Planta (2001) 213: 788–793
DOI 10.1007/s004250100555

ORIGINAL ARTICLE

Qisen Zhang · F. Andrew Smith · Hitoshi Sekimoto
Robert J. Reid

Effect of membrane surface charge on nickel uptake by purified mung bean root protoplasts

Received: 1 December 2000 / Accepted: 10 January 2001 / Published online: 8 May 2001

Abstract The influence of membrane surface charge on cation uptake was investigated in protoplasts prepared from roots of mung bean (*Vigna radiata* L.). Confocal laser scanning microscopy showed that a fluorescent trivalent cation accumulated to very high concentrations at the surface of the protoplasts when they were incubated in medium containing low concentrations of Ca or other cations, but that this accumulation could be completely reversed by suppression of membrane surface negativity by high cation concentrations. Influx of ^{63}Ni was strongly reduced by a range of divalent cations. Increasing the Ca concentration in the medium from 25 μM to 10 mM inhibited ^{63}Ni influx by more than 85%. ^{63}Ni influx was also inhibited by 85% by reducing the pH from 7 to 4. Computation of the activity of Ni at the membrane surface under the various treatment conditions showed that Ni uptake was closely correlated with its activity at the membrane surface but not with its concentration in the bulk medium. It was concluded that the effects on Ni uptake of addition of monovalent, divalent and trivalent cations, and of variations in pH are all consistent with the proposition that the activity of Ni at the membrane surface is the major determinant of the rate of Ni influx into mung bean protoplasts. It is proposed that the surface charge on the plasma membrane will influence the membrane transport of most charged molecules into cells.

Keywords Cation transport · Membrane surface charge · Nickel uptake · Protoplast (Ni uptake) · *Vigna* (Ni uptake)

Q. Zhang · F.A. Smith · R.J. Reid (✉)
Department of Environmental Biology,
University of Adelaide, SA 5005, Australia
E-mail: robert.reid@adelaide.edu.au
Fax: +61-8-83036222

H. Sekimoto
Laboratory of Plant Nutrition and Fertilizer,
Utsunomiya University, 350, Mine, Utsunomiya 321, Japan

Abbreviations PWS: protoplast washing solution · $TDCC^{3+}$: N,N′-di(3-trimethylammoniumpropyl) thiadicarbocyanine tribromide

Introduction

Membrane transport systems in plant cells are commonly described in terms of their substrate affinity and maximum rate using a formalism borrowed from enzyme kinetics. For any one transported solute, the high variability that is observed in different plant cell types for these two parameters is usually interpreted as meaning that although the transporters involved may be performing the same function, they are quite different proteins (see for example, the tabulation of reported kinetic constants for NH_4 and nitrate transporters in Reid 1999). An alternative is that much of the variability is due to the lack of constancy in the experimental conditions used for the measurement of these parameters (e.g. differences in pH, ionic strength or the presence or absence of competing molecules). Kinraide and his co-workers (Kinraide et al 1992; Kinraide 1994; Yermiyahu et al. 1997) have proposed that the variation in solution composition will have a marked effect on the concentration of charged solutes at the membrane surface, by altering the surface electrical potential on the membrane. According to this view, it is the concentration (more correctly the activity) of the transported substrate at the membrane surface that is more closely related to the transport kinetics, rather than the concentration in the bulk medium. Theoretical calculations based on measured surface charge densities show that under low ionic conditions the concentration of a divalent ion at the membrane surface can be several orders of magnitude higher than in the bulk medium, whereas at high ionic strength the membrane surface concentration more closely reflects that in the medium (Kinraide 1994). Earlier measurements by Gage et al. (1985) support this view. They demonstrated that in yeast, the K_m for Rb uptake was closely related to the Ca concentration in the

medium and that the apparent K_m for Rb correlated with the Ca-induced changes in membrane surface potential measured using a cationic fluorescent dye. Reid et al. (1997) also showed that the apparent K_m for Ca uptake in the giant alga *Chara* increased from 0.5 mM to 1.2 mM when the K concentration was increased from 0.1 mM to 20 mM.

In this paper we test the hypothesis that the electrostatic nature of plant cell membranes is a major determinant of rates of membrane transport of charged molecules, using Ni as the test element.

