2 Electrochemical Methods and Measuring Transmembrane Ion Gradients

ANTHONY J. MILLER, DARREN M. WELLS

2.1 Methods for electrical recordings from plants

2.1.1 Making contact

The measurement of a voltage requires a complete electrical circuit or ring that includes the measuring device, a voltmeter or electrometer. The electrical contact to the biological material is provided by an electrode. This interface between the biological specimen and electrometer is very important, as ideally it should provide a low electrical resistance pathway that does not interfere with the cells or tissues being measured. The word *microelectrode* is commonly used to describe a glass micropipette which is pulled into a fine tip at one end and filled with an aqueous salt solution. The junction between the salt solution inside the microelectrode and the input to the electrometer amplifier is provided by a half-cell. There are different types of half-cell, but usually the metal contact is AgCl-coated Ag wire and the salt solution is 0.1 M KCl (e.g. World Precision Instruments, Sarasota, Fla., USA: http://www.wpiinc.com/). The micropipette provides a salt bridge between the inside of a living cell and the metal contact in the half-cell. The simplest microelectrodes measure voltage and when inserted into cells measure the membrane potential, in mV, between the inside and outside of the cell. The metal contact can be made directly to the cell or tissue surface, but this type of electrode can be subject to various types of interference as the surface can be coated by plant material that will influence the stability and size of the electrical potential reported. This problem is much less likely to occur when the tip is constructed from glass that has been heated and pulled into a small fine tipped microelectrode. A small tip also provides less intrusion and interference for the biological tissue or cells being examined.

An *ion-selective* microelectrode contains an ion-selective membrane in the tip of the glass micropipette and is responsive both to the membrane potential and the activity (not concentration) of the ion sensed by the selective membrane. To make intracellular measurements, it is necessary to also simultaneously measure the membrane potential either by insertion of a

Crop Performance and Improvement Division, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK

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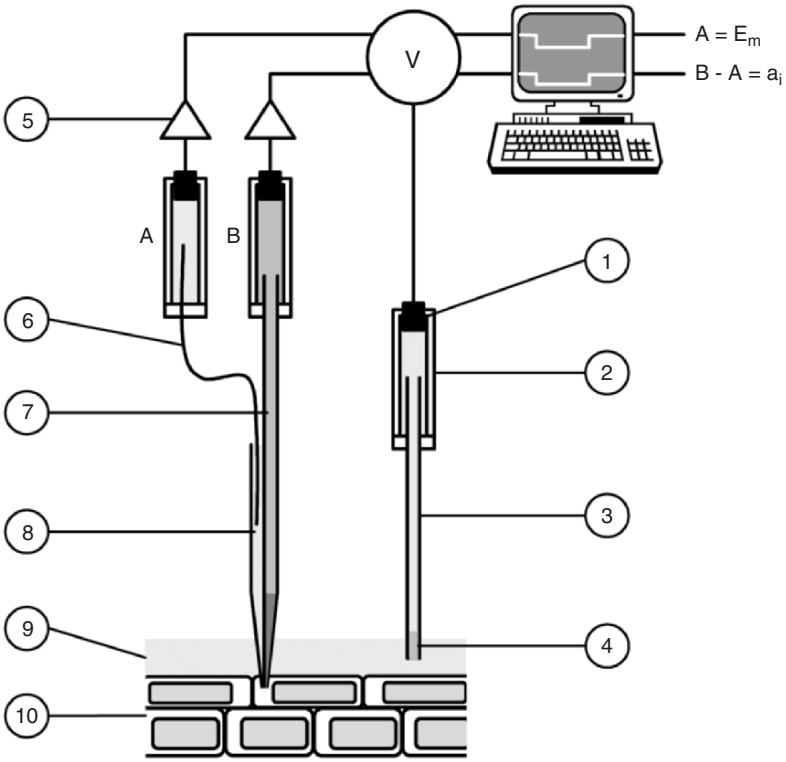

Fig. 2.1. Diagrammatic representation of a double-barreled ion-selective microelectrode. *1* Ag/AgCl chloride coated pellet, *2* half-cell, *3* salt bridge, *4* porous glass frit or agar plug, *5* head-stage signal amplifier, *6* Ag/AgCl coated Ag wire, *7* ion-selective barrel with sensor plug in the tip, *8* cell membrane potential barrel filled with 0.1 M KCl, *9* nutrient solution bathing plant, *10* plant tissue with microelectrode tip in a cell (cytoplasm)

second electrode or, for small cells, by combining the ion-selective and voltage-measuring electrodes into a *double-barreled microelectrode* (see Fig. 2.1). In order to be able to measure several different ions, it may be necessary to combine together several different electrodes to make multi-barreled electrodes (e.g. Walker et al. 1995).

Solid metal electrodes have been used to directly report from plant material and for some types of specialist uses such as measurements of electrical current in oxygen electrodes. Metal electrodes are usually made from Ag or platinum and these solid state electrodes have been used to make ion-selective microelectrodes (see section 2.2). Metal electrodes have also been used for direct recording from the surface of plants to measure extracellular transient electrical signals such as those elicited by external signals, e.g. wounding. The interface between the plant material and a metal recording electrode may also

be made by a salt bridge using a wick electrode (e.g. Wildon et al. 1992). The wick can be made of fiber, for example paper or cotton thread soaked in salt solution.

