

Overcoming the Problem of Non-Ideal Liquid Ion Exchanger Selectivity in Microelectrode Ion Flux Measurements

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Abstract. Ion-selective microelectrodes are a powerful tool in studies on various aspects of cell membrane biology in both animal and plant tissues. Further application of this technique is, however, limited to a large extent by the problem of non-ideal selectivity of the liquid ion exchanger used in the preparation of microelectrodes for ion flux measurements. Because of this problem, which is persistent in many commercial liquid ion exchangers, the microelectrode does not discriminate between the ion of interest and other interfering ions (for example, Mg^{2+} and Ca^{2+} ; Na^+ and K^+), thereby leading to inaccurate concentration readings and, consequently, inaccurate flux calculations. In this work we show that the existing analytical procedure to overcome this problem, using the inverted Nicolsky-Eisenman equation, is inadequate, and suggest an alternative analytical procedure that can be applied directly to the data obtained with commercially available liquid ion exchangers. We show that this alternative procedure allows accurate measurement of ionic concentrations with non-ideal ion-selective microelectrodes in the presence of interfering ions, and illustrate the method by direct experiment using Ca^{2+} and Mg^{2+} as a “case study”. Several more examples are given, further illustrating practical applications of the method for study of plant responses to salinity, osmotic and reactive oxygen species stresses.

Key words: Microelectrodes — Ion fluxes — Selectivity — Liquid ion exchanger — Membrane

Introduction

Trans-membrane ion transport processes are central to the regulation of plant homeostasis and adaptation

(Zimmerman et al., 1999; Shabala, 2003). Ion-selective microelectrodes are a quick, convenient, and relatively inexpensive method to measure net ion fluxes around living tissue, so it is not surprising that they are used in many aspects of modern plant-biological research. There is, however, one factor that severely limits the usefulness of ion-selective microelectrode measurements and creates a major problem for both intracellular measurements of ionic concentrations and non-invasive ion flux measurements: the non-ideal selectivity of the liquid ion exchangers (LIX) used to form the membranes of the microelectrodes (Ammann, 1986). A microelectrode with an ideally selective membrane responds to only one ion, with the electrical potential measured by the electrode related simply to the activity of the ion on the outer surface of the membrane. On the other hand, a microelectrode with a non-ideally selective membrane responds both to the ion of interest and to other ions, known as interfering ions, and there is not a simple relation between the electrical potential measured by the electrode and the external ionic activity. Physiologically important examples of this non-ideal LIX selectivity include Na^+ ionophores being responsive to K^+ , and Mg^{2+} to Ca^{2+} and, to a lesser extent, K^+ . In the case of Na^+ -selective microelectrodes, the high background K^+ concentration in the plant cell and the poor discrimination between Na^+ and K^+ of the microelectrode LIX molecule severely limit the range of Na^+ measurements (Carden, Diamond & Miller, 2001). Similarly, most Mg^{2+} carriers suffer from serious K^+ interference when used for intracellular measurements, while in extracellular potentiometric Mg^{2+} assays, interference from Ca^{2+} is a major drawback (Zhang, X.J., Fakler & Spichiger, 1998).

One way of overcoming this problem is to find better (i.e., more selective) ionophores. To date, however, only a few carriers have been successfully used in practical applications since, under realistic

conditions, their selectivity and reliability are generally limited (Zhang, X.J., et al., 1998). For example, major efforts have been focused on designing Mg^{2+} -selective neutral carriers for electrodes that reject alkaline and alkaline-earth metal cations or, at least, exhibit high selectivities for Mg^{2+} over Na^+ , K^+ , and Ca^{2+} , but even the most improved Mg^{2+} carrier still has rather poor discrimination between Mg^{2+} and Ca^{2+} , with $\log K^{\text{pot}}_{\text{MgCa}} = -0.7$ (Schaller, Spichiger & Simon, 1993; Zhang, X.J., et al., 1998). Furthermore, it is important to note that any search for new, more selective LIX is a slow process due to the difficulty involved in designing, fabricating and testing new chemical compounds. Many researchers do not have the resources to identify and/or fabricate these novel LIX, or they may require quantities beyond their own manufacturing capabilities, say, for large-scale plant screening. Such researchers are forced to use commercially available LIX and work around its attendant foibles.

A simpler approach to compensate for the problem of non-ideal LIX selectivity is to apply an analytical procedure to the data obtained with commercially available LIX to remove the confounding information. The accepted method for dealing with non-ideal ion-selective electrodes uses the Nicolsky-Eisenman (N-E) equation. In this paper, we demonstrate that this existing method is inadequate. We suggest an alternative analytical procedure, which allows accurate determination of ionic concentrations in the presence of an interfering ion, whence ion fluxes may be routinely calculated. We illustrate the use of this procedure by applying it to experiments measuring Mg^{2+} fluxes in the presence and absence of Ca^{2+} , and Na^+ fluxes in the presence of K^+ , neither of which has been presented previously, to our knowledge.

Materials and Methods

PLANT MATERIAL

Wheat (*Triticum aestivum* L. cv. ES8) seeds were kindly supplied by Dr P. Ryan (CSIRO Plant Industry, Canberra), and were germinated hydroponically, essentially as described in Shabala & Knowles (2002). *Arabidopsis thaliana* (Heyn) cv. Columbia seeds were from our laboratory stock, and were grown aseptically at 22°C for 8 to 10 days (16 h day length; 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance) on standard medium comprising 0.3% (w/v) Phytigel (Sigma), full-strength Murashige-Skoog medium (Duchefa, Haarlem, Netherlands) and 1% (w/v) sucrose (Demidchik et al., 2002).

