A Weakly Voltage-Dependent, Nonselective Cation Channel Mediates Toxic Sodium Influx in Wheat[1]

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To determine the transporters responsible for toxic Na^+ influx in wheat (*Triticum aestivum*), root plasma membrane preparations were screened using the planar lipid bilayer technique as an assay for Na^+-permeable ion channel activity. The predominant channel in the bilayer was a 44-pS channel that we called the nonselective cation (NSC) channel, which was nonselective for monovalent cations and weakly voltage dependent. Single channel characteristics of the NSC channel were compared with $^{22}Na^+$ influx into excised root segments. Na^+ influx through the NSC channel resembled $^{22}Na^+$ influx in its partial sensitivity to inhibition by Ca^{2+}, Mg^{2+}, and Gd^{3+}, and its insensitivity to all other inhibitors tested (tetraethylammonium, quinine, Cs^+, tetrodotoxin, verapamil, amiloride, and flufenamate). Na^+ influx through the NSC channel also closely resembled an instantaneous current in wheat root protoplasts (S.D. Tyerman, M. Skerrett, A. Garill, G.P. Findlay, R. Leigh [1997] J Exp Bot 48: 459–480) in its permeability sequence, selectivity for K^+ over Na^+ (approximately 1.25), insensitivity to tetraethylammonium, voltage independence, and partial sensitivity to Ca^{2+}. Comparison of tissue, protoplast (S.D. Tyerman, M. Skerrett, A. Garill, G.P. Findlay, R. Leigh [1997] J Exp Bot 48: 459–480), and single-channel data indicate that toxic Na^+ influx is catalyzed by a single transporter, and this is likely to be the NSC channel identified in planar lipid bilayers.

Soil salinity is a global problem estimated to affect crop productivity in one-quarter to one-third of all agricultural land (Squires, 1994). After several decades of research, the mechanisms underlying salt toxicity in crop plants remain undiscovered. In wheat (*Triticum aestivum*), varietal sensitivity to Na^+ (measured as whole plant biomass reduction) has been shown to correlate with Na^+ accumulation in the shoot (Schachtman et al., 1989; Gorham, 1990; Schachtman and Munns, 1992). Exclusion of Na^+ from the shoot can be achieved by limiting uptake into the root (by reducing influx or increasing efflux) or by prevention of translocation to the shoot (requiring compartmentation in the roots). However, the range of tolerance in wheat is very small. Biomass is reduced by over 90% even in so-called "tolerant" varieties grown in 200 mM NaCl (Kingsbury and Epstein, 1984). Thus, whatever the mechanisms by which the tolerant differ from the sensitive varieties, they are unlikely to resemble those of true halophytes, and confer only small increases in ability to withstand saline conditions.

One important factor limiting the potential tolerance of all wheat varieties may be the high unidirectional influx of Na^+ into the root, which is very similar in both tolerant and sensitive varieties (Davis, 1984; Davenport et al., 1997). This is confirmed by patch-clamp data showing no differences in the channel populations of the same varieties (Schachtman et al., 1991; Findlay et al., 1994). Na^+ influx is much higher than net uptake in these plants, and so requires a high rate of active Na^+ extrusion from the roots. Therefore, the growth of both so-called tolerant and sensitive species may be limited by the energetic cost, and incompleteness, of this Na^+ expulsion, accounting for the relatively small varietal differences observed. Thus, it is likely that the identification and modification of toxic Na^+ uptake mechanisms at the root plasma membrane will be necessary before any dramatic improvements in the salinity tolerance of wheat can be expected.

The transporter(s) responsible for toxic Na^+ influx into wheat have not been identified, although several molecular candidates have been partially characterized. HKT1, a high-affinity Na^+/K^+ cotransporter from wheat, can also function as a relatively low-affinity Na^+/Na^+ cotransporter at high Na^+ concentrations (Rubio et al., 1995; Gassmann et al., 1996). However, the membrane location of HKT1 and evidence for its role in the nutritional uptake of K^+ have not been established (Walker et al., 1996). Moreover, HKT1 is apparently insensitive to inhibition by Ca^{2+} (Schachtman et al., 1997), and therefore cannot account for the Ca^{2+}-sensitive portion of toxic Na^+ influx observed in wheat. Recently, a second, putative low-affinity cation transporter (LCT1) has been cloned from wheat, which catalyzes low rates of monovalent and divalent cation transport from millimolar salt solutions when expressed in yeast (Schachtman et al., 1997; Clemens et al., 1998). The physiological role of the protein and its mechanism of transport remain to be elucidated.