2.1.2 Recording from plants

Electrophysiology recordings from plants require very solid anchoring of the plant material, while at the same time preserving the normal state of the material as far as possible. It is best to avoid dissecting the plant material as this is likely to lead to local wounding that is known to have major effects on gene expression (Delessert et al. 2004). Plants grown in hydroponic culture can be easily transferred to the microscope stage for electrode impalements of either root or leaf tissue. The hydroponic environment for roots is easily maintained on the stage of a microscope but leaves are more difficult, requiring some wet contact between the tissue and the bathing solution. Microelectrode impalements are usually made under a microscope using long working distance objectives that allow sufficient space for microelectrode access. Although they are generally used for patch-clamp experiments, inverted microscopes are not so suitable for this type of work. Dissecting microscopes can be used for microelectrode impalements, but they usually do not have sufficient magnification to see individual cells. They can be used for impalements by letting the electrical recording show when tissue contact has been made and a successful impalement can be gauged by the size of the membrane potential measured. Microelectrodes are mounted on micromanipulators for cellular impalement, to allow the delicate movement of the tip into a cell. There are a range of different types, and hand control of tip movement is achieved by either joystick or rotational manipulation. The size and fine movement axis should be chosen so that the micromanipulator can be conveniently positioned alongside the microscope stage for tissue impalement.

Plant tissue is usually mounted in a purpose built chamber for microelectrode impalements. The chamber is usually made from Plexiglas and is constructed so that the tissue can be perfused with nutrient solution throughout the experiment. This perfusion through the chamber helps prevent large local concentration gradients (unstirred layers) of ions developing around cells. Treatments can be applied to the tissue during a recording by changing the composition of the nutrient solution bathing the tissue. The chamber design is very important, and it is worth investing time in this aspect of the experimental system. If the tissue is not well anchored in position, it is impossible to achieve good electrical recordings. Each type of tissue usually requires a purpose built chamber but published work often does not report the details of this key aspect of the experimental system. The general principles of chamber construction have been reviewed previously (see Blatt 1991) and a chamber for leaf measurements has been described (Miller et al. 2001).

Some of the equipment required for microelectrode recording is shown in Fig. 2.1 but a more complete list is as follows:

- Voltmeter (also known as an electrometer)
- Microscope (with long working distance objectives)
- Micromanipulator
- Tissue chamber (for holding and perfusion)
- Data logging system (e.g. computer or chart recorder)
- Vibration free table (for microscope and micromanipulator to avoid interference from external vibration sources)
- Faraday cage (electrical screening around the microscope and micromanipulator especially necessary for high resistance electrodes)
- Oscilloscope (not essential but useful for fixing recording noise problems)

2.2 Manufacture and use of ion-selective electrodes

Ion-selective microelectrodes are used to measure ion gradients across membranes. These measurements can be made outside and inside cells. For example, ion fluxes at the surface of roots can be measured directly using ion-selective microelectrodes (Henriksen et al. 1990) or by using an ion-selective vibrating probe (Kochian et al. 1992; see Chapter 3 by Sergey Shabala). Intracellular measurements have been used to give important information on the compartmentation of nutrients, dynamics of cellular ion activities (e.g. in intracellular signaling) and transport mechanisms, particularly the energy gradients for ion transport. The main criticism of intracellular measurements made with microelectrodes is that they report the ion activity at a single point within the cell. This will result in incomplete information if there are significant ion gradients within the cytoplasm of a single cell as may occur in some situations. Overall, the chief advantages of using ion-selective microelectrodes are that:

- They offer a non-destructive method of measuring ions within cells
- They do not change the activity of the ion being measured
- They permit simultaneous measurement of the electrical and chemical gradients across membranes
- They are relatively cheap when compared to other methods for measuring intracellular ions and once purchased the same equipment can be used to measure a range of different ions.

2.2.1 Theory of ISEs

The theoretical background has already been described by many authors (e.g. Ammann 1986, and references therein) and will only be outlined here. The properties of an ion-selective microelectrode are defined by several characteristics:

- Detection limit
- Selectivity
- Slope
- Response time

The ideal relationship between electrode output (mV) and the activity (a_i) of the ion of interest (i) is log-linear and is described mathematically by the Nernst equation. Calibration of the electrode against a range of standard solutions should ideally yield a slope (s) of 59 mV (at 25 °C) per decade change in the activity of a monovalent ion. In practice, however, the situation is more complicated than this because no ion-selective electrode (ISE) has ideal selectivity for one particular ion and under most conditions there is more than one ion present in the sample solution. Hence contributions to the overall electromotive force (EMF) made by each interfering ion, j, must be taken into account. In this situation, the Nicolsky–Eisenman equation, a modified Nernst equation, describes the EMF:

$$EMF = E + s \cdot \log\left[a_i + K_{ij}^{pot}\left(a_j\right)^{zi/zj}\right] \quad (1)$$

where K_{ij}^{pot} is the selectivity coefficient of the electrode for the ion i with respect to ion j. This term expresses, on a molar basis, the relative contribution of ions i and j to the measured potential.

The parameters s and K_{ij}^{pot} are the two main characteristics defining any type of ion-selective electrode. The slope should be a near ideal Nernstian response when an electrode is calibrated against ion activity, but s is temperature sensitive (see section 2.4). The selectivity coefficient measures the preference of the sensor for the detected ion i relative to the interfering ion, j. It can be determined by the separate solution method, the fixed interference method or the fixed primary ion method. For ideally-selective membranes, or for samples containing no other ions with the same net charge as the ion in question, K_{ij}^{pot} must be zero. A log selectivity coefficient <1 indicates a preference for the measuring ion i relative to the interfering ion j, and vice versa for a selectivity coefficient >1. The K_{ij}^{pot} values should not be considered to be constant parameters that characterize membrane selectivity under all conditions; the values are dependent on both the method used for determination, and on the conditions under which the calibrations are made. The fixed interference method is most commonly used to calculate the selectivity coefficient and it is the method recommended by the International Union of Pure and Applied Chemistry (Inczédy et al. 1998). Whichever type of method is chosen, the one used should always be quoted.

