated patch’ technique will enable the experimenter to study how various signal transduction pathways impact upon transport functions in an otherwise intact cell. Improved analytical methods and modelling of ion permeation will assist our understanding of such physiologically important parameters as channel gating and selectivity.

A broader familiarity and practical application to plant electrophysiology of these and other developments constituted the main aim of the workshop on ‘Patch clamp technology’ during the 11th International Workshop on Plant Membrane Biology. This paper summarizes the proceedings of the ‘Patch clamp technology workshop’ and discusses both practical and conceptual aspects of: (1) controlling experimental solutions by pipette perfusion, (2) GFP-based cell marking for patch-clamp studies, (3) the perforated patch technique, (4) the macropatch technique, (5) the analysis of single-channel activities, (6) the action potential clamp technique, and (7) assayng exocytosis and endocytosis by membrane capacitance.

Controlling experimental solutions by pipette perfusion

High current resolution in the sub-picoampere range can only be achieved after the formation of a high resistance seal to minimize background current noise. A tight membrane seal allows mechanical manipulation leading to four different patch clamp configurations: cell attached (CA), inside-out patch (IOP), outside-out patch (OOP), and whole-cell (WC). In principle, this allows the experimenter to access and modify experimental solutions on both sides of the membrane. However, in reality, experimental possibilities are restricted when using conventional (closed) patch-clamp pipette holders for several reasons: First, not all configurations can be obtained in one experiment. The experimenter may follow the sequence CA→WC→OOP or the sequence CA→IOP. In the former sequence the experimenter can modify the solution only on the external side of the membrane and in the latter sequence only the cytoplasmic solution can be changed. Second, the composition of physiological solutions is clearly different on either side of the membrane and, since it may often be impossible to obtain the desired configuration, one may end up with an inappropriate solution facing the membrane. Third, the WC configuration is frequently preferred to study factors modulating membrane currents, but the possibility of altering the composition of the cytoplasmic compartment in this configuration is limited. In theory, however, all these limitations are overcome by the perfusion of experimental solutions through the patch-pipette.

Maathuis et al. (1997b) modified commercially available patch-pipette holders (Biologic, Claix, France; Clark Electromedical, Pangbourne, UK; List, Darmstadt, Germany) to fabricate a perfusion pipette based on designs in the literature (Lapointe and Szabo, 1987; Bahinski et al., 1988; Neher and Eckert, 1988; Tang et al., 1992). They created an additional side port at an angle of approximately 30° (Fig. 1). This allowed the insertion of a capillary close to the tip that delivered a perfusing medium. Suction to the original port was used to obtain seals and to draw solution through the capillary. Typical gas chromatography columns were used as perfusion capillaries (e.g. fused silica, polyamide coated 150/230 μm ID/OD capillaries from Scientific Glass Engineering.

Fig. 1. Design of a perfusion pipette. A commercial pipette holder was modified by adding an additional side port for the insertion and manipulation of a capillary which delivers perfusion solution. Before experiments the capillary tip is placed near the pipette tip and grease is used to seal the extra side port. After formation of a seal, the tubing can be transferred to other reservoirs containing different solutions. This figure is reproduced from Maathuis et al. (1997b) by permission of Blackwell Science Ltd.
Milton Keynes, UK). Such capillaries are flexible and can be used many times without breakage. They can be hand-pulled over a Bunsen burner and broken back to the desired tip diameter. Tip diameter and capillary length will determine the rate of pipette perfusion, with small tips (<30 μm) creating a higher flow resistance and a higher risk of bubble formation at the tip. A large capillary tip will allow high flow rates, shortening the perfusion time, but cause more turbulence which potentially disrupts the seal. Maathuis et al. (1997b) attached the external end of the capillary to tubing which could be transferred from one perfusion solution reservoir to another. Both capillary and tubing were filled with solution prior to seal formation to prevent air bubbles occurring. The capillary tip was positioned within 100 μm of the pipette tip by slowly pushing it through the side port while viewing through the microscope. After positioning, the perfusion side port was sealed with vacuum grease to maintain an air-tight system. Obtaining a seal when the perfusion capillary is in place does not differ greatly from conventional procedures. However, large capillary tip diameters may create a lack of vacuum at the patch-pipette tip and cause turbulent flows of solution through the perfusion capillary. Between 5–10 mm Hg vacuum is needed to perfuse the pipette which takes between 30 s and 10 min (depending on the tip diameter of the perfusion capillary). Prolonged perfusion will ultimately fill the electrode holder with solution and this tends to increase noise levels.

Few reports have described the exchange of the pipette solution during a patch-clamp experiment on plant cells. Reifarth et al. (1994) used a small diameter teflon tube and a peristaltic pump to perfuse the pipette solution. In this way they were able to modify cytoplasmic Ca$^{2+}$ to assess the Ca$^{2+}$ dependence of the SV-type tonoplast channels of *Chenopodium rubrum* suspension cells. Maathuis et al. (1997b) used the system described above to study ion channels in the tonoplast and plasma membranes of *Allium cepa*, *Vicia faba* and *Arabidopsis thaliana*. In one experiment on the tonoplast of *Allium cepa* the initial (sealing) solution contained 50 mM KCl and large outward currents, typical for SV-type channels, were observed (Fig. 2). Removal of K$^+$ from the cytoplasmic side by pipette perfusion, reduced the single-channel conductance to near zero (some residual current may be carried by Ca$^{2+}$). Perfusion with K$^+$-containing and K$^+$-free solution could be repeated several times, with stable channel conductances being reached within 3 min. In another experiment it was shown that intracellular perfusion of the K$^+$ channel blocker tetraethylammonium (TEA$^+$) led to an almost complete inhibition of time-dependent K$^+$ currents in *Vicia faba* guard cell protoplasts (Maathuis et al., 1997b). In a separate study, Taylor and Assmann (1995) were able to perfuse the cytoplasm of *Vicia faba* guard cells with vanadate (5 mM) and inhibit the H$^+$-ATPase.

Although patch clamping using a perfusion pipette is technically more demanding and, therefore, more time-consuming, than the use of a conventional pipette, there are many benefits. First, since the basic biophysical characterization of a large number of ion channels has been completed, questions regarding their regulation and physiological functioning are becoming more pertinent. It is therefore imperative that experimenters are able to modify the cytoplasmic compartment with potential transport regulators, especially during WC recordings. The perfusion pipette will allow such manipulations. Second, in the same WC configuration, pipette perfusion will allow the delivery (or removal) of substrate for carrier-mediated transport on the cytoplasmic side. Therefore, simultaneous changes in substrate level on both sides of the membrane become feasible, circumventing the occurrence of artefacts associated with current versus voltage (I/V) curve subtraction when substrates are modified on one side of the membrane only (Blatt, 1986). A third benefit of pipette perfusion stems from the ability to use compositions of the pipette solution that improve the frequency of sealing, but are not necessarily compatible with subsequent experimental requirements, since the initial pipette solution can be exchanged after a seal is obtained.

