Chapter 13

Microelectrode techniques in plant cells and microorganisms

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1. Introduction

This chapter will review recent progress in the application of microelectrode techniques to the study of a range of cell physiological problems in plant cells and microorganisms. Plant cells present a special set of problems for electrophysiology and other microelectrode methods, related principally to cell structure (Fig. 1). The past five years have been witness to an great expansion in the application of microelectrode techniques and great progress towards overcoming some of the most significant problems facing plant electrophysiologists, particularly those associated with the presence of the cell wall and intracellular compartmentalization. Consequently, a wide range of plant cell types are now amenable to investigation using modern electrophysiological techniques and this is reflected by the rapidly increasing literature pertaining to plant cell electrophysiology. Virtually all techniques applied to animal cells are now possible with many plant cell types. Here I will outline some of the most significant recent advances in plant cell electrophysiology and discuss problems which remain to be overcome, indicating some of the likely advances in the near future.

2. Voltage clamp

While conventional voltage clamp of large plant cells, particularly the giant algae, using two or three electrodes, has provided the foundations for much of the current plant electrophysiology, (see e.g. Findlay, 1961; Kishimoto, 1961; Beilby, 1982; Lunevsky *et al.* 1983; Gradmann, 1978), technological developments in voltage and patch clamp have allowed more wide-ranging studies. One of the most significant advances in conventional two-electrode voltage clamp studies has been the application of double-barrelled electrodes to single small cells of higher plants. In this configuration, one barrel of the electrode measures voltage while current is injected through the other barrel. This is perhaps best exemplified by the work of Blatt and co-workers on the stomatal guard cell (e.g. Blatt, 1987, 1991a,b, 1992). The stomatal

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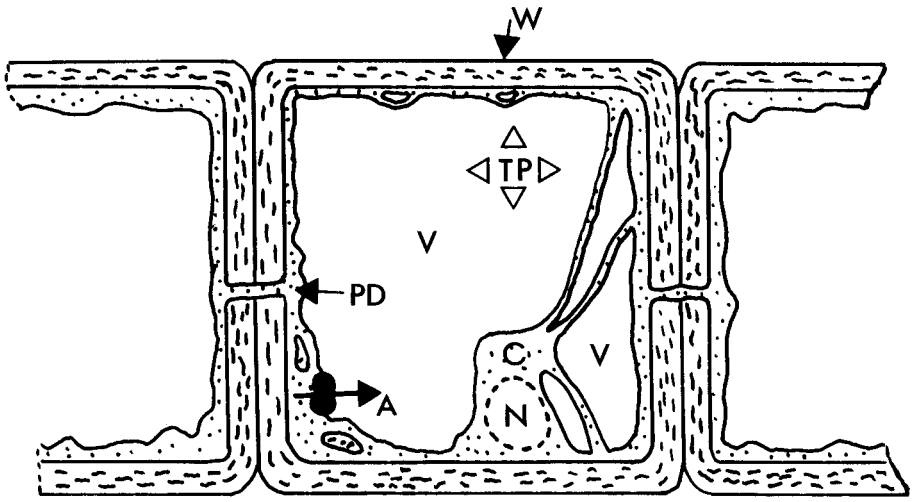

Fig. 1. Diagrammatic representation of a typical higher plant cell, showing particular features relevant to the application of microelectrode methods. W, cell wall; C, cytoplasm; V, vacuole; A, anion transporters in vacuolar and organelle membranes; PD, intercellular electrical continuity via plasmodesmata; TP, turgor pressure.

guard cell is probably the most intensively studied higher plant cell from an electrophysiological aspect. In addition to playing a major role in plant-water relations, stomatal guard cells show very clear responses (stomatal opening or closure, corresponding to changes in cell shape resulting from changes in ion and water fluxes across the plasma membrane (Mansfield *et al.* 1990)) to well-defined signals, including plant hormones (abscisic acid, auxin), light and CO_2. In the studies of Blatt and co-workers, electrodes were filled with 200 mM K^+-acetate to minimise the effects of Cl^- leakage into the relatively small cytoplasmic volume. Typically, steady-state I/V relations were obtained using bipolar staircase voltage clamp protocols. This work has concentrated largely on currents through inward- and outward-rectifying K^+ channels involved in the response to abscisic acid, particularly their control by pH and Ca^{2+} (e.g. Blatt, 1990; 1992; Blatt & Armstrong, 1993). This method has been extended further to allow loading of the cells with caged compounds (caged Ins(1,4,5)P_3 and caged ATP) which were introduced through the recording barrel of the electrode (Blatt *et al.* 1990). Advantages of the double-barrelled voltage clamp method over patch clamping of plant protoplasts (see below) are that it can be used *in situ* on intact cells inside their cell walls and probably reflects a more physiologically relevant situation. Complications arise where the cells are electrically coupled to other cells (which is the case for many plant cell types), restricting the application of this technique. Other disadvantages of this method are that it is often technically difficult to impale the cytoplasm of small highly vacuolated plant cells without damage.