A schematic representation showing an ideal ion-selective microelectrode calibration curve is given in Fig 2.2. The slope s, is the change in EMF per decade change in activity of a monovalent anion i, which is equivalent to 59.2 mV at 25 °C; the limit of detection is defined as described in the text and is also indicated.

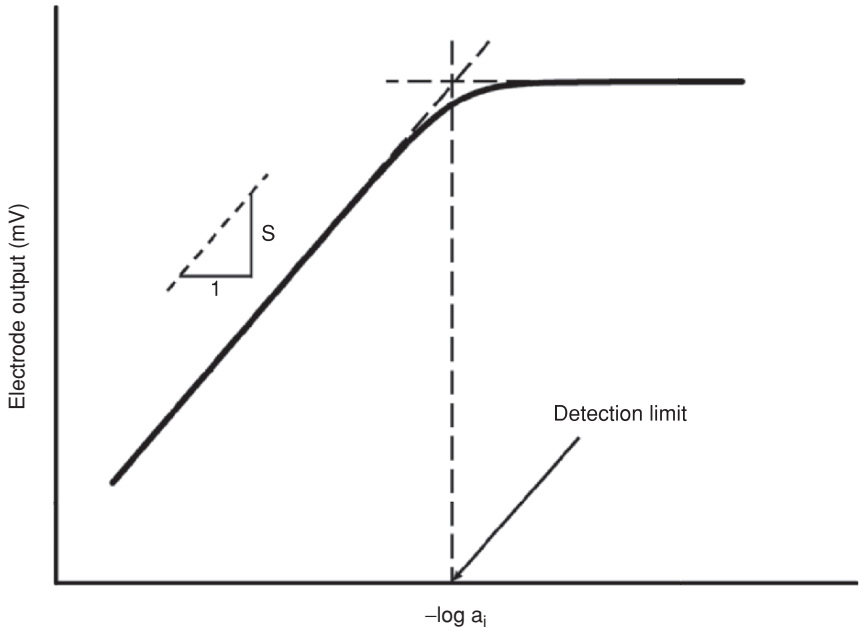

Fig. 2.2. Calibration of an ion-selective microelectrode to show detection limit and how it is calculated

Another important parameter of an ion-selective microelectrode is the detection limit, which is the lowest ion activity that can be detected with confidence and is defined by the intercept of the two asymptotes of the Nicolsky response curve (see Fig. 2.2). In practice, the detection limit seems to depend on the tip geometry and composition of the microelectrode's ion-selective membrane. Finer or smaller diameter tips have higher detection limits; while composition affects detection in ways that can only be determined experimentally (see section 2.4). The presence of interfering ions alters the detection limit (e.g. chloride for nitrate-selective microelectrodes, see Miller and Zhen 1991). Electrodes provide no useful information below their detection limits and for maximum benefit should be used in the linear portion of their calibration curves. The response time of ISEs can be important when measuring changes in ion activities. This microelectrode parameter is dependent on many factors, including tip geometry, membrane composition and resistance. Response time can be measured during the calibration as the time taken for the voltage to adjust when ion activity at the tip is changed.

2.2.2 Types of ISEs

There are three major types of ISE, all of which can be miniaturized for use in plant cells. These are solid state, glass, and liquid (or fluid) membrane

electrodes. Solid-state microelectrodes have been used to measure pH or Cl^- inside plants cells (e.g. Coster 1966) and recessed tip glass microelectrodes have been made using pH-selective glass (Sanders and Slayman 1982). These two types of microelectrode have largely been superseded for intracellular measurements by liquid-membrane electrodes, so only the latter will be described here. Liquid ion-selective membranes are composed of the sensor molecule dissolved in a plasticizer (membrane solvent). The membrane may also contain a lipophilic additive and a matrix. Liquid membrane sensors are commercially available for a wide range of ions (e.g. see Sigma; http://www.sigmaaldrich.com/).

To make an ion-selective microelectrode, the tip of the electrode is filled with an ion-sensing chemical cocktail which gives a voltage output of different values when placed in solutions containing different activities of the ion. Therefore when an electrode is inserted into a cell, the voltage measured gives a direct indication of the intracellular ion activity. This situation is complicated by the voltage across the cell membrane; the ion-selective electrode will sense this in addition to voltage due to the activity of the ion of interest. To obtain the output for the ion alone, the cell membrane potential must be subtracted. This is done by using either two single electrodes or a double-barreled electrode in which the ion-sensing electrode is combined with a cell-voltage-measuring electrode (see Fig. 2.1). Both output voltages are measured against a reference ground electrode in the external solution. The ion activity is determined from the calibration curve after subtracting the membrane potential.

2.2.3 Making ISEs

The preparation of ion-selective microelectrodes can be divided into four main stages:

A. Pulling of glass micropipettes
B. Silanization of the inside of surface of the ion-selective electrode or barrel
C. Backfilling
D. Calibration

The preparation of a nitrate-selective cocktail for backfilling microelectrodes is described by Miller and Zhen (1991) and a generalized method which is suitable for all different types of ion-selective microelectrode has been described previously (Miller 1995). The background to each stage is described here.