**GFP-based cell marking for patch-clamp studies**

To patch-clamp plant cells, cell walls have to be removed to gain access to the membrane. Frequently this involves
enzymatic digestion of bulk tissues, such as entire roots or leaves. Clearly, this results in a preparation that contains protoplasts or vacuoles from a large range of different cell types. In a number of cases cell identification can be based on easily recognizable morphological features, such as cell size (Maathuis and Sanders, 1995) or the presence of chloroplasts (Elzenga et al., 1991), or tissues can be preselected by physical separation, such as stripping of the root cortex (Roberts and Tester, 1995; Wegner and Raschke, 1994) or the leaf epidermis (Schroeder et al., 1994). However, these approaches are frequently too crude to allow isolation of individual cell types.

The green fluorescent protein (GFP) from the jellyfish Aequorea victoria has been used as a ‘reporter gene’ in plants (Haseloff et al., 1997). By harnessing the expression of GFP to cell-type specific promoters, an approach is available to identify protoplasts derived from particular tissues. Promoters from well-characterized genes expressed in specific cell-types can be used to control the expression of GFP. Alternatively, ‘promoter trapped’ and ‘enhancer trapped’ mutants, which express GFP in specific cell-types, can be selected.

Maathuis et al. (1998) have pioneered the use of a GFP-based cell-marking technique for patch-clamp studies. They transformed Arabidopsis thaliana with a plasmid containing a GFP construct (m-gfp5-ER; Haseloff et al., 1997) under the control of a CaMV35S promoter. Transgenic plants expressing GFP showed no visible signs of deficiency or damage, but their growth rates were generally lower than that of wild-type Arabidopsis thaliana. The expression of GFP in transgenic plants was verified using an epi-fluorescence microscope with appropriate filters (excitation light wavelength 460–490 nm, emission light wavelength 520 nm) and a transgenic line (mGFP3), in which visible GFP fluorescence was restricted to stelar cells in root tissues (Fig. 3), was selected for study. High resolution confocal microscopy indicated that the intracellular location of the GFP signal was strongest in endoplasmic reticulum. The brightness and tissue location of GFP fluorescence was found to be largely independent of physiological plant age, with the exception that, in very young plants, some additional fluorescence was found in the root tips. Enzymatic digestion of roots from the mGFP3 transgenic line yielded fluorescent and non-fluorescent protoplasts (Fig. 4). This showed that the GFP signal observed in planta was strong enough to be detected at a cellular level. Approximately 22% (n = 137) of the protoplasts derived from roots of the mGFP transgenic line were fluorescent. The fluorescent and non-fluorescent protoplasts had average diameters of 11 μm (n = 49) and 24 μm (n = 52), respectively, which reflects the relative sizes of stelar and cortical cells in intact Arabidopsis roots and strongly supports a stelar origin for the fluorescent protoplasts.

The ability to differentiate between stelar (small, fluorescent in line mGFP3) and non-stelar (large, non-fluorescent in line mGFP3) protoplasts enabled Maathuis et al. (1998) to compare K⁺ currents in stelar and the transgenic mGFP3 line using the patch-clamp technique. The presence of GFP in the cytoplasm of fluorescent protoplasts did not appear to affect seal formation. In a previous study Maathuis and Sanders (1995) had characterized the main inward rectifying (IRC) and outward rectifying (ORC), time-dependent...
K⁺ currents in large protoplasts (diameter > 20 µm) from presumptive non-stelar cells of *Arabidopsis* roots. In these protoplasts ORC activity (unitary conductance of 38 pS in 100/10 [K⁺]ᵢ/[K⁺]ₒ) was observed in most cells, whereas the whole-cell patch-clamp technique su

vers from aprotoplasts ORC activity (unitary conductance of 38 pS phenomenon called 'run-down' or 'wash-out'. This isin 100/[K⁺]ᵢ/[K⁺]ₒ out) was observed in most cells, manifested as a change in membrane current during the where the dominant, time-dependent, IRC (unitary course of an experiment due to the exchange of the conductance of 6 pS in 100/[K⁺]ᵢ/[K⁺]ₒ out) was less cytoplasm and the pipette solution. The change in whole-frequently observed (Maathuis and Sanders, 1995). In whereas the incidence of ORC activity was comparable in small protoplasts (approximately 35% of all cells), with no significant difference being observed in the incidence of ORC activity between small and large protoplasts (Maathuis et al., 1998). Thus, the occurrence of ORC activity in stelar cells was reflected in both single-channel and whole-cell recordings. In contrast, the incidence of IRC activity was comparable in protoplasts derived from stelar and non-stelar tissue (approximately 55% of all cells). However, a corresponding comparison of IRC and ORC activities in small (average diameter 12 µm), presumably stelar, and large (average diameter 19 µm), presumably non-stelar, protoplasts derived from roots of wild-type *Arabidopsis* showed the same pattern: the incidence of IRC activity was greater in small (stelar) protoplasts compared to large (non-stelar) protoplasts (approximately 35% and 60% of all cells, respectively), with no significant difference being observed in the incidence of ORC activity between small and large protoplasts (Maathuis et al., 1998). Thus, the phenomenon observed in the transgenic mGFP3 line was genuine and not an artefact created by (high levels of) GFP expression. Although the physiological significance of this observation is not yet clear (Maathuis et al., 1998) a high prevalence of IRC activity in stelar cells was previously found in the xylem parenchyma of barley (Wegner and Raschke, 1994).

There is great potential in combining GFP-based cell marking and patch-clamp technology. It can be anticipated that this approach will be developed in many laboratories to target cell-types of particular interest. With an epi-fluorescence facility, GFP-expressing protoplasts of known origin can be assayed directly by patch-clamping, without the need for morphological uniqueness or physical dissection. Furthermore, as an ever-increasing number of transgenic plants showing a multitude of spatial and temporal patterns of GFP-expression are made available, the characterization of ion transport activities in many specific cell-types, and of contrasting physiological or developmental status, will become possible.

**The perforated patch technique**

The whole-cell patch-clamp technique suffers from a phenomenon called 'run-down' or 'wash-out'. This is manifested as a change in membrane current during the course of an experiment due to the exchange of the cytoplasm and the pipette solution. The change in whole-cell currents is caused, for instance, by loss of second messengers, changes in enzyme activity or modification of the phosphorylation state essential for the activation of ion channels. To prevent 'wash-out' the perforated patch technique was developed (Horn and Marty, 1988). The perforation of the patch of membrane under the pipette is established by pore-forming compounds added to the pipette solution. Most often polyenes, such as nystatin and amphotericin B (AmB), are used. The pores formed allow the passage of monovalent ions, thus establishing electrical access to the cell interior, but limit the diffusive exchange of divalent ions, compounds with a molecular weight greater than 200 (thus including most intracellular biochemicals and metabolites, second messengers and Ca²⁺). The perforated patch technique allowed recording of stable whole-cell currents for up to 3 h in animal cells, much longer than was possible with the normal whole cell configuration.