The single electrode discontinuous voltage clamp (see Chapter 2) has been applied to eggs of the marine alga, *Fucus serratus* (Taylor & Brownlee, 1993). With this

method, a single electrode performs both the voltage recording and current injection tasks with a rapid switching frequency, determined by the time constants of the electrode (τe) and cell membrane (τc), allowing any voltage associated with charging of the electrode capacitance during the current injection cycle to decay before membrane potential is sampled. The relatively large size (~70 μm diameter) of *Fucus* eggs ensured a sufficiently high τc despite a relatively low input resistance (100 MΩ). Electrode switching frequency was optimised by bevelling the tip of the electrode (Kaila & Voipa, 1985) and dipping in mineral oil to minimize stray capacitance. Limitations on the range of the voltage clamp potentials that could be used arose from the relatively small τc/τe ratio (typically around 150), resulting in clamp error or oscillations induced by extreme command voltages (Finkel & Redman, 1984). Nevertheless, this study provided evidence for the existence of a voltage-activated inward Ca^{2+} current and a slowly-activating outward K^+ current which were postulated to be involved in the generation of the fertilization potential (Taylor & Brownlee, 1993). As far as we know, this technique has not been applied to other plant cell types, though the relatively high input resistance of many plant cells may render them suitable where other voltage clamp methods may not be feasible.

3. Patch clamp

Plasma membrane

The application of the patch clamp technique to plant cells has undergone a major expansion, significantly improving our understanding of the nature and roles of ion transport in plant cells, relating to nutrient transport, osmoregulation, and intracellular signalling. (For recent reviews on plant cell ion channels and pumps, see Brownlee & Sanders, 1992; Hedrich & Schroeder, 1989; Schroeder & Thuleau, 1991, Tester, 1990). The primary, and most crucial step in patch clamping plant cells is the removal of the cell wall to reveal a clean plasma membrane on which giga-ohm seals can be obtained with a patch pipette. To date, most patch clamp studies on plant cells have utilized protoplasts in which the cell wall has been removed with enzyme cocktails based primarily on cellulase. A variety of enzyme methods have been developed for the production of viable plant cell protoplasts. Methods vary considerably between different cells and tissue types. A major problem with protoplast production is the isolation of particular identified cell types from tissue which may contain several different kinds of cell. Isolation of a particular cell type may involve initial dissection of the tissue, followed by preferential disruption of the unwanted cells and selective enzymatic digestion of the walls of the cells of interest. A good example of the successful isolation of a particular protoplast type is the preparation of stomatal guard cell protoplasts from leaf tissue (Kruse *et al.* 1989). The technique essentially involves mechanical removal of the epidermal tissue containing the guard cells, enzyme treatment to liberate the protoplasts, followed by centrifugation for removal of debris and purification of protoplast fractions. Details

of the methods for production of a variety of guard cell protoplasts suitable for patch clamping are given by Raschke & Hedrich (1989). Separation techniques of this kind, however are only applicable to a few cell types and the often drastic treatments involved may have unwanted side effects, such as alteration of the plasma membrane properties by the enzyme treatment. An indication of the physiologically altered state of protoplasts comes from the few measurements of protoplast membrane potential that have been made. For example, tobacco mesophyll protoplast membrane potential has been measured at around −10 to −15 mV (Venis *et al.* 1992), whereas membrane potentials from most mesophyll cells are very negative (−100 to −150 mV), reflecting the activity of the plasma membrane proton pump (e.g. Senn & Goldsmith, 1988). The depolarized state of the protoplast membrane may reflect, in part, increased leakage arising from electrode impalement, though this would tend to produce variable membrane potentials, depending on the quality of impalement, which is not generally observed. The other possibility is that the activity of the proton pump is reduced in protoplasts for reasons related to the protoplast isolation procedure. This uncertainty awaits resolution. Enzymatically produced protoplasts also lack the plasma membrane/extracellular matrix connections as a result of wall removal. The cell wall performs many functions which may have significant bearing on the physiology of the cell. For example, plasma membrane-wall connections are known to be essential for polar axis fixation in *Fucus* zygotes (Kropf *et al.* 1988). Loss of turgor and non-physiological osmotic environment are unavoidable factors in the application of patch clamp techniques to most plant cells.