2.2.3.1 Pulling of glass micropipettes

Microelectrodes should be prepared to give dimensions suitable for impaling the target cell type. Double-barreled microelectrodes can be prepared by

twisting together two single pieces of filamented glass tubing or using glass which is already fused. Filamented glass has a glass fiber attached to the inner wall; this fiber assists backfilling by providing a hydraulic conduit along which the solution can flow by capillarity. Twisting is done using an electrode puller which both heats the glass and pulls it in a way pre-determined by the operator. The heating is paused for the two barrels to be twisted around one another and then the heating and pulling continues. There are various different types of microelectrode puller and the most important feature is reproducibility; this ensures that when an optimum microelectrode shape for a particular cell type has been prepared, it can be exactly duplicated many times. Before or after pulling, glue or heat shrink tubing can be used to provide support and additional strength to hold together the two or three pieces of glass.

Microelectrodes are usually made from borosilicate glass although the harder aluminosilicate glass is sometimes used. Multi-barreled glass of varying dimensions can be purchased from suppliers (e.g. Hilgenberg; http://www.hilgenberg-gmbh.de/). This type of glass seems to be the best for ion-selective microelectrode work. An alternative type of double-barreled glass called "theta" glass can be used; this has a single thin glass wall between the two pre-formed barrels. Adjacent ion-selective barrels may mutually interfere because the thin glass walls at the electrode tip have electrical impedance that may be as low as the impedances of the liquid ion-exchangers so that the measured potential depends on the potential across the glass as well as the potential across the liquid ion-exchanger. This problem is more acute when "theta" glass is used because the final glass partition in the tip is much thinner. Both barrels of glass should have an internal filament to assist with backfilling. Identification of the different barrels can be done by using different diameter glass, marking with a pen, cutting to various lengths or bending the blunt end of one barrel to give an obvious angle. The advantage of each of the latter two methods is that they make it easier to insert Ag wire (see Fig. 2.1). Wear safety glasses at all times when pulling and breaking glass.

Before preparing the ion-selective microelectrode it is important to determine that glass microelectrodes filled with 0.1 M KCl can be used to impale cells and measure stable resting membrane potentials sensitive to metabolic inhibitors (in the usual range for the cell type, in the bathing solution used). An estimate of the tip geometry of the microelectrode is provided by measuring its electrical resistance when filled with KCl, larger tips having lower resistances. For tips of 2–0.1 μm diameter the electrical resistances of ion-selective microelectrodes are usually in the GΩ range, while microelectrodes filled with 0.1 M KCl have 10^3 smaller resistances in the MΩ range. Electrical resistance does depend on the salt concentration of the backfilling solution. The dimensions of the microelectrodes are usually a compromise between obtaining a stable membrane potential and a good calibration response (detection limit).

2.2.3.2 Silanization of glass surface

The inside of the glass micropipettes must be given a hydrophobic coating to allow the formation of a high resistance seal between the glass and the hydrophobic ion-selective membrane. The barrel designated to be ion-selective is heat dried, then silanized by placing a few drops of a solution of 2% (w/v) silanizing agent in chloroform on its blunt open end. There are a range of different silanizing agents which can be used at this concentration but dimethyldichlorosilane or tributylchlorosilane are most common. Care must be taken to ensure that the reagent does not enter the membrane potential-measuring barrel. Beware: silanizing agents are highly corrosive and toxic, protective glasses and gloves must be worn and glass must be treated in a fume hood. The microelectrode is then placed under a heating lamp giving a temperature of 140 °C at the micropipette surface. After 30 min drying the silanizing solution is added and quickly vaporizes, giving the ion-selective barrel a hydrophobic coating. There should be no visible residue remaining in the microelectrode tip before the next step, backfilling.

2.2.3.3 Backfilling

There are actually two steps to backfilling; the first uses a cocktail to form the ion-selective membrane in the microelectrode tip and the second step, usually a minimum of 48 h later, uses an aqueous salt solution to provide contact between this membrane and the Ag/AgCl metal electrode (in the base of the microelectrode holder). Both steps are made much simpler by using filamented glass to make the microelectrodes and can be achieved using a syringe and fine all metal needle.

Electrodes are back-filled with a sensor cocktail containing several different components:

- An ion-selective molecule, sensor or exchanger
- Membrane solvent or plasticizer
- Additives, e.g. lipophilic cation/anion
- A membrane matrix to solidify the ion-selective membrane. This is essential for measurements in cells possessing turgor.

For many ions, the membrane cocktail can be purchased already mixed and it is advisable to start by using the commercial mixture. However, preparing the cocktail from the individual components is cheaper and these can be bought from chemical suppliers. For commercially available liquid membrane cocktails the membrane matrix is not normally included. A matrix is needed if microelectrodes are to be used in plant cells because turgor will displace a liquid membrane from the electrode tip, thereby changing or eliminating the sensitivity to the measuring ion (Miller 1995). The matrix

used is normally a high molecular weight poly(vinyl chloride) (PVC) polymer, but can also include nitrocellulose for additional strength.

2.2.3.4 Cocktail components

The various components of an ion-selective membrane are mixed together to form a sensor cocktail. Commercial cocktails are available pre-mixed for many ions (e.g. the Selectophore® range from Fluka now sold by Sigma; http://www.sigmaaldrich.com/). If a matrix is present, the cocktail is usually dissolved in a solvent such as tetrahydrofuran. If the cocktail does not include a solvent, it can be introduced to the tip of a micropipette for immediate use. Membranes formed from solubilized cocktails are produced by solvent-casting. The mixture is introduced into a micropipette and the solvent is allowed to slowly evaporate to leave a solid or semi-solid membrane at the micropipette tip. The choice and relative proportions of the components of a cocktail determine the properties of the ion-selective membrane. Cocktails are optimized by a process of informed experimental trial and error, adjusting components and proportions until the desired properties are achieved.