MICROELECTRODE FABRICATION

Fluxes of Mg^{2+} , Ca^{2+} , K^+ and Na^+ were measured in this study. Microelectrodes were fabricated essentially as described in our previous publications (Shabala, Newman & Morris, 1997; Shabala & Lew, 2002). Briefly, electrodes with a tip diameter of about 2 μm

were pulled from borosilicate glass capillaries, dried in an oven for at least 5 h at 220°C, and silanized with tributylchlorosilane (Sigma-Aldrich, Milwaukee, WI). Electrodes were first back-filled with an appropriate solution (0.5 M CaCl_2 for Ca^{2+} , 0.2 M KCl for K^+ , 0.5 M NaCl for Na^+ , and 0.5 M MgCl_2 for Mg^{2+}), then the electrode tips were front-filled with commercially available ion-selective cocktails (K^+ , 60031; Ca^{2+} , 21048; Mg^{2+} , 63048; Na^+ , 71176; all from Sigma-Aldrich). After some time required for conditioning, the electrodes were calibrated in a set of single-ion standards (concentrations of 100 μM , 200 μM and 500 μM of the ion of interest). Electrodes with a response of less than 50 mV per decade for monovalent ions or 25 mV per decade for divalent ions, and correlation $R < 0.999$, were discarded. The reference electrode was a glass capillary filled with 500 mM KCl in 2% agar.

ELECTRODE RESPONSE TESTING

The voltage responses of Mg^{2+} - or Na^+ -selective electrodes in the presence of interfering ions were measured using standard solutions containing known concentrations (0 μM , 200 μM and 500 μM) and combinations of the chloride salts of Mg^{2+} and Ca^{2+} , or Na^+ and K^+ , as appropriate. These voltage responses were used for comparison with the Nicolsky-Eisenman equation and in the determination of equations (7), (10) and (11). Further, the inverted Nicolsky-Eisenman equation and equation (12) were tested using these voltage responses.

ION FLUX MEASUREMENTS

The microelectrode ion flux estimation (MIFE) technique is described in detail elsewhere (Shabala et al., 1997; Shabala, 2000; Newman, 2001). Each plant was mounted either in a Perspex holder by an agar drop (*Arabidopsis* roots) or by a cotton thread (wheat), and roots were immersed in assay solution (as specified in figure legends). Two microelectrodes (one, the non-ideal electrode, the other selective only to the interfering ion; that is, either Mg^{2+} - and Ca^{2+} -, or Na^+ - and K^+ -selective electrodes, depending upon the experiment) were collocated 20 μm above the root surface. Net ion fluxes were measured for 5–10 min in steady-state conditions, then a treatment was given and transient ion fluxes were measured for 30–40 min. The flux calculations were then performed using the suggested algorithm (see Appendix).

ABBREVIATIONS

KIR: Potassium inwards rectifying channel; KOR: Potassium outwards rectifying channel; LIX: Liquid ion exchanger; N-E: Nicolsky-Eisenman; ROS: Reactive oxygen species; NSCC: Non-specific cation channel; MIFE: microelectrode ion flux estimation.

Theory

CALCULATION OF NET FLUXES AROUND PLANT ROOTS

In the absence of significant electric gradients around plant roots, the net flux of an ionic species, j , at the surface of a cylindrical root of radius r can be calculated using an expression derived from Fick's First Law of Diffusion (Henriksen et al., 1992):

$$J_j = u_j RT \left(\frac{[j](r_2) - [j](r_1)}{\ln(r_2/r_1)} \right) \frac{1}{r}, \quad (1)$$

where J_j is the net flux; u_j , the mobility of the ionic species j ; $[j](r_1)$ and $[j](r_2)$ are the concentrations of the ionic species j at two radii r_1 and r_2 from the center of the root. That is, by measuring the concentration of an ion at two points radially distant from the surface of a root, it is possible to estimate the flux of the ion at the surface of the root. Adaptation of this equation to other geometries (planar for biofilms or leaf surfaces; spherical for protoplasts) is quite straightforward and is discussed in, amongst other places, Newman (2001).

DETERMINATION OF ION CONCENTRATIONS IN THE ABSENCE OF INTERFERING IONS

Potentiometric ion-selective membrane electrodes respond to concentration differences between the solution inside the electrode and the solution outside the electrode by producing a potential difference across the ion-selective membrane that is measured by the system voltmeter. In a simple system with an ideal ion-selective membrane the potential difference is given by the Nernst equation, and is proportional to the ratio of the activities of the ions of interest on either side of the membrane:

$$V^{\text{membrane}} = V^{\text{in}} - V^{\text{out}} = \frac{RT}{zF} \ln \frac{a^{\text{out}}}{a^{\text{in}}} \quad (2)$$

where V^{in} and V^{out} are the electrical potentials on the electrode-interior side and solution side of the ion-selective membrane, respectively; R is the universal gas constant; T , the temperature of the system in Kelvin; F , the Faraday constant; z , the valence of the ion to which the membrane is “selective” and a^{out} and a^{in} are the activities of the ions outside and inside the microelectrode, respectively.

Under standard experimental conditions discussed in Newman (2001) this may be rewritten in terms of the concentration of the sample solution and two system-specific parameters:

$$V = V_0 + s \log[c^{\text{out}}] \quad (3)$$

where $s = \frac{RT}{zF}$ (known as the Nernst slope) and V_0 (the sum of the constant potentials within the system) are the slope and intercept of the microelectrode calibration line, respectively, and can be subsequently rearranged and used to determine sample concentrations from electrode electrical responses:

$$c^{\text{out}} = 10^{\frac{V-V_0}{s}} \quad (4)$$

EFFECT OF INTERFERING IONS ON SUSCEPTIBLE ELECTRODES

In a non-ideal system, when the ion-selective electrode responds to more than one ion and interfering ions are present, the response of the electrode is not

Table 1. The nature of the problem caused by non-ideal selectivity.