Materials and methods

Chemicals

Cellulase "Onozuka" RS and pectolyase y-23 were purchased from Yakult Houska Co. and Kikkoman Corporation, Japan, respectively. N,N′-di(3-trimethylammoniumpropyl)thiadicarbocyanine tribromide ($TDCC^{3+}$) was purchased from Molecular Probes, Eugene, Ore., USA.

Preparation of mung bean protoplasts

Mung bean seedlings were grown at 25 °C in vermiculite in pots (32 cm × 28 cm × 8 cm) for 7–14 days. Mung bean roots were harvested, washed with de-ionized water and cut into 1- to 2-mm sections with a razor blade. The root material was incubated for 3–4 h with 2 vol. of an enzyme solution containing 0.4 M sucrose, 2 mM Mes, 2 mM $CaCl_2$, 20 μM $ZnCl_2$, 1% bovine serum albumin (w/v), 1% polyvinylpyrrolidone (w/v), 1% cellulase and 1% pectolyase. The mixture of protoplasts and enzymes was filtered through two layers of Miracloth and the filtrate was centrifuged at 21,000 *g* for 15 min. The protoplasts in the supernatant were concentrated to about 5 ml by filtration on a Millipore filter (8 μm pore size) and washed by six successive additions of 30 ml of a protoplast washing solution (PWS) containing 1 mM Mes, 0.4 M mannitol and 25 μM $CaCl_2$ pH 6. After each addition, 30 ml of PWS was slowly sucked through the filter with gently shaking of the flask to keep the protoplasts in suspension. Care was taken to ensure that approximately 5 ml of the solution always remained on top of the filter because suction on to the filter caused rupture of the protoplasts. This washing procedure gave a dilution of the original solution of more than 10^5-fold, lowering the ionic strength close to that of PWS. The final preparation contained at least 4×10^5 protoplasts/ml.

Imaging of protoplasts

Viability of the protoplasts was examined by incubating them for 20 min in 1.25% Evans Blue, which is excluded by protoplasts with intact membranes. When viewed under a light microscope, these protoplasts appeared white against the blue medium background. Electrostatic attraction of cations to the surface of the protoplasts was observed by incubating the protoplasts in a solution containing 2 μM $TDCC^{3+}$, which is a fluorescent trivalent cation. Images of protoplasts treated with $TDCC^{3+}$ were obtained with a Bio-Rad MRC-1000 confocal microscope (Bio-Rad, Hercules, Calif., USA) using an excitation wavelength of 580 nm and an emission filter collecting a bandwidth of 32 nm around 580 nm.

Assay of Ni uptake

Protoplasts (1.8×10^5/ml) were incubated in PWS containing ^{63}Ni in the absence or presence of other cations and were then rinsed by carefully layering onto a small Millipore filter (0.45 μm) and successively rinsed (six times over 1.5–2 min) with PWS containing 2 mM $CaCl_2$ as described in the protocol for protoplast preparation. The final remaining protoplast suspension and the filter were transferred to a scintillation vial and counted for radioactivity. A control sample without addition of protoplasts was processed in the same way in order to obtain a background (blank) reading. Unless specified otherwise uptake experiments were conducted at pH 6.

Computation of membrane surface activities

The activity of Ni at the surface of protoplasts was calculated according to Kinraide et al. (1998) using a surface charge density of 0.3074 μmol m^{-2}.

Results

Protoplast integrity

Conventional techniques used to prepare leaf protoplasts (e.g. Obi et al. 1989; Ivanov and DiCosmo 1995) were found to be unsuitable for root protoplasts because the sedimentation and re-suspension caused a high degree of rupture. Additionally, there was a significant amount of cell debris, which could potentially bind Ni and thereby distort the results. The more gentle filtration/dilution technique used here resulted in relatively clean preparations with more than 95% intact protoplasts, as judged by Evans Blue staining.