Of all of the components, the ion-selective sensor is the main factor determining electrode characteristics (e.g. slope, selectivity, limit of detection); however the plasticizer can alter properties such as lifetime, stability and selectivity. Additionally, membrane additives, such as lipophilic ions, can be used to improve the performance of microelectrodes. Sometimes these additives can introduce changes in ion selectivity, for example the plasticizer can introduce nitrate sensitivity (Cuin et al. 1999). The final optimum cocktail is found by varying the composition of each component to find electrodes with the best performance. Good electrodes should have a low detection limit, a near ideal slope, and a small selectivity coefficient for physiologically important interfering ions. The roles played by each cocktail component are now described in more detail.

Ion-selective sensor. The sensor is the most important component of the membrane in determining electrode characteristics (Miller 1995). Sensor molecules employed in ion-selective membranes may be ion exchangers or neutral or charged carriers. The discovery of the ion-selective ionophores has lead to the development of a large range of sensors for ion-selective microelectrodes. Sensors are now available for a wide-range of cations and anions (see Miller 1995) and improved sensors are always being reported (e.g. for Na^+, Carden et al. 2001).

Plasticizer. The plasticizer forms the bulk of an ion-selective membrane (typically 60–90 wt %), and can substantially influence membrane properties such as selectivity and lifetime (Ammann 1986). The main function of the plasticizer is to solubilize the ion-selective sensor and any lipophilic additives.

Other important properties include lipophilicity, viscosity, and the ability to plasticize the matrix material (if any). Commonly used plasticizers include: dibutyl sebacate, bis(2-ethylhexyl) sebacate and 2-nitrophenyl octyl ether.

Additives. The performance of most cation-selective neutral carrier membranes can be enhanced by the addition of lipophilic additives. The most common additives are alkali metal salts of lipophilic anions such as sodium tetraphenylborate and potassium tetrakis(4-chlorophenyl) borate. These additives introduce mobile cation-exchange sites into the membrane which can produce many useful effects. They reduce or remove any interference from lipophilic anions in the sample, reduce electrode response time, lower the electrical membrane resistance, and improve cation sensitivity and selectivity (Ammann 1986). A lipophilic cation (methyltriphenylphosphonium bromide) has been used to improve the properties of an anion-selective membrane based on a charged carrier (Miller and Zhen 1991).

Matrix. The membrane matrix provides mechanical stability to a liquid membrane. The most widely used matrix is poly(vinyl chloride), but many other compounds have been used, including: silicone rubber, polyurethane, polystyrene, poly(methyl methacrylate), and nitrocellulose. If a microelectrode is to be used for recording from a cell with turgor, the inclusion of a matrix in the membrane is essential.

2.2.4 Calibration and storage

Ion-selective microelectrodes can be calibrated using concentration or activity, although they actually respond to changes in activity (see section 1.2). Furthermore, activity is actually the important parameter for all biochemical reactions. Calibrating with ion activity gives a microelectrode output which can be used directly without any assumptions of the intracellular activity coefficient for the ion. For these reasons the calibration of microelectrodes generally uses solutions which resemble the intracellular environment in terms of interfering ions, and ionic strength. Calibration of pH microelectrodes is easy because standard pH buffers can be used and simply checked with a pH meter. For other types of ion-selective microelectrode, the calibration solutions may need to contain a pH buffer and a background salt solution to give an ionic strength approximately equivalent to that inside the cell. Care must be taken in the choice of these additional ions; they must not give significant interference over the range of measurements. In other words, the microelectrodes must have very small selectivity coefficients for these background ions. Calibration solutions are usually chosen to be approximately 0.14 M ionic strength. There are very few examples of detailed whole cell sap analysis to suggest what an appropriate figure might be, but for giant algal cells this value would seem reasonable (Okihara and Kiyosawa 1988). The use of computer

programs to calculate ion activity and the availability of a wide range of ion-selective macroelectrodes make it easier to prepare calibration solutions for all types of ion-selective microelectrodes. Furthermore, calibration solution recipes have been published for some ion-selective microelectrodes, (Ca^{2+}, Tsien and Rink 1981; Mg^{2+}, Blatter and McGuigan 1988; Na^{+} Carden et al. 2001; NO_3^-; Miller and Zhen 1991). Some calibration solutions use concentration not activity, and also the term "free" ion usually means concentration of unbound ion and not activity, particularly for Ca^{2+} and Mg^{2+}. The calibration of calcium-selective microelectrodes for intracellular measurements requires the use of calcium buffering agents such as EGTA because of the very low concentrations being measured (Tsien and Rink 1981).

Ion-selective microelectrodes can be calibrated in the microscope chamber where intracellular measurements will be made or in a U-shaped glass funnel alongside the microscope. The slope of the calibration curve is temperature sensitive and both calibrations and intracellular measurements should be done at the same temperature. If the temperature of the calibration solutions is 4 °C and the cell is at 20 °C, the slope of the electrode calibration for a monovalent ion will be 55 mV per decade change in activity, not the 58 mV expected at 20 °C.