Supplied Mg^{2+} (μM)	Supplied Ca^{2+} (μM)	Calculated Mg^{2+} (μM)
0	200	1433 \pm 112
0	500	4221 \pm 419
200	0	191 \pm 4
200	200	1919 \pm 144
200	500	4897 \pm 523
500	0	527 \pm 17
500	200	2484 \pm 162
500	500	5713 \pm 514

Samples ($n = 4$) containing known concentrations of Mg^{2+} and Ca^{2+} were measured using MIFE. The alleged Mg concentration (column 3) was calculated from the electrode potential response to the sample solutions using the standard MIFE concentration equation 4. As can be seen, the presence of Ca^{2+} in the sample solution dramatically increases the amount of Mg^{2+} “measured” by the Mg electrode; effectively, the electrode is selective for Ca^{2+} over Mg^{2+} .

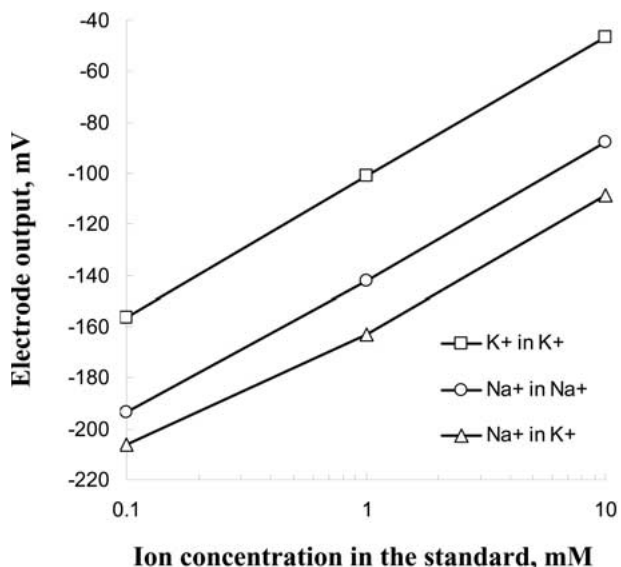


Fig. 1. Calibration curves of K^+ and Na^+ ion-selective microelectrodes. One representative example (out of 15) is shown. Fabricated electrodes, their tips filled with specific LIX, were calibrated in an appropriate set of standards, ranging from 0.1 to 10 mM. In addition, the Na^+ electrode was calibrated in a set of K^+ standards (triangles). Electrode characteristics were as follows. K^+ LIX in K^+ standard: slope -54.90 mV/decade, intercept 63.24 mV, correlation -0.9999 . Na^+ LIX in Na^+ standard: -52.95 mV/decade; 17.61 mV; -0.9999 . Na^+ LIX in K^+ standard: -49.76 mV/decade, -9.3 mV, -0.9998 .

Nernstian (Morf, 1981). That is, the potential of the electrode in the presence of interfering ions cannot be described by Eq. 3. For example, when samples containing known concentrations of Mg^{2+} and Ca^{2+} were measured using MIFE, the concentration of Mg^{2+} in the samples estimated from the voltage response of the Mg^{2+} electrode using the standard MIFE concentration equation (Eq. 4) was 5 to 10

times greater than the actual ion concentration (Table 1). Why this should occur is illustrated in Fig. 1: as can be seen, the Nernst slope for a Na^+ -selective microelectrode calibrated in K^+ is nearly -50 mV/decade with a 0.9998 correlation, i.e., almost “perfect”; such an electrode will clearly be responsive to any K^+ concentration changes in the system, and an analogous situation, albeit much more extreme, as the Mg^{2+} resin used is at least 10 times more sensitive to Ca^{2+} than to Mg^{2+} , occurs with a Mg^{2+} -selective electrode in the presence of Ca^{2+} . Now, from Eq. 1 it is evident that if the estimation of the concentrations is in error, the flux calculations will be in error by a similar factor. Consequently, it is necessary to correct the confounding effect of the interfering ions.

APPLICABILITY OF THE NICOLSKY-EISENMAN EQUATION TO CORRECT FOR THE PRESENCE OF INTERFERING IONS

The accepted method for correcting this non-ideal electrode response in the presence of an interfering ion is the Nicolsky-Eisenman equation (Macca, 2003)

$$V = V_0 + \frac{RT}{z_i F} \ln \left[a_i^{\text{out}} + \sum_{j \neq i} K_{ij}^{\text{pot}} (a_j^{\text{out}})^{\frac{z_i}{z_j}} \right] \quad (5)$$

where R , T , F and V_0 have their usual meanings; a_i^{out} and z_i are the activity of the ion of interest in the solution outside the ion-selective electrode and the valence of same, respectively; a_j^{out} and z_j are the activity of the interfering ions in the solution outside the ion-selective electrode and the valence of same, respectively; and K_{ij}^{pot} is known as the selectivity constant of the membrane.