Electrophysiological studies have suggested that Na^+ influx into cereals is mediated by voltage-independent, nonselective cation channels. Studies of Na^+ currents in the cortex of wheat (Tyerman et al., 1997), maize (Roberts and Tester, 1997), and suspension-cultured barley (Amtmann et al., 1997) indicated that Na^+ influx was predominantly via an instantaneous current of low selectivity among monovalent cations (for review, see Amtmann and Sanders, 1999; Tyerman et al., 1999; White, 1999). In wheat, this current had a permeability sequence for monovalent cations of $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ (calculated from

conductance at E_{Cl}), with a P_{Na}:P_K of 0.8. Na^+ influx was unaffected by tetraethylammonium (TEA^+) or verapamil, but was inhibited to a maximum of 50% by 20 mM Ca^{2+} in 100 mM extracellular NaCl, with a K_i for half-inhibition of 0.31 mM. Single-channel recordings from excised patches occasionally demonstrated the existence of Na^+-permeable channels; however, these channels were always present at high density and appeared to be "clustered," making analysis difficult. Noise analyses gave an average single channel conductance of 30 pS (in 102.5:2 mM NaCl, bath:cytosolic solution) (Tyerman et al., 1997).

The application of patch-clamp techniques to the study of toxic Na^+ influx is limited by the nature of the transporters involved. Patch-clamping favors the identification of voltage-gated and abundant or high-conductance channels. Instantaneous, nonselective currents resemble leaks and are difficult to assign to a particular transporter (particularly in the complex solutions typically used to optimize sealing and channel activity in patch-clamp studies). Rare channels are masked by other conductances in whole-cell mode, and are laborious to locate in single-channel patches. In wheat the Na^+-permeable channels are clustered in the membrane, making them difficult to analyze (Tyerman et al., 1997). To elucidate the molecular mechanism(s) underlying the observed Na^+ currents in wheat roots, we applied the planar lipid bilayer technique, which allows the resolution of small, time-independent, rare currents at the single-channel level. In the minimal solutions used in bilayer studies, the ATP-dependent K^+ channels that dominate whole-cell conductance in protoplasts are not observed (probably due to inactivation during the isolation process, since the addition of ATP to bilayer solutions did not elicit novel channel activity in the present study), allowing rarer channels to be identified. Interestingly, the first patch-clamp study conducted in plants used very simple NaCl solutions without Mg-ATP and identified four classes of Na^+-permeable cation channels in the plasma membrane of wheat leaf cells (Moran et al., 1984).

To determine the range of possible candidates for Na^+ transport, we screened plasma membrane preparations from wheat roots for Na^+-permeable channel activity in the bilayer in simple salt solutions. We also characterized $^{22}Na^+$ influx at the tissue level in wheat roots to determine whether Na^+ influx was likely to be catalyzed by several transporters with differing selectivity and pharmacological characteristics, and whether influx characteristics were affected by growth in high NaCl. Microelectrode measurements of membrane potentials of intact root cortical cells were used to assess the likely contribution of the single channel to whole-root influx under physiologically realistic conditions. We also tested whether Na^+ influx into roots showed any pharmacological similarities with known channel types, which would serve as diagnostic indicators of Na^+ influx mechanisms at the single-channel level.

Several recent reviews have concluded that in saline conditions, the bulk of toxic Na^+ influx into cereal roots is likely to be via nonselective cation (NSC) channels in the plasma membranes of root cortical cells (Amtmann and Sanders, 1999; Tyerman and Skerrett, 1999; White, 1999). We present comprehensive data for root Na^+ influx and single-channel Na^+ transport and compare these with cortical cell patch-clamp data (Tyerman et al., 1997) to demonstrate the role of the NSC channel in Na^+ toxicity in wheat and to establish diagnostic criteria for the isolation of the channel protein.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wheat (*Triticum aestivum* cv Hunter) were obtained from Plant Breeding International (Cambridge, UK). One-hundred-fifty grams of seed was surface-sterilized for 10 min in 2% (v/v) sodium hypochlorite and germinated overnight in reverse osmosis water. Germinated seeds were grown on plastic mesh over hydroponic solution in covered trays, in a growth cabinet on a 16-h/9-h light/dark, 25°C/15°C cycle. Solutions were bubbled with filtered air. Photon irradiance was 200 μmol m^{-2} s^{-1}. Plants were grown for 7 d after planting out before being harvested. Low-salt seedlings were grown in 0.5 mM $CaSO_4$. NaCl-grown seedlings were initially grown in 0.5 mM $CaSO_4$ and then gradually exposed to NaCl in daily increments of 25 mM plus 2.5 mM $CaCl_2$ (i.e. Na^+:Ca^{2+} was maintained at 1:10) commencing on d 4 after planting out (so seedlings were exposed to the highest level of NaCl [100 mM] for 24 h before harvesting). High nutrient-grown seedlings were grown in 0.5 mM $CaSO_4$ for 5 d and then transferred to 0.1× modified Hoaglands solution (1.5 mM KNO_3, 0.5 mM $Ca[NO_3]_2$, 0.2 mM $MgSO_4$, and 0.1 mM KH_2PO_4 plus micronutrients, pH of unbuffered solution approximately 6.5).