Curve fitting software such as the Marquardt curve-fitting algorithm within Sigmaplot (SPSS; http://www.spss.com/) can be used to fit the experimental data to an equation of the form:

$$\mathrm{EMF} = \mathrm{P1} + \mathrm{P2} \cdot \log\left(a_i + \mathrm{P3}\right) \tag{2}$$

where P1, P2 and P3 are constants. Equation (1) can be simplified to (2) and it is then apparent that P2 represents the Nernstian slope, s, and that P3 represents $K_{ij}^{pot} \cdot (a_j)^{zi/zj}$. The term K_{ij}^{pot} is defined as $a_i'/(a_j)^{zi/zj}$ and therefore P3 is equivalent to a_i', the activity at the intersection of the two linear portions of the response curve in Fig. 2.2—the IUPAC definition of detection limit (Inczédy et al. 1998). Constants P2 and P3 from Equation (2) can thus be used to determine the slope, detection limit, and selectivity coefficient (if a_j is known) of ion-selective microelectrodes without recourse to graphical techniques.

Ideally, the detection limits for ion-selective microelectrodes should be calculated using calibration solutions approximating to the cytosolic composition. In practice this is not so easy, because our knowledge of the ionic environment within a cell is very limited, although we do have some information on sap collected from giant algal cells (e.g. Okihara and Kiyosawa 1988). The detection limit of some macroelectrodes is very small, with values of around only 10^{-9} M for some types of ion (Bakker and Pretsch 2005). These limits are quoted by chemists developing new types of sensor molecules but the practical limits for microelectrodes are likely to be several orders of magnitude greater because values depend on the tip diameter and interfering ion concentrations. This means that lower detection limits are possible for extracellular measurements where larger tip diameters can be used.

For long-term storage, ion-selective microelectrodes should be stored without backfilling, in a silica-gel dried sealed container in the dark. This can be done in a screw-cap glass jar containing dry silica gel, with the microelectrodes attached to the inner wall using plasticine or Blu-tack (Bostik, Stafford, UK). Some types of ion-selective microelectrodes can be stored this way for several years and will still give a reasonable performance when back-filled.

2.3 Data analysis, interpretation and presentation

The high electrical resistance of ion-selective microelectrodes requires the use of a high input impedance ($>10^{15}$ Ω) electrometer to measure electrode voltages. In contrast, the electrometer output is of much lower impedance and can thus be monitored and recorded using less specialized equipment. The simplest method is to use a chart recorder, but this requires laborious subsequent processing and it is more convenient to collect data via an analogue to digital (A/D) converter connected to a personal computer. Most modern A/D converters are compatible with software that allows real-time display of the recorded data, reproducing the functionality of a chart recorder whilst storing the raw data in a format that can be easily exported to statistical and graphing software. A useful feature of any data collection software is the ability to fit calibration curves and use the fitted parameters to display real-time ion activities as the data are being collected.

Ion-selective macroelectrodes have a lower resistance and can be used with lower input impedance ($\sim 10^{12}$ Ω) devices such as benchtop pH/mV meters. The output from such meters can again be recorded using an A/D converter and PC or alternatively a simple data logger may be used. Connecting a battery-powered amplifier to a data logger creates a portable system. Portable macroelectrodes can be used for extracellular measurements in the field (Miller et al. 2003) and allow uptake studies to be made in controlled-environment conditions rather than the laboratory.

One point regarding statistical analysis of data concerns the calculation of mean values. These should be calculated using the data which is distributed normally, that is using the log activity or output voltages not the actual activities (Fry et al. 1990). Therefore, when mean activity value is used it can only be expressed within 95% confidence limits, whereas –log [activity] can be given standard errors or standard deviations.

When measuring changes in intracellular ion concentrations, artifacts can be caused by the differential response times of the two barrels; the ion-selective barrel usually has a slower response time than the membrane potential-sensing barrel. Response of the membrane-potential barrel is almost instantaneous whilst that of the ion-selective is usually in the order of 5–20 s depending on the measured activity (Fluka 1996). As ion activity is calculated from the voltage difference between the two barrels, an incorrect activity can

be reported for this time, limiting detection of rapid changes in ion activity. This can be corrected for when the response time of the electrode is known (Sanders and Slayman 1982).

2.4 Finding problems with ion-selective microelectrodes and a comparison with other methods

2.4.1 Troubleshooting guide

The best approach is to solve problems by a process of elimination. Firstly, establish whether a problem occurs in the circuitry or is specific to the ion-selective microelectrodes. The circuitry can be tested by putting a broken-tipped KCl-filled microelectrode in place of the ion-selective microelectrode. The broken-tipped should give a stable zero output. It may be necessary to recoat Ag/AgCl contact in the half-cell or there may be a wiring problem. Noisy recordings can be caused by poor earthing or air bubbles in backfilling solutions. If the circuitry has no problems then the ion-selective microelectrode must be the cause. When the ion-selective microelectrode does not respond to the calibration solutions then the membrane can be checked by deliberately breaking the tip to expose a larger area of ion-selective membrane. Breaking the tip can displace the ion-selective membrane from the tip so it is important to measure the resistance to check it is still in the GΩ range. If the broken tip gives a good response to changes in ion activity then the problem is independent of the composition of the membrane. When the microelectrode tip diameter becomes too fine the output from the ion-selective electrode will no longer respond to changes in ion activity.

Several criteria for acceptable measurements can be defined. After impalement the ion-selective microelectrode should be recalibrated and should give a very similar response to that shown before the cell impalement particularly at activities similar to those measured in vivo. Sometimes the detection limit of the ion-selective microelectrode has changed but provided the measurement was on the linear response range of the electrode calibration curve this is not usually a reason to disregard the result. The performance of the ion selective microelectrode can even improve with the detection limit actually becoming lower. For this reason, it may be best to quickly impale a cell with a new tip before calibrating prior to measuring the activity in the cell. A comparison between the electrical resistance of the ion-selective microelectrode before and after impalement provides a good indicator of whether the tip will recalibrate. If the resistance decreases below 1 GΩ, the ion-selective membrane has probably been displaced during impalement and the electrode will not recalibrate. Throughout the recording the state of the cell can be assessed by monitoring the membrane potential (which should remain stable unless deliberately perturbed) or processes such as cytoplasmic streaming.