The determination of the selectivity coefficient presents some practical problems: its value varies according to the experimental method of evaluation (Vaughan-Jones & Aickin, 1987; Zhang, W., et al., 1998; Bakker, Pretsch & Buhlmann, 2000), and also with the activities of the ions present in the solution (Ren, 2000). Nonetheless, with carefully chosen values of K_{ij}^{pot} , the N-E equation approximates quite closely the voltage response of an ion-selective membrane electrode. For example, within a physiological range of concentrations of external Ca^{2+} and Mg^{2+} ions the N-E equation, used with the Fluka-provided value for the selectivity coefficient ($\log K_{\text{MgCa}}^{\text{pot}} = 1.1$; note that the coefficient currently provided on the Fluka web-site is incorrectly given as $\log K_{\text{MgCa}}^{\text{pot}} = -1.1$) for the LIX, predicted the voltage response of the Mg^{2+} electrode to within 5% (Table 2), which is quite acceptable.

But while the N-E equation adequately approximates the voltage response of an ion-selective electrode in the presence of an interfering ion, when the equation is inverted for practical use in a potentiometric electrode system the results provided by the equation are not only far from accurate, but

Table 2. The accuracy of the Nicolsky-Eisenman equation 5 in predicting the voltage response of ion-selective membrane electrodes in the presence of an interfering ion.

Supplied Mg^{2+} (μM)	Supplied Ca^{2+} (μM)	Fraction
0	200	0.930 ± 0.009
0	500	0.940 ± 0.013
200	0	0.995 ± 0.002
200	200	0.955 ± 0.009
200	500	0.953 ± 0.014
500	0	1.004 ± 0.003
500	200	0.971 ± 0.008
500	500	0.967 ± 0.012

Samples ($n = 4$) containing known concentrations of Mg^{2+} and Ca^{2+} were measured using MIFE, the measured voltage being compared with the expected response calculated with Eq. 4 using the known Mg^{2+} and Ca^{2+} concentrations. Column “Fraction” = theoretical response \div actual response.

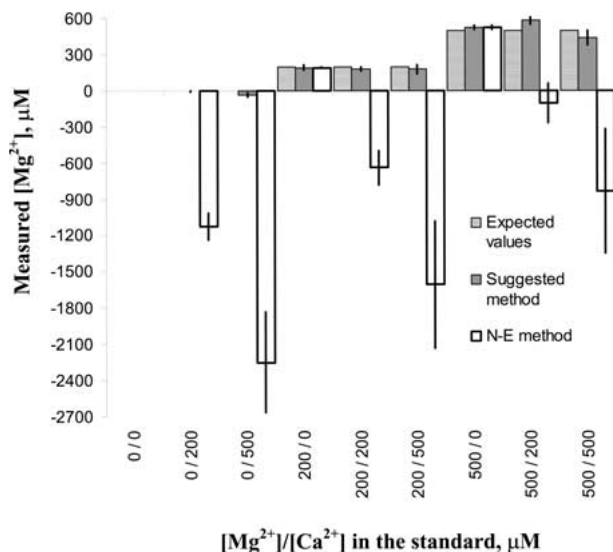


Fig. 2. Measurements of free Mg^{2+} concentrations in experimental solution at different $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratios, using traditional (Nicolsky-Eisenman, N-E) and suggested methods. Samples containing known concentrations of Mg^{2+} and Ca^{2+} were measured using the MIFE technique, and the concentration of Mg^{2+} in the samples was estimated from the voltage response of the electrode using the inverted extended Nicolsky equation and published values for the selectivity coefficient. White bars are expected concentrations of Mg^{2+} in experimental solution. Data are average \pm SEM ($n = 4$). Please note negative values for measured Mg^{2+} concentrations at low $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratios.

also contain some implausible values, such as negative ion concentrations (Fig. 2). Now, under the standard conditions described in Newman (2001), Eq. 5, when inverted, becomes

$$c_i = 10^{\frac{V-V_0}{s}} - \sum_{j \neq i} K_{ij}^{\text{pot}} (c_j^{\text{out}})^{\frac{z_i}{z_j}} \quad (6)$$

This equation, when used with the appropriate Fluka selectivity coefficient, interfering ion concentration, Mg^{2+} electrode calibration parameters and measured Mg^{2+} -electrode voltage response to a known sample solution of 0.2 mM MgCl_2 and 0.2 mM CaCl_2 , calculates the Mg^{2+} concentration to be a ridiculous -0.65 mM. Furthermore, the higher is the Ca^{2+} to Mg^{2+} ratio, the bigger is the inaccuracy in Mg^{2+} concentration measurements (Fig. 2).

Recall that the very same value of the selectivity coefficient, when used in the non-inverted N-E equation (5) under similar experimental conditions, gave very good correspondence between the theoretical and actual electrode voltage responses in the presence of an interfering ion (Table 2). This implies that the problem is with the actual form of equation 5. To proceed, it is necessary to find an equation to describe the voltage response of an ion-selective electrode in the presence of an interfering ion that has a form that will allow it to be inverted to allow determination of the concentration of an ion from the electrode voltage response.

A NOVEL APPROACH TO OVERCOME THE PROBLEM

Ion-selective microelectrodes usually respond differently (in terms of the electrode calibration slope and intercept) to the ion of interest and the interfering ion if measured in single-ion solutions (e.g., Fig. 1). In the presence of both ions the response of the electrode should be treated as a function of both ions, with the electrode response being described as a combination of the electrode responses to the individual ions present. A further restriction on the expression is that, in the absence of one of the ions, the expression should describe the electrode response to the other ion. In a system containing the two ions Mg^{2+} and Ca^{2+} (for illustrative purposes), an equation that meets these criteria is

$$V = \phi_{\text{Mg}}\{V_{0\text{Mg}} + s_{\text{Mg}} \log([\text{Mg}^{2+}] + [\text{Ca}^{2+}])\} + \phi_{\text{Ca}}\{V_{0\text{Ca}} + s_{\text{Ca}} \log([\text{Mg}^{2+}] + [\text{Ca}^{2+}])\} \quad (7)$$

where ϕ_{Mg} and ϕ_{Ca} are coefficients; $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ are the activities or concentrations of the ions Mg^{2+} and Ca^{2+} , and V_0 and s are the offset and slope of the electrode calibration graph, respectively, with the subscript referring to the single-ion solution in which the electrode was calibrated to get the values for V_0 and s . Note that this and subsequent equations apply equally to Na^+/K^+ .