Radio-Isotopic Measurements in Roots

Excised root segments were used to reduce the complexities of shoot interactions (shoot nutrient demand, feedback, and transpiration), which could mask membrane transport processes and make comparison with single-channel data more difficult. Roots were excised 2 cm from the seed and cut into 2-cm sections. Low-salt-grown root segments were rinsed in deionized water and transferred to aerated 0.5 mM $CaSO_4$ solution at 25°C for 1 h to recover from the effects of excision (Gronewald et al., 1979). Segments were then transferred to 0.5 mM $CaSO_4$ plus 200 mM sorbitol solutions and aerated at 25°C for 3 h before experiments (to separate temporally effects of sudden changes in osmolarity and salt concentration).

NaCl-grown plants were rinsed in deionized water and transferred to aerated 0.5 mM $CaSO_4$ plus 200 mM sorbitol solution at 25°C for 1 h, then transferred back to 100 mM NaCl plus 10 mM $CaCl_2$, and aerated at 25°C for 3 h before experiments (to maintain adaptations to growth conditions). Before $^{22}Na^+$ uptake, root segments were rinsed in deionized water (to remove surface $CaSO_4$ or $CaCl_2$) and pretreated in unlabeled solutions identical to uptake solutions for 10 min to equilibrate cell wall Ca^{2+} and osmolarity. Segments were then transferred to $^{22}Na^+$-labeled uptake solutions, approximately 0.2 to 0.8 g tissue/25 mL and 0.01 to 0.05 μCi mL^{-1}. Solutions were unbuffered, since the

pH was found to remain unchanged (approximately pH 5.5) after 20-min uptake periods. There was no significant depletion of salt solutions over 5-min uptake periods (monitored by measurements of the radioactivity of the solutions). Solutions were not re-used. Uptake solutions were contained in shallow evaporation dishes and were gently shaken during uptake to stir and aerate the root segments.

A time course of uptake in 100 mM NaCl was determined in low-salt roots. ^{22}Na$^+$ influx appeared to approach saturation over 20 min in 100 mM NaCl. The first minute of uptake was assumed to comprise a significant amount of apoplastic binding, so 5 min was chosen as the period of uptake for all subsequent experiments. At the end of 5 min, the segments were gently removed from the labeled solution using tweezers, transferred to sieves, and rinsed by gentle agitation in two successive 1-min rinses of ice-cold 10 mM NaCl plus 10 mM CaCl$_2$ osmotically adjusted with sorbitol to match the uptake solution. The rinses were designed to displace apoplastic ^{22}Na$^+$ while inhibiting efflux from the cells. Roots were then blotted gently with blotting paper, weighed, and transferred to plastic vials with 4 mL of scintillation cocktail (Optiphase HiSafe, Fisher Chemicals, Loughborough, UK). Samples were counted on a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Data were fitted using FigP version 2.2 software (BioSoft, Cambridge, UK). Hill dose-response curves were fitted using the equation $y = \text{min} + ([\text{max} - \text{min}]/[1 + \{Ca^{2+}/K_i\}^{-P}])$, where min refers to influx in the absence of the inhibitor, max refers to influx when the inhibitory effect is saturating, and P is the Hill coefficient.

Microelectrode Impalement of Root Segments

Roots of 7-d-old seedlings were excised 2 cm from the seed and pretreated as for ^{22}Na$^+$ uptake experiments. Electrodes were pulled from triangular cross-section borosilicate glass on a vertical electrode puller (model PE-2, Narishige, Tokyo), and were filled with 300 mM KCl. Roots were secured horizontally with silicon grease in an 8-mL Perspex chamber, which was constantly perfused using a peristaltic pump. Outer cortical cells were impaled 2 to 3 cm from the root tip. The potential difference between the intracellular electrode and the bath solution was measured and displayed on a chart recorder.

Preparation of Plasma Membrane Vesicles

Roots were excised 2 cm from the seed with scissors into chilled deionized water, strained, blotted, and weighed. Preparation was carried out at 4°C. Roots were chopped with a herb chopper in 300 mM Glc, 100 mM K-gluconate (except for preparations in which tonoplast pyrophosphatase activity was measured, when K-gluconate was replaced with 200 mM Glc), 2 mM EGTA, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-1,3-bis(Tris[hydroxymethyl]methylamino) propane (BTP), pH 7.4 (2 mL g^{-1} root fresh weight), and strained through three layers of cheesecloth. This solution was centrifuged for 15 min at 13,000g in a rotor (JA20, Beckman Instruments) to sediment organelles. The supernatant was centrifuged at 45,000g in an ultracentrifuge rotor (Ti70, Beckman Instruments). The pellets were resuspended in 500 mM Glc and 5 mM HEPES-BTP, pH 7.8 (the "microsomal fraction").