Uptake and surface binding of Ni by protoplasts

To distinguish surface binding from actual uptake across the membrane, ^{63}Ni-loaded protoplasts were rinsed with either PWS or PWS plus 2 mM $CaCl_2$ at the end of the radioactive incubation period. Table 1 shows the distribution of Ni in/on protoplasts after rinsing with or without $CaCl_2$. This experiment showed that about 80% of the ^{63}Ni was on the surface of the protoplasts and that 20% ^{63}Ni remained after rinsing with 2 mM $CaCl_2$. The remaining activity was assumed to be due to uptake of ^{63}Ni into the protoplasts. In order to confirm that the

Table 1 Uptake and surface binding of Ni in mung bean (*Vigna radiata*) root protoplasts. Uptake of Ni was measured by incubating protoplasts in10 μM ^{63}Ni for 5 min and rinsing with 2 mM $CaCl_2$ in PWS to remove surface ^{63}Ni activity. Surface binding was estimated by running parallel experiments in which $CaCl_2$ was omitted from the rinse solution (except for 20 μM Ca). To verify that the activity in the protoplasts after rinsing with $CaCl_2$ was intracellular, protoplasts were osmotically lysed by diluting the rinse solution. The data represent the mean ± SE of three independent and identical experiments. *nd* Not determined

Protoplasts	Uptake + surface binding [pmol (10^5 protoplasts^{-1}) h^{-1}]	Uptake [pmol (10^5 protoplasts^{-1}) h^{-1}]
Intact	166 ± 3	48 ± 10
Lysed	nd	2 ± 1

remaining Ni was intracellular and not due to inefficient desorption of the membrane surface, a comparison was made between intact protoplasts and protoplasts that were lysed after the rinse period. This showed that 95% of the ^{63}Ni was released by lysis (Table 1), indicating that very little of the original activity had been bound to the surface after rinsing with 2 mM $CaCl_2$. The percent distribution of Ni was approximately the same at 1 and 10 μM Ni (data not shown).

Time-course of Ni uptake by protoplasts

Uptake of Ni by protoplasts was rapid over the first 10 min and then tended to equilibrium (Fig. 1). The slope of the uptake curve was almost linear over about the first 10 min and should therefore reflect unidirectional influx into the protoplasts. In the experiments described below, influx measurements were made over the first 5 min of incubation in Ni medium.

Effects of divalent cations and ionic strength on Ni uptake

The base medium for uptake experiments contained a background concentration of 25 μM Ca to maintain protoplast membrane integrity and up to 0.5 mM Na used in the adjustment of pH. Addition of cations to this low-ionic-strength medium had a strong influence on Ni uptake. Divalent cations Ca, Mg, Ba and Sr at 0.2 mM reduced Ni influx by more than 55% (Fig. 2). Methyl viologen (0.2 mM) inhibited Ni uptake by approximately 45%. Addition of 10 mM of Ca, Mg, Ba or Sr caused more than 90% inhibition (data not shown). There were no appreciable differences in effect among Ca, Mg and Sr, but Ba seemed to cause a slightly larger reduction. Addition of K and Na at higher concentrations also reduced Ni influx (data not shown).

Effect of Ca on Ni uptake on Ni surface activity

The effect of Ca on Ni influx was examined in more detail. Increasing the Ca concentration from 25 μM to 10 mM reduced Ni uptake approximately 10-fold (Fig. 3a). The decrease in Ni uptake with increasing Ca in the medium was correlated with the decrease in the activity of Ni at the membrane surface (Fig. 3a). When Ni influx was plotted as a function of the Ni surface activity, the relationship could be described by a Michaelis-Menten curve with $K_m = 100$ μM and $V_{max} = 35$ pmol $(10^5$ protoplasts$)^{-1}$ h^{-1} (Fig. 3b).

Effect of pH on Ni uptake

Ni uptake showed a pH optimum around 7 and was strongly inhibited by low pH (Fig. 4). There was a close correlation between uptake and the surface activity of Ni, suggesting that much of the effect of pH is not directly on the activity of the membrane transporter but on the concentration of the transport substrate at the membrane surface. The deviation of influx from surface activity around pH 6 indicates that surface activity is not the only determinant of Ni influx is this range.

Response of Ni uptake to multiple variables

Figure 5 summarises the results of a large number of experimental treatments designed to alter the activity of Ni at the membrane surface. These treatments included variations in mono-, di- and tri-valent cations, varying the concentration of Ni in the medium plus changes of pH (Table 2). The points are more scattered than those for a single treatment variable such as shown in Fig. 3 for Ca, and this may be due to secondary effects such as competition between cations and errors in calculation of Ni