The values for the concentrations of both Mg^{2+} and Ca^{2+} must be contained within the argument for the logarithm or the expression will become unde-

fined when either of the two ions is absent. For example, if Eq. 7 was

$$V = \phi_{\text{Mg}}\{V_{0\text{Mg}} + s_{\text{Mg}} \log([\text{Mg}^{2+}])\} + \phi_{\text{Ca}}\{V_{0\text{Ca}} + s_{\text{Ca}} \log([\text{Ca}^{2+}])\} \quad (8)$$

then, in the event that Ca^{2+} were absent from the solution, the second term of the right-hand side would become $\phi_{\text{Ca}}\{V_{0\text{Ca}} + s_{\text{Ca}} \log(0)\}$, which is not possible, as logarithms are not defined at zero.

Given the requirement for both the Mg^{2+} and Ca^{2+} concentrations to be included in the arguments of the logarithms, the coefficients ϕ_{Mg} and ϕ_{Ca} must be “shaped” so that, in the absence of one ion in solution, the term describing the component of the electrode’s response to that ion vanishes; the expression then describes the electrode response to the existing ion. For example, in the absence of any Mg^{2+} in solution, the coefficient ϕ_{Mg} in Eq. 7 must become zero so that the term giving the component of the electrode response to Mg^{2+} vanishes and the coefficient ϕ_{Ca} must become unity so that the expression describes the reaction of a simple Ca^{2+} electrode:

$$V = \{V_{0\text{Ca}} + s_{\text{Ca}} \log(0 + [\text{Ca}^{2+}])\} \quad (9)$$

The simplest mathematical form to provide this property is to define the coefficients ϕ_{Mg} and ϕ_{Ca} to be the mole fractions of the ions to which the membrane is responsive. However, empirical validation showed that better agreement is reached when the coefficients were defined as

$$\phi_{\text{Mg}} = \left(\frac{[\text{Mg}^{2+}]}{[\text{Mg}^{2+}] + [\text{Ca}^{2+}]} \right)^2 \quad (10)$$

and

$$\phi_{\text{Ca}} = 1 - \phi_{\text{Mg}} \quad (11)$$

when the electrode potential predicted by equation (7) was within 2% of the measured electrode potential (*data not shown*).

Now, while Eq. 7 adequately describes the voltage response of the Mg^{2+} ion-selective membrane electrode in the presence of the interfering ion Ca^{2+} , it must be inverted to be used to determine the Mg^{2+} concentration of a sample from the potential difference measured by the system. That is, Eq. 7 must be rearranged to express the Mg^{2+} concentration in terms of other system parameters:

$$[\text{Mg}^{2+}] = -[\text{Ca}^{2+}] + 10 \left\{ \frac{V - (\phi_{\text{Mg}} V_{0\text{Mg}} + \phi_{\text{Ca}} V_{0\text{Ca}})}{(\phi_{\text{Mg}} s_{\text{Mg}} + \phi_{\text{Ca}} s_{\text{Ca}})} \right\} \quad (12)$$

The corrected concentrations obtained from Eq. 12 can now be used in Eq. 1 to estimate ion fluxes.

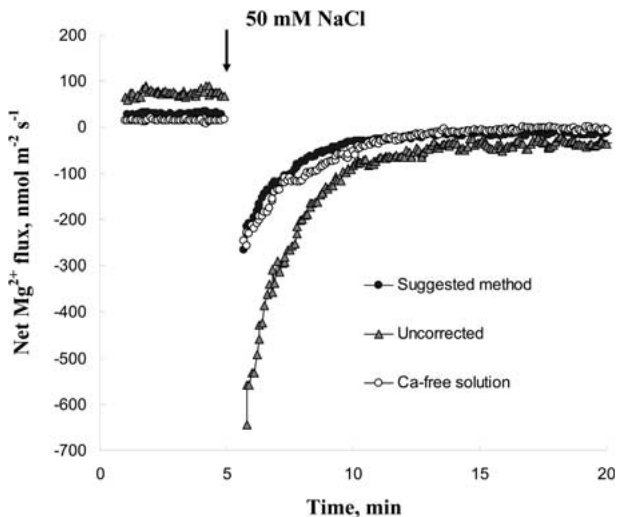


Fig. 3. Validation of the suggested method. Transient Mg^{2+} flux responses were measured from mature root epidermis of wheat (8 mm from the tip) in response to salt stress (50 mM NaCl) in two different types of solution. Solution 1 (*empty symbols*) was Ca^{2+} -free solution (0.2 mM MgCl_2 only); solution 2 (*filled symbols*) contained 0.2 mM CaCl_2 + 0.2 mM MgCl_2 . One (out of 4) representative example is shown. Please note a high degree of similarity between plant Mg^{2+} flux response to NaCl in Ca^{2+} solution and corrected values for measurements in the presence of interfering Ca^{2+} ions (*empty and filled circles*, respectively). Negative values correspond to net ion efflux.