In plant cells, identifying in which internal cell compartment (cytoplasm or vacuole) the microelectrode tip is located can be a problem for some ions and it may be necessary to grow the plant under conditions in which two populations of measurements can be identified. Alternatively, a triple-barreled microelectrode can be used where one barrel is pH or Ca^{2+} selective. Large gradients of these two ions are known to exist across the tonoplast, with the cytoplasm maintained at relatively constant values (pH 7.2, Ca^{2+} 100 nM) so compartment identification is possible. Another approach is to use tissues where the two major cell compartments can be identified under the microscope, e.g. root hairs, or cell cultures which have no large vacuole. However, identifying which compartment the electrode is in can still be problematic, particularly if the electrode indents the tonoplast but does not penetrate it.

Leakage of salts from the tip of the membrane potential-sensing barrel has been reported (Blatt and Slayman 1983), and this may particularly be a problem in small cells. Diffusion of ions from the membrane potential-sensing barrel could give high local gradients of ions at the tip of a double-barreled microelectrode. It may be important to try measurements where different types of backfilling solution are used in the reference barrel. Large leaks should affect membrane potential and monitoring this should indicate possible problems.

The Ag/AgCl junctions of electrodes have been found to respond directly to light (Janz 1961) and problems with obtaining stable recordings can result from a poor chloride coating on the Ag of the half-cell. For stable recording, both the metal electrode part of half-cells and the Ag wire contacts (see Fig. 2.1) require regular re-coating with AgCl.

A further possible problem can arise when using ion-selective microelectrodes with inhibitors. Some inhibitor chemicals are highly lipophilic and will readily dissolve in the ion-selective membrane. These chemicals can poison the membrane but this will be demonstrated during the recalibration of the ion-selective microelectrode.

A few practical points:

- Handle microelectrodes with forceps.
- When dispensing THF pour a few ml from the stock bottle into a clean glass beaker, after first rinsing the beaker with a little freshly-dispensed THF. Cover the beaker with Parafilm, then dispense further THF using a glass syringe and needle by piercing the film cover with the needle, this helps to reduce solvent vapor and prevents contamination of THF.
- If more than one type of cocktail is used, employ a different syringe for each type of cocktail. It is best to dedicate a syringe for one particular cocktail only, and thus avoid any contamination by other ion sensors.
- Calibrate starting with the highest concentration, and calibrate only in the range in which you expect to be working. There is no point in exposing electrodes unnecessarily to low ion concentrations as most types of ion-selective membrane respond badly to long exposures at very low concentrations.

2.4.2 Comparison with other methods

No method is ideal for measuring transmembrane ion gradients as all involve some perturbation of the tissue that may directly influence the parameters being measured. Extracellular measurements and biological samples can use ISEs. A recent review of various analytical methods for measuring trace elements in biological samples compared potentiometry (ISEs), voltammetry, atomic spectrometry (e.g. ICP-MS, inductively coupled plasma mass spectrometry) and nuclear techniques (Brown and Milton 2005). For example, ICP-MS and ISE measurement of lead concentrations in the same samples gave excellent agreement. The authors reported that ISEs compared well and provided moderate throughput at a low cost but the detection limits were higher than most other methods, typically 10^{-9} M compared with 10^{-11} M.

There are several different non-destructive methods for measuring transmembrane ion gradients. Ion-selective microelectrodes have an advantage over most other methods because they can be used to report the ion activities in single cells and within the vacuole and cytoplasm. Compartmental tracer ion efflux analysis has frequently been used to measure the cytoplasmic concentrations of ions within living cells (MacRobbie 1971). This technique depends on the fact that the labeled ion or compound is in equilibrium with the unlabelled form in all parts of the cell. The method treats all the different tissues of an organ like the root as a single entity even though the transport properties of each may be different. For the calculation of the intracellular ion concentrations, it is necessary to make assumptions about the volume of each cellular compartment (see Miller and Smith 1996). Dyes can be used to measure intracellular ion concentrations or activities but they first require some method for introducing the dye into the cell. Microinjection, ionophoresis (using electrical current flow to carry charged molecules) and in some cases the cell's transport systems can be used to take up ion reporter dyes into cells (Negulescu and Machen 1990). Once inside the cell, dyes are usually used to monitor ion concentrations in the cytoplasm. The presence of the dye in the cell may influence normal cell processes. In addition, the dyes themselves must bind the ion being detected to function as a reporter and may therefore be influencing the parameter that they are measuring. Nuclear magnetic resonance can also be used to measure transmembrane ion gradients of some ions (e.g. pH and phosphate), but is more commonly used for metabolite molecules (reviewed by Ratcliffe and Shachar-Hill 2001).

Plants can be genetically engineered to express foreign proteins that are sensitive to changes in the local ion environment and these can be used to measure in both the apoplast and the cytoplasm (e.g. Gao et al. 2004). The quantitative use of these reporter proteins requires in vitro calibration and to obtain sufficient signal for the detection system expression is driven by a strong promoter (e.g. CaMV 35S). The use of this promoter gives expression of the reporter protein in many different types of tissue. These proteins, like

the dyes, require physical interaction with the ion and their presence in the cell may cause modifications to normal cellular function.