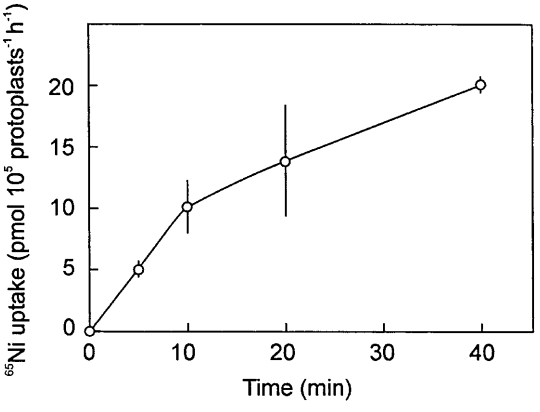

Fig. 1 Time course of Ni uptake by protoplasts. Mung bean (*Vigna radiata*) root protoplasts were incubated with Ni (1 μM) in PWS for the desired times and rinsed with 2 mM $CaCl_2$ in PWS. The data are means of three replicates from a representative of at least two independent experiments and bars indicate standard errors

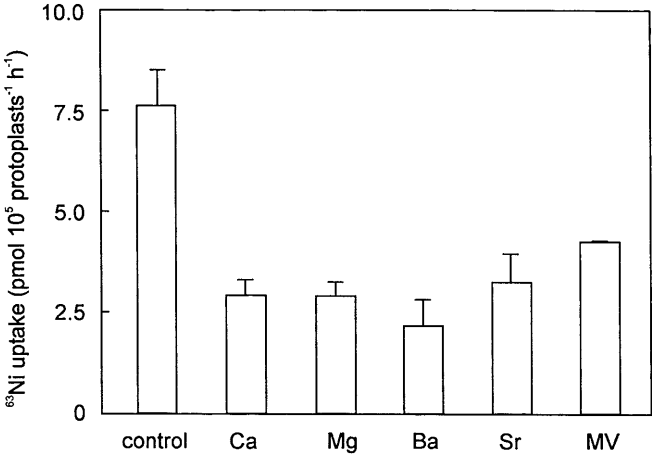

Fig. 2 Effect of other divalent cations on the uptake of ^{63}Ni into mung bean root protoplasts. The control solution was PWS containing 25 μM Ca; other treatments contained 0.2 mM of the divalent cation specified. The data represent the mean ± SE of 3 measurements. Uptake $t = 5$ min. $^{63}Ni = 1$ μM. *MV* Methyl viologen

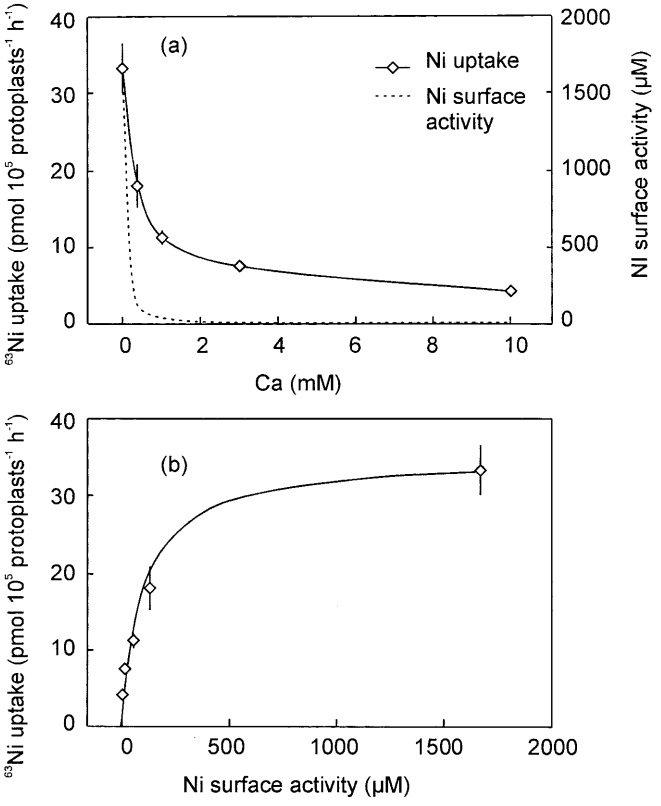

Fig. 3 **a** The effect of Ca on uptake of ^{63}Ni in mung bean protoplasts. Solution Ni = 8 μM. Uptake $t = 5$ min in PWS at pH 6. The *dashed line* is the activity of Ni at the surface of the protoplasts calculated according to Kinraide (1994) using a surface charge density of 0.3074 μmol m^{-2}. **b** Ni uptake values from **a** plotted as a function of the surface activity of Ni. The line is a Michaelis-Menten curve with $V_{max} = 35$ pmol (10^5 protoplasts)$^{-1}$ h^{-1} and $K_m = 100$ μM