Validation and Practical Examples

Equation 12 was developed and tested under conditions similar to the test of the inverted N-E equation (Eq. 6), and the result is shown in Fig. 2. It can be seen that Eq. 12 much more closely approximates the concentration of Mg^{2+} present in the sample. For example, from a known sample of 0.2 mM MgCl_2 and 0.2 mM CaCl_2 , the calculated Mg^{2+} concentration was $183 \pm 22 \mu\text{M}$, which is close to the expected 200 μM . It should be recalled that the use of the inverted N-E equation (currently used in physiological literature) results in an absurd reading of $-650 \mu\text{M}$.

This new method was applied in an experiment measuring net Mg^{2+} and Ca^{2+} ion fluxes from the mature region of wheat roots in response to salt stress (50 mM NaCl) in two different types of solution (Fig. 3). Wheat seedlings germinated and grown in a solution containing 0.2 mM CaCl_2 + 0.2 mM MgCl_2 were pre-treated, and fluxes measured, in one of two solutions, one solution of 0.2 mM CaCl_2 + 0.2 mM MgCl_2 , and the other of 0.2 mM MgCl_2 only. As can be seen in Fig. 3, due to Mg^{2+} LIX sensitivity to Ca^{2+} described above, the measured net Mg^{2+} flux response (calculated with standard MIFEFLUX software; Newman, 2001) was approximately double the actual one.

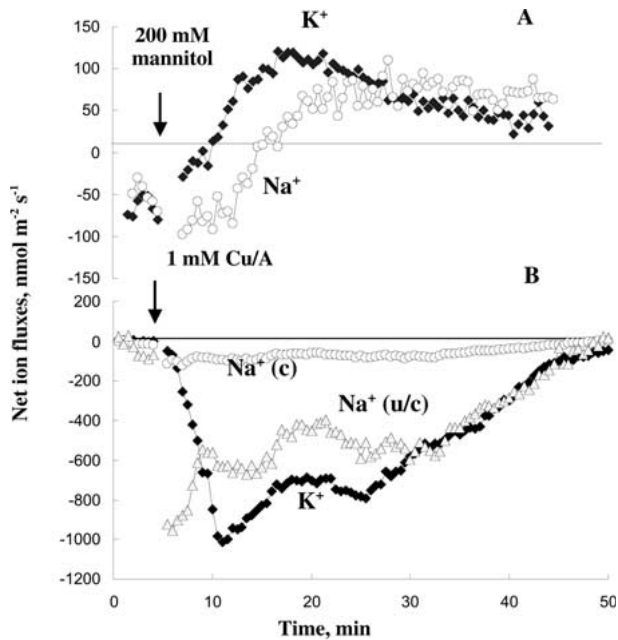


Fig. 4. Resolution of Na^+ and K^+ fluxes in response to various types of stress by suggested method. (A) Transient flux responses from *Arabidopsis* roots in response to hyperosmotic (200 mM mannitol) stress. Fluxes were measured in the mature (4 mm from the tip) zone of 8 d old roots. One representative example (out of 6) is shown. (B) Kinetics of Na^+ and K^+ flux responses to ROS (1 mM copper ascorbate, Cu/A, added at 4 min). One representative example (out of 5) is shown. Na^+ (u/c), uncorrected Na^+ flux values; Na^+ (c), Na^+ flux calculated by the suggested method. Negative values correspond to net ion efflux.

However, when measurements were performed in Ca^{2+} -free solution (*empty circles* in Fig. 3), the measured net Mg^{2+} fluxes were within 5% of those measured in 0.2 mM CaCl_2 + 0.2 mM MgCl_2 solution (*filled circles* in Fig. 3).

Two other practical examples are presented. Following successful testing of the suggested method on the Na^+/K^+ interfering pair (*results not shown*), the suggested method was used to resolve K^+ and Na^+ fluxes from *Arabidopsis* roots in response to hyperosmotic (200 mM mannitol) and ROS (1 mM copper ascorbate, Cu/A) stresses (Fig. 4A and Fig. 4B, respectively). In both cases the standard MIFEFLUX software significantly overestimated the maximum magnitude of Na^+ flux responses (5 to 6 times; Fig. 4B). More importantly, using the suggested method allowed fine resolution of Na^+ and K^+ flux kinetics, which are often stress-specific. For example, the hyperosmotic treatment resulted in rather distinct shifts in the timing of the activation of net K^+ and Na^+ fluxes into the *Arabidopsis* root (Fig. 4A), with the K^+ flux peaking 8 to 10 minutes earlier. This difference would have been overlooked had the “standard” calculation algorithms been used.

Discussion

It appears that the accepted analytical method to correct for non-ideal LIX selectivity, the N-E equation, while adequate to predict the voltage response of an ion-selective electrode in the presence of an interfering ion, is inadequate when inverted to determine the concentration of an ion from the electrode voltage response when there is an interfering ion present. Negative concentration readings (Fig. 2) are absurd and, since the error clearly does not lie with the selectivity coefficient, the problem must be with the form of the equation. The method suggested in this paper eliminates this problem, allowing measurements (within 10% accuracy, Fig. 2) with commercially available ionophore cocktails.

The application of this method to simultaneous measurements of fluxes of “interfering ions” may be of great importance for a wide and growing group of researchers making non-invasive ion flux measurements, or those interested in measuring intracellular ion concentrations with multi-barrelled ion-selective microelectrodes. For example, the kinetics of NaCl-induced Mg^{2+} fluxes are very similar, both qualitatively and quantitatively, to those of Ca^{2+} fluxes (*data not shown*, but *see* Shabala, Babourina & Newman (2000) for an example). Since it has been shown that NaCl-induced Ca^{2+} fluxes originate essentially from the cell wall (Shabala & Newman, 2000), it is reasonable to suggest that a similar scenario applies to NaCl-induced Mg^{2+} fluxes reported here (Fig. 3). An alternative explanation may be that both fluxes are due to activation of non-selective cation channels, NSCC (Demidchik, Davenport & Tester, 2002a). The answer may come from simultaneous measurement of net Mg^{2+} and Ca^{2+} fluxes from roots pre-treated with La^{3+} (a known Ca^{2+} -channel blocker) or Gd^{3+} (a non-selective cation channel blocker) and/or from measurements of Mg^{2+} fluxes from isolated protoplasts.