**Application of the perforated patch technique to plant cells**

The technical details of the perforated patch technique have been described in several papers (Korn et al., 1991; Rae, 1991; Rae et al., 1991; Verheyck, 1998). Although specific procedures may differ, they are all essentially similar and emphasize certain aspects that appear to be critical for the successful application of the perforated patch technique. The two successful protocols devised for plant cells also reiterate these points, namely: (1) that both nystatin and AmB are very photolabile and should be shielded from the light throughout the procedure; (2) that a stock solution in DMSO should be made fresh every 3–4 h, as even at −20 °C the stock solution will lose its activity; and (3) that nystatin and AmB interfere with seal formation and, before filling the pipette with the polyene-containing solution, the tip should be filled with polyene-free solution.

After the successful use of nystatin and, later, AmB on animal cells, several groups attempted to use the perforated patch technique on plant cells. Most efforts were unsuccessful and, apart from a study using a porin (haemolysin) from *Escherichia coli* (Schroeder, 1988), the technique proved enigmatic with plant membranes. However, in a recent survey, two groups reported success in obtaining low access resistance on plant protoplasts (W Gassmann, J Schroeder, B Linder, and K Raschke, personal communications). The few examples of the successful application of the perforated patch technique to...
plant membranes suggest that there is no fundamental difference between plant and animal cells that prevents the formation of pores. However, the general opinion is that the resistance of plant membranes to treatment with polyenes is due to the absence of cholesterol. The basis for this belief is the traditional model for polyene action, in which the binding of the polyene to a sterol in the membrane facilitates the formation of pores. Pores are thought to be formed by a complex of 8–12 polyene molecules and a similar number of sterol molecules. The binding of polyenes to sterols is very specific. For example, the use of nystatin and AmB in the treatment of fungal infections is due to their high affinity for ergosterol (a sterol found exclusively in fungi), but their toxic effects on patients, which limit their application, are caused by a lower, but still significant, affinity for cholesterol. This binding to cholesterol is assumed to be essential for the formation of pores by polyenes in the perforated patch technique in animal cells.

Paradoxically, AmB does not have a higher association constant with cholesterol than with stigmasterol, one of the main sterols in plant membranes (Backes and Rychnovsky, 1992). So, if binding of polyenes to sterols in the membrane is the main factor determining the formation of pores, plant membranes should be compatible targets. More recent models of AmB action might give an indication as to how this paradox can be resolved. To accommodate results from experiments on membrane vesicles that do not agree with the traditional model, AmB has been suggested to interact with the membrane in several different ways (reviewed by Hartsel et al., 1993). In addition to the classical single-sided barrel pore, a pore can be formed by association of two barrels on opposite sites of the membrane and, by a third mechanism, membranes can be made permeable by defects caused by AmB aggregates. The first two mechanisms are assumed to be the primary mechanisms affecting ergosterol containing membranes, while the third mechanism is also proposed to affect membranes lacking ergosterol.

The first two mechanisms are most effective when the AmB in solution is kept under the critical micellar concentration (CMC < 10^{-6} M), while AmB must be present in a concentration above the CMC for the aggregates to form. In perforated patch experiments one normally uses a concentration of 80–180 μM, which is a hundred times higher than the CMC. While the classical models, describing the specific interaction of AmB with ergosterol in a barrel-shaped pore, all relate to the first two mechanisms, the role of sterols in the ‘membrane defect’ mechanism is still unclear.

If the difference in binding properties of cholesterol and stigmasterol is not the reason for the difficulty in obtaining the perforated patch configuration in plant protoplasts, then what is? An obvious difference between experiments on mammalian cells and plant cells is the temperature at which they are performed. Electrophysiologists maintain animal cells at 37 °C, while most plant cells are kept at room temperature, and often much lower, as this facilitates the formation of a gigaOhm seal. Increasing the temperature has a dramatic effect on the ability of polyenes to affect the integrity of plant membranes (Fig. 5). This observation confirms, together with the two successful applications of the perforated patch technique on plant cells, that there is no fundamental difference between animal and plant cells that prevents the interaction of polyenes with plant membranes. By optimizing the experimental conditions, including solute composition and temperature, the perforated patch technique could become a practical tool in plant electrophysiology.

Prospects for the perforated patch technique

If the protocol for the perforated patch technique can be optimized for use on plant cells its value for studying the effects of external stimuli, such as microbial elicitors, light and temperature, on ion transport processes is self-evident. It allows a relatively undisturbed cytoplasmic composition, which is a prerequisite if soluble cytoplasmic second messengers are involved in the signal transduction. Furthermore, using the perforated patch technique on cells that have been preloaded with fluorescent probes enables the simultaneous measurements of membrane currents and cytoplasmic pH or Ca^{2+}. This experimental design has the advantage over the currently used method of loading the protoplast with the fluorescent dye through the pipette, that, as polyene pores are impermeable to fluorescent probes, slow changes in pH or Ca^{2+} could be correlated with changes in membrane conductance.

Application of the macropatch technique to plant vacuoles

Macropatches, or ‘giant’ membrane patches, have been successfully used to study ion channel and pump currents across the plasma membrane of various animal cells, including *Xenopus* oocytes and cardiac myocytes (Stühmer, 1992; Hilgemann, 1994). The macropatch technique allows the fast voltage-clamping of large membrane areas, usually from cells that are too big to voltage-clamp in the whole-cell mode using a conventional patch-clamp configuration (e.g. >50 μm cell diameter for the EPC-7 amplifier from List-Medical, Darmstadt, Germany). Studying membrane patches of between 5 and 15 μm diameter allows good capacitance compensation, and the amplitude of currents remains moderate, thereby keeping voltage errors small. Also, the macropatch technique prevents any space clamp problems (Armstrong and Gilly, 1992).

The low resistance of the pipettes employed (between
Fig. 5. The effect of temperature on membrane integrity of pea (Pisum sativum) epidermal cells treated with polyenes. Photographs: the vitality of pea epidermal cells incubated in a medium containing 150 mM KCl and 10 mM HEPES/Tris pH 7.2 (A) plus 80 μM amphotericin at 37 °C for 40 min, (B) plus 80 μM amphotericin at 0 °C for 40 min, and (C) without amphotericin at 37 °C for 60 min. Vitality of cells was determined by staining with fluorescein diacetate, which stained the cytoplasm of the cells with intact membranes (yellow), and propidium iodide, which stained the nucleus of cells with reduced membrane integrity (orange/red). Graph: the effects of temperature and nystatin on cell viability. Viability was determined by staining after incubating the cells with 80 μM nystatin at 37 °C for different periods. As a control, cells were treated with nystatin at 0 °C and were kept in media without nystatin at 37 °C and at 0 °C. In all controls, little decrease in viability was observed.

300 kΩ and 2 MΩ) together with the small membrane capacitance allows for a very fast voltage clamp, with a potential frequency of several hundred kHz (Hilgemann, 1995). As with single-channel patch-clamping, macropatches provide a quick and effective way of controlling the solute composition at both membrane faces. Obviously there is no need to wait for equilibration of the internal lumen with the pipette solution. The use of macropatches also allows the single-channel analysis of low-abundance channels, providing that they can be separated from more frequently occurring channels.