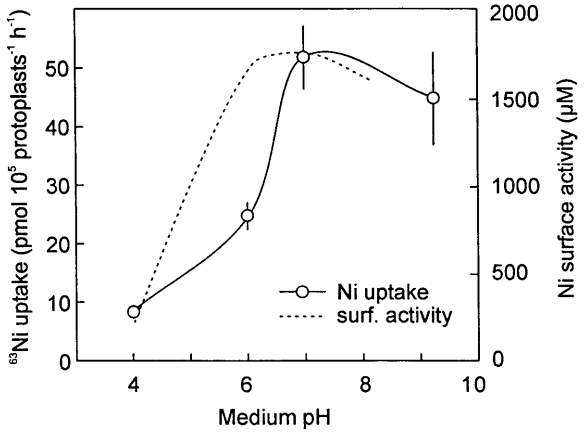

Fig. 4 Comparison of the pH dependence of ^{63}Ni uptake by mung bean root protoplasts and the surface activity of Ni. The surface activity was calculated taking into account the pH-dependent speciation of Ni determined using GEOCHEM-PC (Parker et al. 1995)

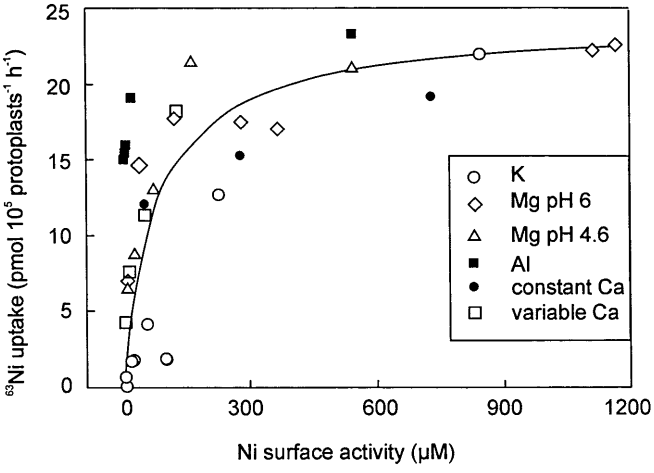

Fig. 5 Computation of kinetic parameters for Ni uptake in different treatment solutions. Rates of Ni uptake by mung bean root protoplasts were measured under a variety of conditions (see Table 2). The line is a Michaelis-Menten curve with $K_m = 80$ μM and $V_{max} = 24$ pmol (10^5 protoplasts)$^{-1}$ h^{-1}

surface activity due to inaccuracies in the estimates of binding constants for different cations. Nevertheless the combined data can be fitted by a monophasic saturating curve of Ni uptake as a function of Ni surface activity (Fig. 5) with a $K_m = 80$ μM and $V_{max} = 24$ pmol (10^5 protoplasts)$^{-1}$ h^{-1}(Fig. 5).

Imaging of cation accumulation at the membrane surface

The existence of electrical potential differences between surfaces and solutions is more difficult to demonstrate than measurements of transmembrane potential differences using microelectrodes and electrometers. Nevertheless, it is relatively easy to demonstrate the accumulation of cations at the membrane using

Table 2 Conditions of assay for the measurement of effect of cations and pH on Ni uptake. Ni uptake by mung bean root protoplasts was assayed in PWS under a variety of conditions designed to alter the activity of Ni at the surface of the protoplasts

Cations	mM	[Ni$_{med}$] (μM)	{Ni$_{surf}$} (μM)	pH	[Ca] (μM)
K	0–50	8	1,658–35	6	25
Mg	0–5	8	1,658–10	6	25
Mg	0–5	8	541–9	4.6	25
Al	0–0.05	8	541–3	4.6	25
Ca (constant) -1	0.025	0–10	0–1976	6	25
Ca (constant) -2	0.25	0–10	0–267	6	250
Ca (varied)	0.025–5	8	1,658–8	6	25–5,000

radioactive or fluorescent tracers. Figure 6a shows a confocal microscope image of protoplasts incubated in a low-ionic-strength solution containing 2 μM of a non-penetrating trivalent cation, $TDCC^{3+}$. The local concentration of dye on the protoplast surface was estimated using fluorescence imaging software to be approximately 1,000-fold higher than in the bulk solution. Addition of 2 mM Mg (or other divalent cations) reversed this accumulation (Fig. 6b) due to the screening and charge reduction from binding of the surface charges.