A wide range of possibilities is presented when net K^+ and Na^+ fluxes are measured simultaneously (Fig. 4). It has been reported that both Na^+ and K^+ ions are taken up in response to hyperosmotic treatment (Shabala et al., 2000; Shabala & Lew, 2002). From Fig. 4A, it appears that Na^+ and K^+ ions make almost equal contributions to cell osmotic adjustment. In contrast, the effects of ROS stress (1 mM Cu/A, Fig. 4B) on net Na^+ and K^+ fluxes from *Arabidopsis* roots were rather different, with K^+ efflux being an order of magnitude higher (after Na^+ fluxes are corrected for non-ideal LIX sensitivity). This is consistent with the idea that the major target for ROS are NSCC (Demidchik et al., 2003). As in normal conditions cytosolic $[K^+]$ is at least two orders of magnitude higher than cytosolic $[Na^+]$, much higher K^+ efflux is expected when NSCC are open in response to oxidative stress.

In summary, the suggested method may significantly improve the accuracy of measuring ion concentrations and net ion fluxes in “natural” conditions (in the presence of interfering ions), thereby widening the application of ion-selective microelectrode techniques in studies on various aspects of plant biology.

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Appendix

ION FLUX CALCULATION ALGORITHM

The procedure for calculating the flux of the ion of interest in the presence of an interfering ion is illustrated for the Mg^{2+}/Ca^{2+} pair of ions. A similar procedure is applicable to deal with the problem of non-ideal selectivity of other ions (Na^+ over K^+ ; NH_4^+ over K^+ , etc.). The suggested algorithm includes three major steps.

Step 1: Simultaneous Measurement of Both Ions

A compulsory condition of the suggested procedure is that the concentrations of both ions (the one of interest and the interfering one) are measured simultaneously. Prior to the concentration measurements, both electrodes should be calibrated in different sets of standards. Assuming the ion of interest is Mg^{2+} , and the interfering ion is Ca^{2+} , the following calibrations are needed: (i) Mg^{2+} electrode in Mg^{2+} standards; (ii) Mg^{2+} electrode in Ca^{2+} standards; (iii) Ca^{2+} electrode in Ca^{2+} standards.

Step 2: Calculation of Correct Values of the Ion Concentration in Solution

In the case of Mg^{2+} , the following equation was used:

$$[Mg^{2+}] = -[Ca_{\text{known}}^{2+}] + \left[1,000,000 \times 10^{\left\{ \frac{V - (\phi_{Mg} V_{0Mg} + \phi_{Ca} V_{0Ca})}{(\phi_{Mg} S_{Mg} + \phi_{Ca} S_{Ca})} \right\}} \right] \quad (A1)$$

Where

$$\phi_{Mg} = \left(\frac{[Mg_{\text{known}}^{2+}]}{[Mg_{\text{known}}^{2+}] + [Ca_{\text{known}}^{2+}]} \right)^2 \quad \text{and} \quad (A2)$$

$$\phi_{Ca} = 1 - \left(\frac{[Mg_{\text{known}}^{2+}]}{[Mg_{\text{known}}^{2+}] + [Ca_{\text{known}}^{2+}]} \right)^2$$

Here $[\text{Mg}^{2+}]$ is the corrected Mg^{2+} concentration (μM); $[\text{Mg}^{2+}_{\text{known}}]$, the known Mg^{2+} concentration in the bath solution; $[\text{Ca}^{2+}_{\text{known}}]$, the known Ca^{2+} concentration, measured with the Ca^{2+} -selective electrode, at this point in the record (μM); V , the measured electrode voltage (mV); $V_{0\text{Mg}}$, the offset of the Mg^{2+} electrode calibrated in magnesium-only solution; s_{Mg} , the slope of the Mg^{2+} electrode calibrated in magnesium-only solution; $V_{0\text{Ca}}$, the offset of the Mg^{2+} electrode calibrated in calcium-only solution; s_{Ca} , the slope of the Mg^{2+} electrode calibrated in calcium-only solution. To calculate ϕ_{Mg} and ϕ_{Ca} , the actual concentration of Ca^{2+} , measured with the Ca^{2+} -selective electrode, and the expected concentration (assume bath concentration) of Mg^{2+} , were used. Note that the factor of 1,000,000 is introduced to convert the value given by the term in square brackets into μM .