However, the fabrication of pipettes for patch-clamping macropatches can be more demanding since it involves an extra step to break the pipette tips to the large sizes needed. In general, larger pipette tips lower the success rate in obtaining good and stable seals. In addition, the seal resistance tends to be lower than with small membrane patches, due to a linear increase of leak currents with increasing patch diameter, thereby increasing the noise in the recordings. Also, excision of the macropatch often leads to the formation of vesicles, which can be detected by changes in capacitance and by ill-defined currents. Furthermore, the membrane capacitance can be difficult to measure, since the actual patch in the pipette often is omega-shaped, and not always visible. As with most electrophysiological techniques, much empirical work needs to be undertaken to optimize the method.

The macropatch technique: a practical example

In plants, recordings from large membrane patches have been used successfully to study malate currents and vacuolar SV channels (Cheffings et al., 1997; Pottosin et al., 1997). The macropatch technique was applied to vacuoles of leaf mesophyll cells of Mesembryanthemum crystallinum, a succulent halophyte (Fig. 6). These vacuoles are between 40 and 200 μm in diameter, and the total membrane resistance is often as low as 200 MΩ. Because of their size, these vacuoles are difficult to perfuse, and the exchange of the luminal contents of the vacuole with the pipette solution is very slow. For example, with a 7 MΩ access resistance of the pipette and a vacuole of 70 μm diameter, the time constant for the exchange of the luminal contents of the vacuole with the vacuole solution is around 22 min. With a 40 μm vacuole, it is less than 5 min (Pusch and Neher, 1988; F Wissing, unpublished data). Large voltage steps often generate currents that can exceed the maximum range of the amplifier and cause significant voltage errors. Since the tonoplast membrane is too plastic to allow impalement with sharp microelectrodes, the macro-
patch technique has been used to record currents that reflect whole-vacuole currents.

A major difference between the macropatch technique and the conventional patch-clamp technique is the diameter of the pipette tips. Pipettes with an opening of up to 15 μm diameter, giving resistances between 300 and 800 kΩ in 100 mM KCl solutions, have been used successfully. While smaller tips with resistances around 2 MΩ or more could easily be pulled on a conventional two-stage electrode puller, for larger openings the method described by Hilgemann (1995) was used. In brief, a bead of soft glass (i.e. with a high lead content) was heated on the platinum wire of a microforge, and then a pipette was pushed into the molten glass. This pipette had been pulled previously just like a conventional patch pipette with a short taper. After 2–3 s, the pipette was retracted swiftly, giving a clean break of the tip. The pipette was then polished, until the rim was clearly rounded off.

The best seals were obtained using thin-walled borosilicate microcapillaries (Sigma Chemicals Co., Poole, UK) with a wide lumen (OD 1.72 mm, ID 1.36 mm). This helps to provide a larger reservoir to prevent the pipette from running dry under positive pressure. Also, it reduces the capillary forces that might otherwise suck the vacuole up into the pipette once positive pressure is released. Various other types of thick-walled borosilicate and softer glass types did not give good seals or were difficult to shape. The pipettes were coated by dipping them into inlay casting wax (Kerr UK Ltd., Peterborough, UK) while maintaining slight positive pressure (Cheffings et al., 1997). Any wax blocking the tip was melted on the microforge using a brief pulse of heat. This method also provides a thin hydrocarbon coating inside the pipette that facilitates sealing to the membrane.

As with conventional patch-clamping, the pipettes were filled with filtered solutions containing 1 mM Ca²⁺, 2 mM Mg²⁺ and high K⁺ to promote seal formation (Maathuis et al., 1997b). Low K⁺, even later on during bath perfusion, often led to poor sealing of the membrane and loss of seal. To obtain a seal, the pipette was placed above the vacuole and, while maintaining slight positive pressure, slowly moved down onto the vacuole. At this stage, vacuoles often started spinning, and with further descent of the pipette the membrane bulged inwards where the outflowing pipette solution hit the membrane surface. Subsequent release of the positive pressure initiated seal formation. In most cases, seal formation took between 2 min and 10 min. A slight negative pressure and a negative pipette potential around −20 mV facilitated sealing. Under the light microscope the appearance of an omega-shaped membrane patch in the pipette could be observed, slowly moving upwards into the pipette (up to 15–25 μm). The final seal resistance typically reached between 0.5 and 2 GΩ. The currents observed were between 0.5 and 5 nA in the vacuole-attached configur-
tion, and no single channels could be resolved (Fig. 6A). On excision, the outward-current amplitudes generally became much smaller, while the inward currents hardly changed (Fig. 6B). The seal resistance remained constant and single-channel currents were not usually resolved.

Occasionally, seals of several $G_0$ were formed within seconds. In these cases, only a small omega-shaped patch, if any, could be seen. The currents observed in these patches were much smaller ($< 100 \text{ pA}$) and single channels with a conductance of around $40 \text{ pS}$ could clearly be resolved (Fig. 6C).

To measure the capacitance of a macropatch, Hilgemann (1995) suggested two methods. First, the total capacitance of pipette and patch is measured using the capacitance compensation. Then the patch is ruptured and the tip sealed with a parafilm-mineral oil mixture. Thus, measuring the pipette capacitance and subtracting it from the total capacitance gives the membrane capacitance. Another way to measure the patch capacitance is to fully compensate the total capacitance with the seal intact and the pipette tip just below the bath surface. By lifting the pipette out of the bath and thereby rupturing the seal, the change in capacitance gives an upper estimate of the patch capacitance.

Future prospects

While the method described here has given some favourable initial results, the conditions need to be further optimized to increase the seal rate and to make the size and shape of the membrane patches more predictable. In particular, the formation of deep omega-shaped membrane patches can lead to problems with efficient perfusion of the vacuolar membrane face. Furthermore, varying the Ca$^{2+}$ concentration can have an effect on the formation of vesicles, as well as on the sealing in general. As our results have shown, this method has great potential for studying ion channel and pump currents from cells and vacuoles that are too large for reliable voltage-clamping with a conventional patch-clamp. This should allow the study, for example, of vacuolar ion transport in the giant epidermal bladder cells of *Mesembryanthemum crystallinum*, which can be up to 2 mm in diameter. The same is true for several types of large algal cells, for which the macropatch technique can provide an alternative to the use of microelectrodes. In addition, the potentially low time constant of the voltage-clamp should allow recordings with very high temporal resolution, allowing more detailed studies of gating currents of ion channels and transporters in plants.

The analysis of single-channel current recordings

The analysis of single ion channel currents has been underexploited in plant sciences. However, this approach alone allows ion channel activities to be resolved at the molecular level, which is required for any detailed understanding of the underlying molecular mechanisms.

Researchers seek to define, under controlled conditions, two basic properties of ion channels: their gating, which may be understood as the kinetics of transitions between discrete conductance states of the channel, and their selective permeability. Temporal analysis of single-channel recordings provides the data for kinetic models and estimates of unitary current are used to develop permeation models. Both can be incorporated into broader models, such as those considering the electrocoupling between ion transporters in membranes, which have physiological implications.