Discussion

The purpose of this work was to determine whether the electrostatic accumulation of cations at the membrane surface was a significant factor in determining their rate of uptake across the membrane. The wide range of treatments applied in these experiments all produced changes in the rate of Ni uptake that were consistent with their expected effects on membrane surface charge and surface accumulation of Ni. The simplest explanation, then, is that Ni uptake is more closely linked to its activity at the membrane surface than to its concentration in the bulk medium. The alternative (more complicated) explanation is that these treatments produce independent effects on Ni uptake (e.g. pH-induced changes in transport activity, competition for Ni uptake by other cations) that are coincidental with the effects on surface charge. The following discussion presents the argument in favour of the simpler explanation.

The images of the attraction of the fluorescent cation to the surface of the protoplasts illustrate the general features of surface electrostatics and the role of membrane solution composition in controlling surface cation concentrations. The accumulation of Ni at the membrane surface would be expected to show a similar pattern although the surface concentration would be less because it is divalent rather than trivalent. The question that needs to be asked is how this accumulation of Ni (or other cations) at the membrane surface might affect transport of Ni into the cell. Apart from regulatory factors operating on the transporter itself, there are two parameters that could control transport. These are (i) the availability of substrate (in this case Ni) and (ii) the magnitude of the electrochemical gradient for passive Ni uptake or alternatively the availability of metabolic energy to drive transport against an outwardly directed electrochemical gradient. Given that most plant cells maintain an electrical potential difference of more than –100 mV between the cytoplasm and the external solution, there will normally be a strong inwardly directed gradient favouring uptake of divalent cations such as Ni. However, the rate of transport is unlikely to be limited by the driving force where the overall reaction operates far from electrochemical equilibrium (i.e. there is much more energy available than is needed for the reaction). In this case, the main limitation will be the chemical activity of the substrate at the external face of the transporter. Clearly, electrostatic attraction of Ni to the membrane will increase this activity, and thereby increase the rate of transport across the membrane. The existence of significant surface charge will therefore be expected to have strong effects on Ni transport, and this is supported by the empirical evidence presented here.

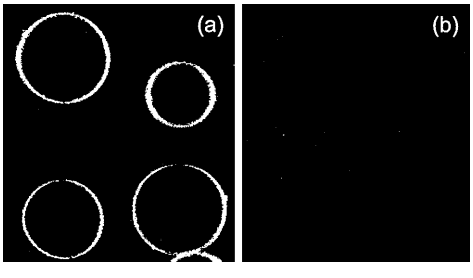

Fig. 6 **a** Electrostatic accumulation of a fluorescent trivalent cation $TDCC^{3+}$ on the surface of mung bean protoplasts incubated in low-ionic-strength medium. **b** Release of $TDCC^{3+}$ following the suppression of membrane surface charge by addition of 2 mM Mg^{2+}

Where the transport reaction operates close to electrochemical equilibrium the influence of surface charge is more difficult to predict. Consider the following two situations, one in which the external solution contains high concentrations of cations so that the surface charge is completely suppressed and one in which the external solution contains low concentrations of cations so that there is a large surface charge. In the first, where there is no surface charge, the concentration of Ni will be the same at the membrane and in the bulk solution. In the second scenario, Ni will accumulate to high concentrations at the membrane surface. In both cases the electrochemical potential difference for Ni will be the same whether considered in terms of the electrochemical potential of Ni in the bulk solution or at the membrane surface, because the higher concentration component at the membrane is offset by a smaller electrical difference across the membrane. In other words, changing the surface charge does not alter the electrochemical gradient for Ni. Yet the two situations are not equivalent. In one case the chemical activity of the Ni at the membrane surface adjacent to the transporter is the same as that in the external solution and in the other it is considerably higher due to the electrostatic attraction. Another consideration is the possible change in the ionic environment of the transporter in the two solutions differing in ionic strength. The surface charge on the membrane is due to ionisation of various groups on phospholipids and membrane proteins and can be suppressed either by screening by cations, or by physical binding of cations to the negative sites. The latter is especially true of polyvalent cations such as Al^{3+} (Kinraide et al. 1998). It is difficult to determine how either of these differences, the higher chemical activity or the different ionic environment, will affect the kinetic characteristics of the transporter.