The success rate of giga seal formation in plant protoplasts prepared by standard enzyme methods varies but is generally low compared with most animal cells. This probably reflects combinations of the lack of cytoskeletal rigidity, incomplete wall removal or protoplast cleaning, and regeneration of the wall. Fairley & Walker (1989) showed that inhibition of wall regeneration in maize protoplasts did not improve electrode-membrane sealing rates. Significant improvements in seal rates have, however, been produced by modifications of the enzyme treatment, particularly by reducing the enzyme treatment to a minimum. Elzenga *et al.* (1991) used a 5 minute enzyme treatment to weaken the cell wall, followed by osmotic adjustment to make the protoplasts swell and pop out of the weakened wall. The method was shown to be successful in several cell types (Table 1). Giga seal (>10 MΩ) formation was obtained in >40% of attempts, usually within 2 minutes. A similar approach has been used to obtain improved seal rates from root cell protoplasts (Vogelzang & Prins, 1992). In this case, protoplasts were released by application of mechanical pressure.

The patch clamp technique has been used in a variety of animal cells to measure capacitance changes in the plasma membrane related to exocytosis (e.g. Lindau & Neher, 1988). So far, there is only one published report of the application of this technique to plant cells (Zorec & Tester, 1992). In that study, capacitance of protoplasts from barley aleurone cells was shown to increase when cells were dialysed through the patch pipette with high (1 μM) Ca^{2+}. Whole cell patch clamp of enzymatically produced protoplasts has also been used in combination with ratio photometry using the fluorescent Ca^{2+}-sensitive dye, fura-2 (Schroeder & Hagiwara,

Table 1. *Success rate of giga-ohm seal formation on protoplasts prepared by brief enzyme treatment*

Protoplast source	Total seals	R>10GΩ	1GΩ>R >1GΩ	Fail/WC	Isolates
Pisum sativum, leaf epidermis	123	67	32	24	29
P. sativum stem epidermis	98	42	31	25	27
Phaseolus vulgaris leaf mesophyll	15	10	3	2	2
Avena sativa coleoptile cortex	15	6	5	4	2
Arabidopsis thaliana cotyledon mesophyll	15	9	5	1	2

Numbers indicate seal formation with resistance >10GΩ; resistance between 1 and 10GΩ; and attempts which resulted in no seal formation or where the patch ruptured during seal formation i.e. went whole cell (fail/WC). "Isolates" represents the number of different protoplast batches used. (Reproduced, with permission from Elzenga *et al.* (1991).)

1990). In this study, dye was introduced into the cell via the patch clamp electrode. Transient elevations of cytosolic Ca^{2+} were observed with simultaneous increases in inward current after the application of abscisic acid.

Mechanical removal of the cell wall presents an alternative to enzymatic treatments for plasma membrane access. This was initially used in giant cells of *Chara australis*, using a knife cannula or fine forceps to cut the wall (Laver, 1991) The patch pipette could be inserted through the hole cut in the cell wall. Development of a microsurgical technique using a U.V. laser arose out of a need to maintain the polarity of rhizoid cells of *Fucus* in studies of the distribution of channel activity during polarized growth (Taylor & Brownlee, 1992). In recent years, the use of lasers as microsurgical tools has become established (see Berns *et al.* (1991); Greulich & Weber (1992); Weber & Greulich, (1992) for recent reviews). The most useful laser for plant cell microsurgery is a pulsed nitrogen laser such as a 337 VSL or 337 ND (Laser Science Inc., U.S.A.). This can be passed via appropriate optics through the fluorescence port of a microscope and focussed to a very small spot by a U.V.-conducting objective. We routinely use a 40, 1.3. N.A. objective (Nikon). The power density of the focussed spot is sufficient to ablate selected regions of the cell wall. Prior osmotic shrinkage of the cytoplasm allows the plasma membrane to be withdrawn from the cell wall during ablation. Careful reflation of the cytosol by adjusting the osmotic potential of the bathing medium results in extrusion of varying amounts of plasma membrane-bound cytoplasm which can be accessed by a patch electrode (Fig. 2). To date, this method has allowed rapid access to the plasma membrane of a growing *Fucus* rhizoid, giving a rate of seal formation (40-80% for 5-15 GΩ seals), not possible with enzymatic protoplasting techniques. Laser microsurgery has also recently been used to expose the plasma membrane of root hairs of *Medicago* (Kurkdjian *et al.* 1993). The high success rate of sealing observed in *Fucus* suggests that the technique may be useful as an alternative for plant cell types that are difficult to patch clamp following enzymatic wall removal. Laser microsurgery should also