Analysis of single channel kinetics

The temporal analysis of single-channel current recordings provides a means to determine the kinetic schemes underlying, for example, the processes of channel gating or ionic blockade. Several methods are available for estimating the life-times of distinct kinetic states, and the rate constants for transitions between each state.

The textbook method of analysis begins by attempting to reconstruct a noise-free record. This can be done by filtering the data with a high-order Bessel filter and using threshold detection (Colquhoun, 1994) or by using a high-order Hinkley detector (Schultze and Draber, 1993). Rate parameters can then be estimated from the distributions of dwell times at various current levels (see Hanson et al., 1997, and Blunck et al., 1998, for a discussion of methods). This approach works well if the mean lifetimes of the kinetic states are lengthy, but as mean lifetimes decrease it eventually becomes unreliable, because there are too many missed events.

Life-times of channel conformations between 1 ms and 1 $\mu$s can be estimated from current-amplitude frequency distributions. These become distorted from a Gaussian to an asymmetric distribution, which approximates a beta distribution, within this temporal range. Life-times of kinetic states can be estimated from current-amplitude frequency distributions both empirically for two-state kinetic models, by comparing simulated and experimental data (Yellen, 1984), and numerically, for multistate kinetic models when data have been filtered by a single-pole filter (Rießner and Hansen, 1995). A method which can incorporate both multiple kinetic states and adjustable filtering of the data using an 8-pole Bessel filter was developed recently by White and Ridout (1998). This method simply compares the observed distributions of current amplitude with theoretical distributions derived by combining several simplifying assumptions about the underlying stochastic process with a model of the filter and electrical noise.
Models of ion permeation

Goldman-Hodgkin-Katz (GHK) electrodiffusion. The GHK current equation describes explicitly the effects of the concentrations of permeant ions on unitary current versus voltage (I/V) relationships, and the GHK voltage equation allows the calculation of apparent permeability ratios from measurements of the current reversal potential ($E_{\text{rev}}$) under defined ionic conditions (Lewis, 1979; Hille, 1992). However, the assumptions embedded in GHK theory are inappropriate for most ion channels (Hille, 1992; Eisenberg, 1996; Gradmann, 1996). Firstly, ion permeation is not independent. There is often no unique diffusion coefficient for an ion and apparent permeability ratios vary with ionic concentrations. Both currents and unidirectional fluxes saturate, and flux ratio coupling factors greater than unity are observed. Second, the electrical field does not drop linearly across the channel since channel structure contributes to the electrical field (for example via dielectric forces, local dipoles and local charges within the pore) and, since the electrical fields of permeating ions perturb local ionic fields, changes in experimental conditions are likely to affect the electrical field across the channel. For these reasons the application of GHK equations to estimate, or even to express, permeability is inappropriate. Permeability can only be described by determining appropriate parameters of more complex models.

The dynamic-pore. The dynamic-pore (cyclic) reaction-kinetic description of ion permeation envisages a selectivity filter within a channel pore in which a single binding site alternates its orientation between one side of the membrane and the other within a fraction of the electrical distance and in a rate-limiting fashion (Gradmann, 1996; Gradmann et al., 1997; Allen et al., 1998). The translocation of the charged substrate is assumed to be the only voltage-dependent reaction step within the selectivity filter.

Dynamic-pore models can fit diverse I/V relationships and have been used to describe Ca$^{2+}$ and K$^+$ permeation through tonoplast cation channels (Gradmann et al., 1997; Allen et al., 1998). They can predict intrinsic current saturation at extreme voltages, but this is not exclusive (Gradmann et al., 1997). Total current, unidirectional fluxes and net fluxes are non-monotonic functions of the applied voltage, and maxima and minima can exceed saturation levels. Their properties can be summarized by two limiting cases (Gradmann, 1996): in dilute solutions, the transport of ions is independent and the slow reorientation of the unoccupied binding-site is significant. In this case the system may obey the GHK constant-field equations. As ionic concentrations are increased, the binding-site is rarely unoccupied and the system behaves as an antiporter. If the system behaves as an antiporter, conventional calculations of permeability ratios from $E_{\text{rev}}$, using the GHK voltage equation may yield inappropriate values and the reversal voltages for ionic fluxes may differ significantly from their Nernst equilibrium voltages.

Software developed at Göttingen (Enzyme Kinetics of Ion Transport) for estimating rate constants for the dynamic-pore model is available through the Internet (http://www.biophysik.uni-goettingen.de/EKIT.shtml).

Free-energy barrier models. These have been termed ‘rigid-pore’ models. They assume that ions pass in single file through a corrugated free-energy profile, which has two additive components: one originating from the structure of the pore and the other from a constant electrical field (Hille, 1992). Two classes of models are distinguished: one-ion pores, which can contain only one ion at any time, and multi-ion pores, which can contain several ions simultaneously. Models of multi-ion pores frequently incorporate a parameter to describe the interaction (repulsion) between ions within the pore. Software to compute multi-barrier, multi-occupancy permeation models for both monovalent and divalent cations from I/V relationships is available (Alvarez et al., 1992; White and Ridout, 1995, 1999).

One-ion pores rarely conform to the GHK equations. However, they obey the rules of independence and conform to Ussing’s flux ratio criterion (Hille, 1992). One-ion pores produce saturation of ionic fluxes at high concentrations and apparent permeability ratios (determined using the GHK equation) which may vary with ionic conditions. By contrast, multi-ion pores do not obey the rules of independence. Interactions between ions within a multi-ion pore can produce a variety of I/V relationships, complex relationships between conductance and ion activity, flux ratio exponents greater than unity, concentration-dependent apparent permeability ratios, anomalous mole fraction effects (where channel conductance or $E_{\text{rev}}$ goes through a minimum as a function of the ratio of ionic concentrations of two permeant ions) and co-operative, steeply voltage-dependent block by small ions. In general, multi-ion pore models are expected to be considerably more versatile, and provide better fits to data, than single-binding site models.

One frequent application of the one-ion pore model is to describe voltage-dependent blockade of channels by both impermeant and permeant ions, following the precedent of Woodhull (1973). Woodhull assumed a pore with a single binding site and energy maxima located halfway between the external solution and the energy minimum. As a result the rate constants for blockade and relief of blockade by impermeant ions had equal and opposite voltage-dependence. This is rarely observed, suggesting that energy maxima are sited asymmetrically between the external solution and energy minimum (Tikhonov and Magazanik, 1998). A voltage-dependence of blockade (1/$K_d$) by an impermeant monovalent ion
greater than $e$-fold ($e = 2.72$) per 25 mV at 20 °C, or of a rate constant greater than twice this, suggests a more complex pore structure. Such voltage-dependencies require interpretation by dynamic-pore or multi-ion pore models. A prominent influence of ion concentration on voltage-dependence is also suggestive of complex pores (Tikhonov and Magazanik, 1998).