The electrostatic nature of plant cell membranes has long been recognized (see Møller and Lundborg 1985) and it is perhaps surprising that this phenomenon has

not been more widely considered in studies of membrane transport, or for that matter in studies of membrane-bound enzymes. Gibrat et al. (1985) showed that in plasma-membrane vesicles the apparent K_m (ATP) of the H^+-ATPase decreased as the ionic strength increased. Their explanation was that the reduction in the negativity of the membrane surface by cations in the medium facilitated the accumulation of the negatively charged substrate MgATP, thereby reducing the apparent K_m. Gage et al. (1985) observed a similar effect of Ca on Rb uptake in yeast and hypothesized that Ca did not directly inhibit Rb uptake, but suppressed the membrane surface potential and thus reduced the activity of Rb at the membrane. The same arguments can be applied to the effects of not only Ca, but also other cations on Ni uptake. We conclude that the effects on Ni uptake of addition of monovalent, divalent and trivalent cations, and of variations in pH are all consistent with the proposition that the activity of Ni at the membrane surface is the major determinant of the rate of Ni influx into mung bean protoplasts. We expect that this will also be true for other cations and suggest that care should be taken in 'characterising' the properties of membrane transporters in terms of K_m and V_{max} while ignoring effects of other ionised solutes or surface charge.

Acknowledgements The authors thank Dr. T. Kinraide for his helpful advice and for his careful reading of the manuscript. This work was supported by the Australian Research Council.

References

Gage RA, van Wijngaarden W, Theuvenet APR, Borst-Pauwels GWFH, Verkleij AJ (1985) Inhibition of Rb^+ uptake in yeast by Ca^{2+} is caused by a reduction in the surface potential and not in the Donnan potential of the cell wall. Biochim Biophys Acta 812:1–8

Gibrat, R, Grouzis J-P, Rigaud J, Gignon C (1985) Electrostatic characteristics of corn root plasmalemma: effect on the Mg^{2+}-ATPase activity. Biochim Biophys Acta 816:349–357

Ivanov AG, DiCosmo F (1995) Microelectrophoretic and 9-aminoacridine fluorescence study of the surface electrical properties of suspension cultured *Catharanthus roseus* cells and isolated protoplasts: effects of abscisic acid treatment. Plant Cell Physiol 38:709–715

Kinraide TB (1994) Use of a Gouy-Chapman-Stern model for membrane-surface electrical potential to interpret some features of mineral rhizotoxicity. Plant Physiol 106:1583–1592

Kinraide TB, Ryan PR, Kochian LV (1992) Interactive effects of Al^{3+}, H^+, and other cations on root elongation considered in terms of cell-surface electrical potential. Plant Physiol 99:1461–1468

Kinraide TB, Yermiyahu U, Rytwo G (1998) Computation of surface electrical potentials of plant cell membranes. Plant Physiol 118:505–512

Møller IM, Lundborg T (1985) Electrostatic surface properties of plasmalemma vesicles from oat and wheat roots. Ion binding and screening investigated by 9-aminoacridine fluorescence. Planta 164:354–361

Obi I, Ichikawa Y, Kokutani T, Senda M (1989) Electrophoresis, zeta potential and surface charges of barley mesophyll protoplasts. Plant Cell Physiol 30:129–135

Parker DR, Norvell WA, Chaney RL (1995) GEOCHEM-PC: a chemical speciation program for IBM and compatible personal computers. In: Loeppert RH, Schwab AP, Goldberg S (eds) Soil chemical equilibrium and reaction models. Soil Science Society of America, Madison, Wis., pp 253–269

Reid RJ (1999) Kinetics of nutrient uptake by plant cells. In: Rengel Z (ed) Mineral nutrition of crops: fundamental mechanisms and implications. Haworth, New York, pp 41–66

Reid RJ, Tester M, Smith FA (1997) Voltage control of calcium influx in plants. Aust J Plant Physiol 24:805–810

Yermiyahu U, Rytwo G, Brauer DK, Kinraide TB (1997) Binding and electrostatic attraction of lanthanum (La^{3+}) and aluminum (Al^{3+}) to wheat root plasma membranes. J Membr Biol 159:239–252