enable studies of other systems where ion channel activity is important in relation to polarity, such as fungal hyphae, root hairs and pollen tubes. It should also be possible to patch clamp membranes of identified cells which are difficult to isolate using bulk protoplasting techniques. Our own experience (unpublished) indicates that laser microsurgery can produce high quality protoplasts from cells types as varied as fungal hyphae and stomatal guard cells.

Organelles

Significant progress has now been made in patch clamp studies of the vacuolar membrane of a variety of plant cells. Vacuolar isolation techniques are relatively straightforward and generally involve washing the surface of a freshly cut tissue slice with buffer to liberate the exposed vacuoles (Coyaud *et al.* 1987; Keller & Hedrich, 1992). Vacuoles can also be produced from protoplasts (e.g. Ping *et al.* 1992). Whole-vacuole and single channel recordings have been obtained from a variety of channel types. These include K^+, Cl^- and Ca^{2+} channels (e.g. Alexandre & Lassalles, 1990; Hedrich & Neher, 1987; Johannes *et al.* 1992; Pantoja *et al.* 1992; Ping *et al.* 1992). The success of vacoular patch clamp recordings is dependent in part on obtaining appropriate osmotic conditions for both bath and electrode filling solutions. Improvement in signal-to-noise ratio has been reported by dipping the pipette in a 30% silane:70% carbon tetrachloride solution after filling, presumably by forming a hydrophobic surface at the pipette shank (Alexandre & Lassalles, 1990).

Patch clamp recordings have been made from giant chloroplasts liberated from osmotically shocked protoplasts of *Peperomia metallica* leaf cells (Schonknecht *et al.* 1988; Keller & Hedrich, 1992). This treatment caused osmotic swelling of the chloroplast and rupturing of the thylakoid envelope, exposing thylakoid membrane blebs which could be patch clamped and allowed studies of the ion fluxes associated with the pH gradient across the thylakoid membrane.

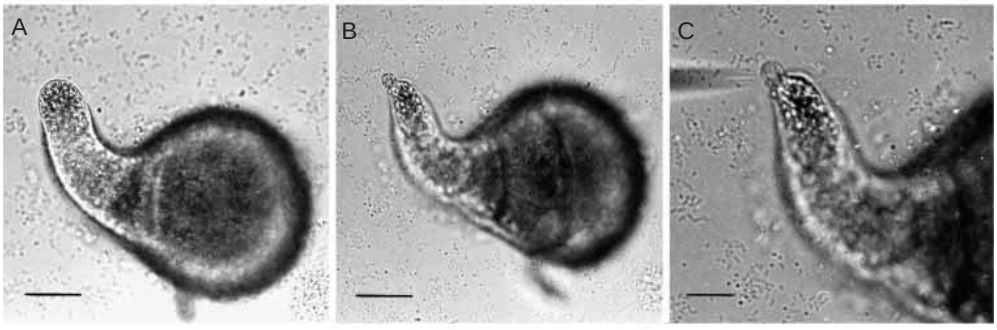

Fig. 2. Localized patch clamping from a *Fucus* rhizoid cell using U.V.-laser microsurgery. (A) 24-h *F. serratus* zygote with developing rhizoid. Scale bar: 10 μm. (B) After laser ablation of the cell wall and reflation of the cytoplasm. Scale bar: 10 μm. (C) Exposed plasma membrane and patch clamp electrode. Scale bar: 5 μm. (Reproduced, with permission from Taylor & Brownlee (1992).)