Poisson-Nernst-Planck (PNP) electrodiffusion. Ultimately, it might be useful to consider permeation models based on molecular structure, such as those involving solutions to the Poisson-Nernst-Planck (PNP) equations (Eisenberg, 1996; Nonner et al., 1998; Nonner and Eisenberg, 1998). The PNP equations provide a self-consistent theory for electrodiffusion. Their application promises to yield detailed information about the permanent charge profile within the pore and unique diffusion coefficients for permeant ions, which are related to the pore's frictional selectivity. Unfortunately, the application of such models is limited at present, not only by the requirement for great computational power but also by the paucity of structural information regarding pore dimensions. However, pharmacological methods of estimating channel length and pore diameter are available and the profile of permanent charge is open to manipulation via molecular-genetic techniques. The PNP equations can be solved numerically, and software is available through the Internet to illustrate PNP theory (http://144.74.27.66/pnp.html).

Electrocoupling ion transport activities

Knowledge of channel gating kinetics and permeability can be used to predict the ionic fluxes and currents passing in physiological conditions, and can also be incorporated into electrocoupling models of membrane electrical activities. All membranes can be viewed as dynamic systems of ion transport mechanisms coupled via the membrane voltage. Ion channels are integral components of this system. Not only do they influence membrane voltage through the ionic currents they mediate, but their activities are also regulated by membrane voltage. Software allowing the exploration of electrocoupling between ion transporters is available (Gradmann and Buschmann, 1997).

The electrocoupling of ion transport mechanisms at the plasma membrane has been studied in the context of osmotic adjustment (Gradmann and Buschmann, 1997) and cell signalling (White and Ridout, 1999). Recent models incorporating five electroenzymes (H\(^+\)-pump, OR and IR K\(^+\) channels, Cl\(^-\) channels and Cl\(^-\)/2H\(^+\) symporter) suggest that, under constant environmental conditions, the system converges either to a steady state (where the membrane potential equals the Nernst potential for K\(^+\) ($E_\text{K}$) and Cl\(^-\) influx equals Cl\(^-\) efflux) or to stable oscillations. Two oscillations are distinguished: the first type, whose frequency is sensitive to the velocities of the gating processes of Cl\(^-\) transporters, occurs without intrinsic concentration changes and the second type, whose frequency is proportional to the surface/volume ratio of the system, has intrinsic concentration changes (Gradmann and Hoffstadt, 1998). Models investigating the coupling between membrane potential and voltage-dependent Ca\(^{2+}\)-permeable channels suggest that these are credible mechanisms for intracellular signalling (White and Ridout, 1999).

Studying ionic currents during an action potential with action potential clamp

The membrane voltage of many plant cells can change by some 100 mV in the time range of a few seconds (for a review see Pickard, 1973). A goal for electrophysiologists is to understand these voltage excursions on the basis of changes in plasma membrane permeability to ions. Following Hodgkin and Huxley’s (1952) investigation of the action potential (AP) in the squid axon, plant physiologists have attempted to uncover the changes in ionic permeabilities which underlie the AP or AP-like voltage changes in plant cells. In voltage-clamped cells rectangular test voltage steps cause current changes with characteristic activation and inactivation kinetics (Beilby and Coster, 1979a). From the voltage-dependent time-course of these currents their contribution to the AP could be anticipated. The kinetic information contained in the time-course of the rise and decay of individual currents at various voltages has been used in a few cases to reconstruct the time-course of the free running voltage and the dynamics of permeability changes underlying the AP mathematically (Beilby and Coster, 1979b; Mummert and Gradmann, 1991).

Animal physiologists developed an experimental method, termed action potential clamp (AP-clamp), which circumvents the elaborate mathematical approach. This allows the time-course of the changes in current and permeability which occur during an AP to be determined in a more straightforward manner (Starzak and Starzak, 1976; Doerr et al., 1989; De Haas and Vogel, 1989; Ibarra et al., 1991).

The action-potential-clamp technique: theory and practice

The underlying observation behind the AP-clamp method is that the net current through the membrane at the free running voltage is always zero. When, for example, during an AP the permeability of the membrane is transiently increased to ions, both inward and outward current increase in an antiparallel and symmetrical fashion, and the sum of the currents is at all times zero (Hodgkin and Huxley, 1952). Thus, no clamp current is required to clamp the cell to the voltage-course of its own AP. If an
ion-transport pathway is blocked pharmacologically, the clamp circuit compensates for the missing current through the blocked ion-transport pathway. Hence, in the presence of a specific blocker the time-course of the clamp current mirrors the current which would occur during an AP through the blocked ion-transport pathway.

The first step in AP-clamp is to record an AP. This AP may be spontaneous or externally stimulated. The nature of the stimulus is not important. Digital storing can be accomplished either by a computer or on a video- or DAT-recorder. When the cell is at rest, the stored AP is fed into the voltage command input of the clamp circuit. Originally the AP-clamp method was used with conventional two (intracellular) electrode voltage-clamp techniques (Starzak and Starzak, 1976), but recent reports demonstrate that patch-clamped cells can also be investigated by AP-clamp (Doerr et al., 1989; Ibarra et al., 1991).

Both practical and analytical features of the method are best explained in the context of an example (Fig. 7). In the experiment illustrated, an AP was stimulated in a *Chara* cell by a 100 ms long voltage-clamp step from the resting voltage to +25 mV (Fig. 7A). The AP was recorded with a high impedance amplifier and stored digitally. After repolarization of the cell, the stored AP was fed into the clamp circuit as command voltage. During any one AP-clamp cycle, the feedback circuit clamped the membrane to the shape of the stored AP. This resulted in an initial transient current that resembled the current input which stimulated the original AP (Fig. 7B, inset). This is to be expected. Most significantly, however, during the remaining time in which the cell was clamped to the recorded form of the AP, the clamp current was close to zero (Fig. 7B). This shows that the shape of the AP in *Chara* is highly reproducible. Cells in which the shape of the AP is not reproducible are not suitable for investigation with the AP-clamp technique.

If the clamp current is small under control conditions (e.g. 5% of the current under investigation), an inhibitor may be added to the bathing medium to block a conductance of interest. The best results are obtained with rapidly acting channel blockers with high specificity (de Haas and Vogel, 1989; Thiel, 1995), but antagonists which act indirectly on the activity of an ion-transport pathway also appear to be suitable. In our example, the *Chara* cell was challenged with 200 μM neomycin sulphate. This inhibitor is known to block the activity of phospholipase C in animals and plants (Epstein et al., 1985; Chen and Boss, 1991) and, thereby, to interfere with Ca\(^{2+}\) dynamics (Franklin-Tong et al., 1996). In test experiments neomycin prevented electrical excitation in *Chara*. Some minutes after the application of neomycin, an outward current became apparent when clamping the cell to the stored AP (Fig. 7C). Subtraction of the current recorded in the presence of neomycin (Fig. 7C) from the control (Fig. 7B)
gave the time-course and the magnitude of the neomycin-suppressed current which passes the plasma membrane during the AP (Fig. 7D). A common problem in these experiments is the great scatter of currents during the period of AP-stimulation (dotted line in Fig. 7D), which probably results from a loss of accurate voltage clamp control during the large command signal. For this reason, currents during this period were systematically discarded. The neomycin-suppressed inward current (Fig. 7C) was similar in its kinetics and magnitude to the niflumic acid blockable Cl\(^{-}\) current which passes the membrane during the AP in *Chara* (Thiel, 1995; Thiel et al., 1997). It can, therefore be argued that neomycin inhibits the activation of Cl\(^{-}\) channels in *Chara*, probably by interfering with Ca\(^{2+}\)-dynamics.