Energy dispersive X-ray microanalysis (EDAX) is a destructive method for measuring intracellular ion concentrations and the method requires chemical fixation of the tissue prior to the measurement. This treatment may lead to changes in transmembrane ion gradients before the fixation is complete. The precise area of tissue sampled for this analysis is difficult to control and so compartmental assignment of the measurement can be difficult. The method gives total elemental analysis of the tissue and for many ions this figure may be very different from the more biologically relevant value, the ion activity. Another example of a destructive technique is cell fractionation; this involves breaking the tissue into protoplasts and then vacuoles and measuring the concentrations of ions in each fraction. Unfortunately, the preparation of the cell fractions requires incubation for several hours in tissue degrading enzymes that might lead to changes in transmembrane ion gradients.

There are very few comparisons of techniques for intracellular measurements. Intracellular ion-selective microelectrode measurements of vacuolar nitrate have been compared with whole tissue analysis and single cell sap sampling methods (Zhen et al. 1991). In a more recent paper, nitrate-selective microelectrode measurements of cytosolic nitrate were compared with values obtained using NMR on the same tissue (Radcliffe et al. 2005). In both these examples, there was good agreement between the different methods employed. The best approach to making intracellular measurements of ion concentrations is to use several different methods to obtain a consensus value.

2.5 Transport and transmembrane ion gradients

Ion-selective microelectrode data give information as to both intracellular activities and the electrical gradients across the plasma membrane and tonoplast. This information on the intracellular electrochemical gradients can be used to determine the likely mechanisms of transport across cell membranes. One example is the use of pH and nitrate-selective microelectrode measurements to determine the thermodynamics of nitrate transport systems across the plasma membrane (Miller and Smith 1996) and tonoplast (Miller and Smith 1992). Compartmental measurements of the transmembrane ion gradients of NO_3^- and H^+ enabled the energetic feasibility of different co-transporter stoichiometries to be calculated. This type of measurement can also be used to show the activity of proton-coupled transport systems at the plasma membrane as an acidification of cytosolic pH can be measured when nitrate is supplied outside the cell.

Figure 2.3 shows the distribution of NH_4^+ in an internodal cell of the freshwater alga *Chara corallina* measured using NH_4^+-selective microelectrodes (Wells and Miller 2000). Published data for vacuolar and cytoplasmic pH

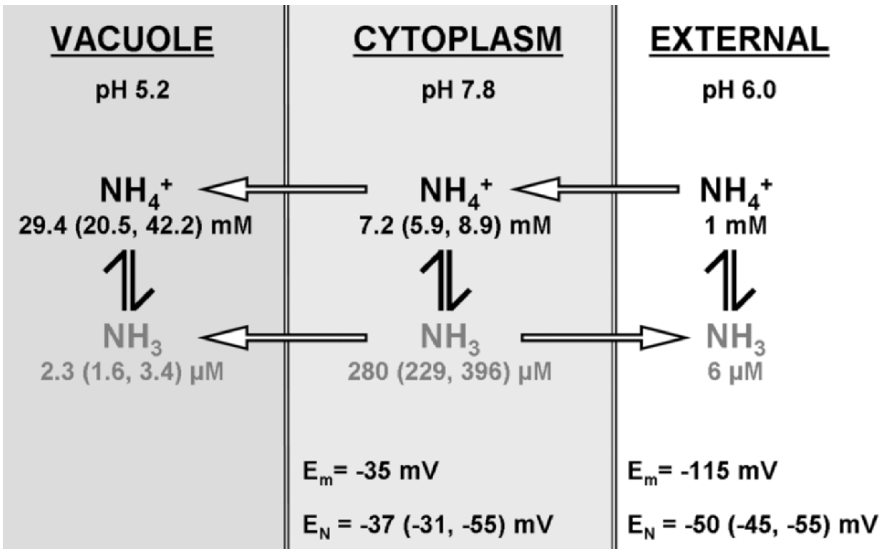

Fig. 2.3. Diagrammatic representation of the transmembrane pH, NH_3 and NH_4^+ gradients in an internodal cell of *Chara corallina*. E_N is the Nernstian equilibrium voltage for the measured NH_4^+ distribution. *Arrows* show direction of the chemical gradient for NH_3 and the electrochemical gradient for NH_4^+. Microelectrode and derived data are given as means (1 SD range). Data from Wells and Miller (2000 and unpublished)

allows a calculation of the compartmental concentrations of NH_3 using the Henderson–Hasselbalch equation. Microelectrodes also measure the transtonoplast and plasma membrane potentials, allowing the mechanism of transport of the two forms of ammonium to be modeled. The Nernstian equilibrium voltage for the observed distribution of NH_4^+ across the plasma membrane is –50 mV, lower than the –115 mV measured, suggesting the passive uptake of NH_4^+ at this external concentration. Similarly, the observed vacuolar levels of NH_4^+ may be explained by passive transport driven by the transtonoplast potential. Calculated NH_3 concentration based on the experimental data is higher in the cytoplasm than either the vacuole or the external solution. NH_3 is freely diffusible across biological membranes and passive diffusion of NH_3 along this concentration gradient will thus be out of the cytoplasm into both the vacuole and the external solution.

Ion-selective microelectrodes can be used to directly measure the ion concentrations in other biological situations such as depletion from a nutrient solution for net uptake studies or on very small samples such as plant exudates. Micro- and macro-electrodes can also be used directly in soil where they provide a direct measure of the nutrient concentration that is available at the surface of plant roots and allow mapping of spatial and temporal nutrient heterogeneity. Soil electrode data can also be used to shed light on important soil

processes such as N mineralization, which can be quantified by the simultaneous use of nitrate- and ammonium-selective electrodes. Combining such field data with laboratory measurement of apoplastic and intracellular ion concentrations allows models of transport to be developed.

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