Patch clamp and microorganisms

Progress in patch clamp studies of microbial membranes has been reviewed recently (Siami *et al.* 1992). The protozoan, *Paramecium* can be induced to form plasma membrane blisters under conditions of low Ca^{2+} and shear which are accessible for patch clamping. Ion channels have also been recorded in isolated *Paramecium* cilia (Siama *et al.* 1992). Developments in the production of protoplasts from yeast has enabled detailed patch clamp studies of both the plasma membrane and tonoplast. Bertl & Slayman (1990) and Bertl *et al.* (1992), for example, used a tetraploid strain of *Saccharomyces cerevisiae* (YCC78) as starting material for protoplasts from which both vacuolar and plasma membrane channels have been characterized. Both voltage- and Ca^{2+}-dependent cation channels were found in the plasma membrane and tonoplast. Methods for the production of yeast protoplasts (spheroplasts) are described in detail by Siami *et al.* (1992).

Giant spheroplasts can also be produced from bacteria, such as *E. coli* and *B. subtilis* (Siami *et al.* 1992). Various treatments have been utilised to effect wall loosening and osmotic swelling of the underlying protoplast. These include cephalexin or U.V. treatment to produce unseptated filaments, followed by lysozyme treatment and osmotic swelling. Magnesium chloride coupled with cephalexin has also been used to produce giant cells. Mutants lacking certain cell wall components, or osmotically sensitive mutants have also been used (Siami *et al.* 1992; Criado & Keller, 1987). Fusion of bacterial membrane vesicles with liposomes followed by unilamellar blister formation in the presence of 20 mM $MgCl_2$ has yielded recordings from a variety of reconstituted channels, including a mechanosensitive channel, a voltage-sensitive channel and a cation-selective channel (Delcour *et al.* 1989a,b).

Protoplasts have also been obtained from fungal hyphae, allowing patch clamp recordings and preliminary characterisation of channel distribution between protoplasts isolated from different regions of the hypha of *Saprolegnia ferax* (Garril *et al.* 1992a). Problems were encountered in obtaining high resistance seals, limiting studies to cell-attached recording mode. However, both whole cell and excised patch recordings were made from protoplasts of the fungus, *Uromyces* (Zhou *et al.* 1991), though Garril *et al.* (1992b) have questioned the origin of the membrane in these studies, suggesting that it may be tonoplast. It is likely that the application of laser microsurgery will improve the quality of giga-seal formation on the fungal plasma membrane. Our preliminary experiments show that high resistance seals (>10 GΩ) can be obtained relatively easily with protoplasts extruded from *Phytophora* following laser microsurgery of the cell wall (A. Taylor, P. Whiting, D. Sanders & C. Brownlee, unpublished observations).

Wall-free mutants of the unicellular green alga, *Chlamydomonas* have been used in a patch clamp study of the rhodopsin photoreceptor current (Harz & Hegemann, 1991; Harz *et al.* 1992). In these experiments whole cells were gently sucked into fire-polished pipettes, forming seals with resistances up to 250 MΩ, allowing cell-attached recordings from a relatively large membrane area, though higher resistance seals were not achieved.

4. Microinjection

Studies employing microinjection based on both iontophoresis and pressure are now widespread in plants. Examples include microinjection of fluorescent antibodies for microtubules and actin into living cells (Cleary *et al.* 1992), caged compounds (Blatt *et al.* 1990; Gilroy *et al.* 1990) and Ca^{2+} indicators (e.g. Brownlee & Pulsford, 1988; Gilroy *et al.* 1990; McAinsh *et al.* 1990; Miller *et al.* 1992; Rathore *et al.* 1991) Microinjection has proven to be essential in several cell types for the use of fluorescent dyes for monitoring cytoplasmic Ca^{2+} and pH. Plant cells generally do not take up and hydrolyse the acetoxymethyl esters of these dyes which are commonly used in many animal cell studies. Even when this does occur, compartmentalization of the free acid form of the dye into vacuoles and intracellular vesicles and loss from the cell across the plasma membrane is frequent (Brownlee & Pulsford, 1988; Bush & Jones, 1987; reviewed by Read *et al.* 1992). Circumvention of these problems has recently been achieved by the use of fluorescent dyes linked to high relative molecular mass (M_r) dextran (10,000 or more: Miller *et al.* 1992). This usually necessitates the use of pressure and may be problematic in highly turgid plant cells. Reduction of the cell turgor pressure was found to facilitate injection and reduce cell damage during microinjection of mixtures of the Ca^{2+} indicator, Calcium Green-dextran and the pH indicator SNARF-dextran into *Fucus* zygotes (Berger & Brownlee, 1993). In this study it was found that 10,000 M_r Calcium Green-dextran, though excluded from intracellular vacuoles and vesicles and retained in the cytoplasm, did enter the nucleus. 70,000 M_r Calcium Green-dextran, however, was excluded from the nucleus, except during cell division when nuclear envelope breakdown occurred. Using these long wavelength-excitable dextran-linked dyes enabled the acquisition of confocal sections of Ca^{2+} distribution, followed over a period of 3 days, without any significant loss of dye signal or compartmentalization. It is possible to microinject 10,000 M_r dextran dyes iontophoretically using positive current pulses. Negative current, however, causes preferential injection of any non-dextran linked dye molecules present as impurities and should be avoided.