Further information on the AP-associated Cl\(^{-}\) conductance can be extracted from the data presented in the example: first, the time-course of the current reflects the kinetics of single-channel activation and inactivation during an AP (Thiel, 1995). From the voltage and current data, the time-course for the changes in permeability of the membrane to Cl\(^{-}\) can be estimated using the constant-field equation (de Haas and Vogel, 1989; Thiel, 1995; Thiel et al., 1997). In addition, the current integral (the area beneath the solid line in Fig. 7D) is a measure of the amount of Cl\(^{-}\) translocated during an AP. The values calculated from AP-clamp experiments match those estimated from radiotracer fluxes during excitation (Thiel, 1995). Finally, the current obtained by means of the AP-clamp can be plotted directly versus voltage. This gives a time-averaged \(I/V\) relation for the contribution of the inhibitor-sensitive current to the AP (Fig. 7E). In the example, the current does not reverse. But the curve can be tentatively extrapolated (dashed line) to a reversal voltage at about zero mV. This is the probable reversal voltage for Cl\(^{-}\) currents across the plasma membrane in these cells (Thiel et al., 1997).

In combination with other inhibitors, the same procedures can be used to unravel the time-course of changes in other ionic currents, for example K\(^{+}\) or Ca\(^{2+}\) currents during the AP. The sequential application of inhibitors, which block different types of ion-transport pathways, can be used to uncover differences in the kinetics of permeability changes for different ion species (de Haas and Vogel, 1989; Thiel, 1995).

**The limitations and applications of AP-clamp**

One obvious limitation for resolving currents with the AP-clamp technique is the specificity of the inhibitor. Also, resolution of currents is determined by the relative magnitude of the inhibitor suppressible current compared to the background clamp current under control conditions (e.g. the difference between the currents in Fig. 7C and Fig. 7B). The more reproducible an AP is, the smaller will be the background clamp current and the better will be the ability to resolve small currents.

In the conventional form described here, the AP-clamp has limited application to plant cells. It has been mentioned throughout that a voltage change based, at least macroscopically, on a strictly voltage-dependent opening and closing of ion channels is an essential requirement of the technique. In other words, repetitively triggered APs, or AP-like voltage fluctuations, should be reproducible, and should always have the same shape. The voltage changes observed in plant cells rarely meet this criterion (Pickard, 1973). However, the averaging of many APs could be usefully employed, the mean AP then being used as command voltage in the clamp circuit to improve the resolution of currents (de Haas and Vogel, 1989).

The idea behind the AP-clamp technique may be used for other purposes too. One example, provided by Grabov and Blatt (1998) is to use the AP-clamp technique to investigate whether Ca\(^{2+}\) oscillations in guard cells are the cause or the result of voltage oscillations. Another example would be to test, and improve, theoretical models (Gradmann et al., 1993) that explain voltage relaxations in cells on the basis of activation and inactivation of voltage-sensitive channels, by the application of this technique in vivo.

**Assaying exocytosis and endocytosis by membrane capacitance**

In addition to investigations of ion movement across the plasma membrane and vacuolar membrane, the patch-clamp technique has also been used to study exocytosis and endocytosis by monitoring membrane capacitance (Battey et al., 1996; Homann and Tester, 1998).

The surface area of the plasma membrane depends on the balance between exocytosis and endocytosis. Exocytosis results in the incorporation of vesicular membrane into the plasma membrane, leading to an increase in cell surface area. During endocytosis, parts of the plasma membrane are retrieved, resulting in a decrease in surface area. Changes in surface area can be monitored by measuring membrane capacitance (\(C_m\)). In protoplasts, accessed using a whole-cell patch-clamp configuration, it can be shown \(C_m\) is proportional to the surface area of the plasma membrane (Fig. 8A). The relationship between \(C_m\) and area is linear and, for the guard cell protoplasts shown here (Fig. 8A), a specific capacitance (capacitance per unit area of membrane, \(C_{spc}\)) of 8.1 mF m\(^{-2}\) was calculated. Similar results have been found for barley aleurone protoplasts (\(C_{spc}=7.5 \text{ mF m}^{-2}\); Zorec and Tester, 1992), maize coleoptile protoplasts (\(C_{spc}=8 \text{ mF m}^{-2}\); Thiel et al., 1994) and maize root cap protoplasts (\(C_{spc}=7.5 \text{ mF m}^{-2}\); Carroll et al., 1998). Providing \(C_{spc}\) remains constant, the changes in plasma membrane surface area resulting from
Fig. 8. Patch-clamp capacitance measurements to monitor exocytosis and endocytosis. (A) Correlation between optically determined surface area and measured membrane capacitance ($C_m$) in guard cell protoplasts under isotonic (filled symbols) and hypotonic (open symbols) conditions. (B) Time-course of $C_m$, access conductance ($G_a$) and membrane conductance ($G_m$) under isotonic and hypotonic (osmotic gradient of 80 mOsmol kg$^{-1}$) conditions (indicated by the bar). Capacitance was measured in the whole-cell configuration with a two-phase lock-in amplifier (SWAM II C, Henigman, Portoroz, Slovenia). (C) Time-course of $C_m$ measured in the whole-cell configuration where the whole-cell membrane capacitance has been compensated. Arrows indicate discrete upward steps in capacitance which reveal the fusion of a single vesicle. Measurements were carried out under hypoosmotic conditions (osmotic potential difference: 100 mOsmol kg$^{-1}$). $C_m$ was recorded with a two-phase lock-in amplifier using a 56 mV sine wave at a frequency of 1.6 kHz. (D) Distribution of exocytotic steps obtained from five measurements under hypoosmotic conditions (as in C). Scale of vesicle diameter was calculated with a specific capacitance of 8 mF m$^{-2}$.

Exocytotic and endocytotic activity can be monitored by measuring membrane capacitance.