Step 3: Conversion of the Measured (Corrected) Ionic Concentration Gradients into Net Ion Fluxes

These calculations use equation 1, adapted to meet the requirements of consecutive Valid Data Intervals as described elsewhere (Shabala et al., 1997; Newman, 2001). In the case of Mg^{2+} , the following equation was used:

$$J = (1 \times 10^9) \times u_{\text{Mg}} \times RT \times \frac{\left\{ \left(\frac{[\text{Mg}_{\text{before}}] + [\text{Mg}_{\text{next}}]}{2} \right) - [\text{Mg}_{\text{now}}] \right\} \times 0.001}{\left\{ \ln \left(\frac{r_{\text{next}}}{r_{\text{now}}} \right) \right\} \times \left\{ \frac{r_{\text{root}}}{1000000} \right\}} \quad (\text{A3})$$

where J is the flux of Mg^{2+} in $\text{nmol m}^{-2}\text{s}^{-1}$ (influx positive); u_{Mg} , the mobility of the ion ($u_{\text{Mg}} = 2.9 \times 10^{-13}$ units); $R = 8.31$, the universal gas constant; T , the absolute temperature (300°K for a room temperature); $[\text{Mg}_{\text{before}}]$, the preceding corrected Mg^{2+} concentration measurement (μM); $[\text{Mg}_{\text{now}}]$, the current corrected Mg^{2+} concentration measurement (μM); $[\text{Mg}_{\text{next}}]$, the next corrected Mg^{2+} concentration measurement (μM); r_{next} , the vector radius from the next Mg^{2+} concentration measurement (μM); r_{now} , the vector radius from the current Mg^{2+} concentration measurement (μM); r_{root} , the root radius (μM). Note that the 0.001 correction factor in the top line of the fraction is to convert the Mg^{2+} concentrations from μM to mol m^{-3} (as used in most MIFE papers published in recent years).

The above flux calculations assume cylindrical tissue geometry (e.g., ion flux measurements from roots or other plant radial tissues of organs) as it is the most often used. A similar procedure should be applied and validated for spherical (protoplasts or single-cell measurements) or planar geometry, with Eq. A3 modified according to Newman (2001).

References

- Ammann, D. 1986. Ion Selective Micro-Electrodes. Springer-Verlag, New York.
- Bakker, E., Pretsch, E., Buhmann, P. 2000. Selectivity of potentiometric ion sensors. *Anal. Chem.* **72**:1127–1133
- Carden, D.E., Diamond, D., Miller, A.J. 2001. An improved Na^+ -selective microelectrode for intracellular measurements in plant cells. *J. Exp. Bot.* **52**:1353–1359
- Demidchik, V., Davenport, R.J., Tester, M. 2002a. Nonselective cation channels in plants. *Ann. Rev. Plant Biol.* **53**:67–107
- Demidchik, V., Bowen, H.C., Maathuis, F.J.M., Shabala, S.N., Tester, M.A., White, P.J., Davies, J.M. 2002b. *Arabidopsis thaliana* root non-selective cation channels mediate calcium uptake and are involved in growth. *Plant J.* **32**:799–808
- Demidchik, V., Shabala, S.N., Coutts, K.B., Tester, M.A., Davies, J.M. 2003. Free oxygen radicals regulate plasma membrane Ca^{2+} and K^+ -permeable channels in plant root cells. *J. Cell Science* **116**:81–88
- Henriksen, G.H., Raman, D.R., Walker, L.P., Spanswick, R.M. 1992. Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. 2. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. *Plant Physiol.* **99**:734–747
- Macca, C. 2003. The current usage of selectivity coefficients for the characterization of ion-selective electrodes. A critical survey of the 2000/2001 literature. *Electroanalysis* **15**:997–1010
- Morf, W.E. 1981. The Principles of Ion-selective Electrodes and of Membrane Transport. Elsevier Scientific Publishing Company, Amsterdam.
- Newman, I.A. 2001. Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant Cell Environ.* **24**:1–14
- Ren, K. 2000. The ion adsorption effect on selectivity of liquid state, O,O%-didecyldithiophosphate chelate based ion-selective electrodes. *Talanta* **52**:1157–1170
- Schaller, U., Spichiger, U.E., Simon, W. 1993. Novel magnesium ion-selective microelectrodes based on a neutral carrier. *Pfluegers Archiv* **423**:338–342
- Shabala, S. 2000. Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. *Plant Cell Environ.* **23**:825–838
- Shabala, S. 2003. Regulation of potassium transport in leaves: from molecular to tissue level. *Ann. Bot.* **92**:627–634
- Shabala, S., Babourina, O., Newman, I.A. 2000. Ion-specific mechanisms of osmoregulation in bean mesophyll cells. *J. Exp. Bot.* **51**:1243–1253
- Shabala, S., Knowles, A.E. 2002. Rhythmic patterns of nutrient acquisition by wheat roots. *Functional Plant Biol.* **29**:595–605
- Shabala, S., Lew, R.R. 2002. Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. *Plant Physiol.* **129**:290–299
- Shabala, S., Newman, I. 2000. Salinity effects on the activity of plasma membrane H^+ and Ca^{2+} transporters in bean leaf mesophyll: masking role of the cell wall. *Ann. Bot.* **85**:681–686
- Shabala, S., Newman, I.A., Morris, J. 1997. Oscillations in H^+ and Ca^{2+} ion fluxes around the elongation region of corn roots and effects of external pH. *Plant Physiol.* **113**:111–118
- Vaughan-Jones, R.D., Aickin, C.C. 1987. Ion-selective microelectrodes. In: Microelectrode Techniques, The Plymouth Workshop Handbook. N.B. Standen, P.T.A. Gray, M.J. Whitaker,

- editors. pp 137–167, The Company of Biologists, Ltd, Cambridge
- Zhang, W., Fakler, A., Demuth, C., Spichiger, U.E. 1998. Comparison of different methods for determining the selectivity coefficient using a magnesium-selective electrode. *Analytica Chimica Acta* **375**:211–222
- Zhang, X.J., Fakler, A., Spichiger, U.E. 1998. Development of magnesium-ion-selective microelectrodes based on a new neutral carrier ETHT 5504. *Electroanalysis* **10**:1174–1181
- Zimmerman, S., Ehrhardt, T., Plesch, G., Muller-Rober, B. 1999. Ion channels in plant signalling. *Cell Mol. Life Sci.* **55**:183–203