5. The pressure probe

An intracellular electrode technique which appears to be uniquely applied to plant cells is the pressure probe. This was developed by Zimmerman *et al.* (1969) for the study of the large intracellular (turgor) pressures which are a fundamental feature of the water relations of walled plant cells. Following its initial application to giant algal cells, the technique has been adapted for use with much smaller cells of higher plants (Husken *et al.* 1978). An oil-filled micropipette is connected to a microsyringe and a pressure transducer. Impalement of a cell results in displacement of the oil meniscus in the tip of the micropipette which can be observed microscopically. The pressure required to return the meniscus to the pipette tip is a measure of the internal hydrostatic pressure of the cell. The technique has been used in several studies of

water and solute relations of plant cells (e.g. Malone & Tomos, 1990, 1992; Pritchard & Tomos, 1993; Rygol *et al.* 1993; Tomos *et al.* 1992). Modifications of this technique include turgor clamp (Murphy & Smith, 1989; Zhu & Boyer, 1992) which allowed investigation of growth under carefully controlled conditions of cell turgor, and precise pressure injection of fluorescent probes along with simultaneous monitoring of turgor (Oparka *et al.* 1991). A further modification of this technique allows rapid sampling of vacuolar contents of higher plant cells (Leigh & Tomos, 1993; Malone *et al.* 1989, 1991). This has allowed analysis of vacuolar ion concentrations (using X-ray microanalysis), osmotic potential (freezing point method) and metabolites from a variety of identified cell types (see Tomos *et al.* (1993) for experimental details).

6. Ion-selective electrodes

Despite the emergence of fluorescent dyes for measuring intracellular ion concentrations, work with ion-selective microelectrodes has continued to give important insights into a range of processes, particularly those involving Ca^{2+} and pH, though nitrate-selective electrodes have been used in both giant algal and higher plant cells (Miller & Zhen, 1991; Zhen *et al.* 1991). The advantages of ion-selective microelectodes continue to be their relative ease of manufacture and low cost. Provided attention is paid to calibration, they can provide accurate measurements of ion concentration in selected compartments. Improvements in sensor (particularly for Ca^{2+}) and electrode fabrication has largely overcome problems of sensitivity and sensor displacement due to turgor pressure. Problems still exist in re-calibration of Ca^{2+} electrodes after intracellular recording (e.g. Amtmann *et al.* 1992) and in their low sensitivity at physiological free Ca^{2+} concentrations and tip diameters small enough to penetrate most plant cells (Felle, 1993). This, together with the fact that the response of a Ca^{2+} electrode is only half of that of a monovalent ion-selective electrode has limited their use in plant cells (see Felle (1993) for review on the use of Ca^{2+}-selective microelectrodes in plants). In contrast, pH-selective microelectrodes have found considerably wider application. They do not appear to suffer from significant calibration problems and the sensitivity and response time is significantly better than for Ca^{2+} electrodes. Felle (1993) reviews the use of pH microelectrodes in plants and provides a general overview of their role in elucidation of pH relations of plant cells. An additional problem with the use of ion selective electrodes is the determination of the location of the electrode tips in the cytosol or vacuole. This has been discussed by Felle (1993), Felle & Bertl (1986), Miller & Sanders (1987) and Miller & Zhen (1991).

An extracellular vibrating Ca^{2+}-selective electrode has been developed by Kuhtreiber & Jaffe (1990). This electrode contains a Ca^{2+}-selective sensor in the tip and oscillates between two fixed points close to the surface of a cell converting small gradients of Ca^{2+} into voltage differences. Provided external $[Ca^{2+}]$ is low, high sensitivity and low noise are achieved by averaging the oscillating voltage signal,

allowing the detection and quantification of small Ca^{2+} gradients resulting from Ca^{2+} influx around the surface of a variety of plant and animal cells (Kuhtreiber & Jaffe, 1990; Schiefelbein *et al.* 1992).

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