**Measurement of membrane capacitance**

The membrane capacitance of a cell can be determined from current relaxation in response to a voltage step. It requires only standard electrophysiology equipment. It is equally applicable to the slow whole-cell configuration (perforated patch recording) as to normal whole-cell measurements. For the measurement of whole-cell membrane capacitance the two-phase lock-in technique has gained most acceptance since its resolution is highest and it allows capacitance to be measured in real time. For the determination of $C_m$, a sinusoidal voltage command is applied to the cell in the whole-cell configuration. The resulting current response has both resistive and capacitive components and is measured at two orthogonal phases. Because the phase of the capacity current is orthogonal to the resistive current, this allows the calculation of the membrane capacitance (Gillis, 1995). To determine the phase angle of the currents relative to the command signal, a two-phase lock-in amplifier in combination with a patch-clamp amplifier or a software-based phase detector can be used. For an accurate measurement of whole-cell $C_m$ with a two-phase lock-in amplifier a large and stable access conductance is a key requirement and caution must be taken in the analysis of the data when large changes in membrane conductance occur during the experiment. A software-based phase detector automatically determines the appropriate phase settings for the measurement of $C_m$ (phase tracking technique) and thus accounts for phase changes resulting from changes in the
electrical properties of the cell during the measurement. However, this approach may result in slightly higher noise compared to the hardware-based phase detector (Gillis, 1995) and has greater requirements for computer hardware. The example shows a patch-clamp capacitance measurement of a guard cell protoplast under isotonic and hypotonic conditions measured with a two-phase lock-in amplifier (Fig. 8B). Under isotonic conditions hardly any change in $C_m$ was observed. Upon hypotonic treatment $C_m$ increased steadily (5 fF s$^{-1}$) while the access conductance and membrane conductance remained fairly constant. As the specific capacitance did not change during the experiment this result demonstrates the stimulation of exocytosis upon hypotonic treatment.

The high temporal resolution, and the potential of manipulating the cytosolic composition, make patch-clamp capacitance measurements a powerful tool for studying exocytosis and endocytosis. The main limitations of $C_m$ measurements are the general limitations of patch-clamp measurements: the requirement of an accessible membrane (measurements are generally carried out on protoplasts) and possible loss of endogenous substances that are affecting endocytosis and exocytosis during cell dialysis. In addition, changes in $C_m$ represent net changes in surface area. In principle, an increase in $C_m$ could, therefore, be the result of a stimulation of exocytosis or an inhibition of endocytosis or both. Possible solutions to overcome this limitation are described in the following paragraphs.

**Measurement of fusion and retrieval of single vesicles**

Measurements of individual fusion and retrieval events provide a means to separate exocytotic from endocytotic phenomena. These microscopic $C_m$ measurements are performed in the whole-cell configuration (in which the whole-cell capacitance has been compensated), or in the cell-attached mode (in which changes in the small membrane area beneath the patch pipette are measured).

Changes in capacitance recorded from a guard cell protoplast in the compensated whole-cell configuration under hypo-osmotic conditions are shown in Fig. 8C. A stepwise increase in $C_m$ represents the addition of membrane material to the plasma membrane resulting from the fusion of individual vesicles. Under hypo-osmotic conditions downward steps, resulting from the retrieval of single vesicles, were rarely observed. The rms noise level of the capacitance trace was 0.3 fF, allowing the detection of discrete capacitance steps larger than about 0.5 fF. This corresponds to a vesicle diameter of 140 nm. The modal value of capacitance steps was 1.5 fF (Fig. 8D) which is equivalent to a vesicle diameter of 240 nm. Higher resolution can be achieved using cell-attached capacitance measurements. In maize coleoptile protoplasts the fusion and retrieval of vesicles as low as 60 nm could be resolved (Thiel et al., 1998). By comparing the distribution of vesicle sizes estimated from capacitance measurements with microscopic observations, the patch-clamp capacitance measurements of single fusion/retrieval events also provide a connection between electrical signals and morphological structures.

**Simultaneous measurement of membrane capacitance and optical imaging of stained membranes**

The fluorescent dye FM1–43 reversibly partitions into the outer leaflet of exposed membranes, but does not penetrate the membrane bilayer. When it partitions into a membrane its brightness increases by about 350-fold, which allows the imaging of stained membranes while the dye is present in the external solution. In the presence of FM1–43 the fluorescence of the cell surface reflects cumulative exocytosis, whereas the measured capacitance corresponds to the net difference of exocytosis and endocytosis. Fluorescence is unaffected by endocytosis, because endosomes remain fluorescent. The difference between the change in surface area determined from membrane capacitance measurements and from fluorescence measurements provides an estimate of endocytosis (Smith and Betz, 1996). Thus, by combining measurements of membrane capacitance and FM1–43 fluorescence simultaneous and independent estimates of exocytosis and endocytosis can be made.

Treating plant cells with FM1–43 has only recently been undertaken. In protoplasts from guard cells simultaneous fluorescence and capacitance measurements under hypo-osmotic conditions have confirmed the correlation between changes in fluorescence intensity and $C_m$ (Homann et al., unpublished observations). In addition, vesicle trafficking and recycling has been studied by confocal microscopy in cells stained with FM1–43. Internalization of FM1–43-stained plasma membrane has been demonstrated in secretory root cap cells (Carroll et al., 1998), Fucus embryos (Brownlee et al., 1998) and hypertonically treated guard cell protoplasts (Homann et al., unpublished results).

**Investigating the control of exocytosis and endocytosis**

From capacitance measurements obtained by patch-clamp techniques, several factors have been shown to be involved in the regulation of exocytosis and endocytosis in plants (for a review see Thiel and Battey, 1998). Stimulation of exocytosis by increasing cytosolic Ca$^{2+}$ has been demonstrated for barley aleurone (Zorec and Tester, 1992; Homann and Tester, 1997), maize coleoptile (Thiel et al., 1994), and maize root cap protoplasts (Carroll et al., 1998). For barley aleurone, Ca$^{2+}$-independent exocytosis has also been established (Homann and Tester, 1997). In maize root cap protoplasts GTP hydrolysis was found to inhibit Ca$^{2+}$-activated exocytosis (Carroll et al., 1998).
The involvement of GTP-binding proteins in Ca\textsuperscript{2+}-stimulated exocytosis in barley aleurone protoplasts has been suggested by the inhibition of Ca\textsuperscript{2+}-stimulation in cells dialysed with non-hydrolysable guanine nucleotides (Homann and Tester, 1997). The activation of exocytosis in maize root cap cells dialysed with maize annexins provides direct evidence for the role of annexins in the regulation of exocytosis in plants (Carroll et al., 1998).

The patch-clamp technique has also been used for the investigation of the effect of hydrostatic and osmotic pressure, and thus membrane tension, on exocytosis and endocytosis. In barley aleurone protoplasts, hydrostatic pressure was found to cause dramatic changes in exocytotic activity (Zorec and Tester, 1993). In guard cell protoplasts, hypo-osmotic and hyperosmotic conditions have been shown to stimulate exocytosis and endocytosis, respectively, in a Ca\textsuperscript{2+}-independent manner (Homann, 1998).

**Future prospects**

Using the patch-clamp technique to measure membrane capacitance allows the role of potential regulators of exocytosis and endocytosis to be investigated directly. Several factors have already been shown to be involved in the control of exocytosis and endocytosis in plant cells. Future research will determine whether other intracellular factors known to be involved in secretion in animal cells also play a role in plant cells. In addition, regulators of exocytosis and endocytosis unique to plant cells may be discovered. The identification and study of secretory mutants and the application of combined capacitance and fluorescence measurements will add further to the understanding of vesicle fusion and retrieval in plants.

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