A plasma membrane-enriched fraction was obtained from the microsomal preparation by two-phase aqueous partitioning (Larsson et al., 1987). The procedure was optimized to maximize protein yield and purity. The final composition of the two-phase system was 5.8% (w/v) dextran, 5.8% (w/v) PEG 3350, 5 mM KCl, 5 mM HEPES-BTP, pH 7.8, and 500 mM Glc. Two-hundred microliters of microsomes was kept for membrane marker enzymatic assays. Two microliters of microsomal suspension was added to a 14-g two-phase system, gently mixed, and then centrifuged at 1,000g for 5 min in a benchtop centrifuge to promote phase separation. The upper (PEG) phase was collected and loaded onto fresh lower phase and the mixing and separation steps repeated. The upper phase was passed over fresh lower phase and separated a final time, yielding a U3' phase that was then diluted 10-fold in 500 mM Glc, 20 mM HEPES-BTP, pH 7.4, and centrifuged for 90 min at 90,000g in a rotor. Pellets were resuspended in 2 mL of 500 mM Glc, 20 mM HEPES-BTP, pH 7.4, divided into 20-μL aliquots, and snap-frozen in liquid nitrogen.

Measurement of Purity of Plasma Membrane Vesicle Preparations

Protein content was assayed spectrophotometrically using a protein assay with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA). Membrane preparation samples were treated with 1.4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) on ice for 20 min to solubilize protein.

Plasma membrane content was assayed as glucan synthase II activity, using techniques adapted from Piñeros and Tester (1995). The reaction medium consisted of 10 mM cellobiose, 500 mM Glc, 0.05 mM CaCl$_2$, 40 μM spermine, 25 mM HEPES-KOH, pH 7.0, 0.4 mM UDPG, 0.2 μCi [^{14}C]UDPG, and 0.02% (w/v) digitonin. Digitonin was made up as a 2 mg mL^{-1} stock with ethanol for immediate use. One-hundred microliters of reaction medium was added to 100-μL vesicles and samples incubated for 20 min at 25°C. The reaction was stopped by boiling samples for 10 min. A small aliquot of powdered cellulose was added to each sample and samples incubated overnight at 4°C. Samples were filtered under vacuum onto GF/B filters (Whatman, Clifton, NJ) presoaked in rinse solution (350 mM ammonium acetate plus 30% [w/v] ethanol). Filters were rinsed with 5 × 3-mL rinse solution and then transferred to scintillation vials with 4 mL of scintillation cocktail (Optiphase HiSafe, Wallac) and counted in a liquid scintillation counter (Beckman Instruments).

Tonoplast content was assayed as activity of the K$^+$, Mg^{2+}-stimulated pyrophosphatase using techniques adapted from Bencini et al. (1983). The reaction medium consisted of (at final concentrations) 50 mM KCl, 200 μg mL^{-1} Brij-58, 100 mM sodium molybdate, 7.5 mM MgSO$_4$, 0.3 mM Na$_2$EDTA, 0.5 mM sodium pyrophosphate, and 25 mM HEPES-BTP, pH 8.0. One-hundred microliters of assay

medium was added to samples and made up to 200 μL with 500 mM Glc and 20 mM HEPES-BTP, pH 7.4. Samples were incubated for 15 min in a 30°C water bath, then diluted with 600 μL of Bencini's reagent (100 mM zinc acetate and 15 mM ammonium molybdate, adjusted to pH 5.0 with 6 N HCl) and left for 2 min at room temperature before spectrophotometric assay for orthophosphate production (A_{350}). Controls were without KCl in the reaction medium.

ER content was measured by activity of antimycin-A-insensitive NADH-cytochrome c (Cyt c) reductase using the procedure described by Piñeros and Tester (1995) adapted from Lord (1987). The reaction buffer was 235 mM K-phosphate, pH 7.2, 95 μM KCN, 48 μM Cyt c, 9 μM antimycin A, and 189 μM NADH. Vesicles were disrupted with 0.008% (w/v) digitonin, and 50-μL samples added to 900-μL reaction buffer (mixed in a cuvette). The initial rate of reduction was followed as changes in A_{550}.

Mitochondrial membrane content was measured by activity of Cyt c oxidase. Cyt c was reduced in Na-dithionite (0.66 mg of Cyt c in 1 mL of 23 mM K-phosphate buffer, pH 7.4, plus 0.7 mg $Na_2S_2O_4$), and bubbled with O_2 to remove excess dithionite before use. Vesicles were disrupted with 0.008% (w/v) digitonin, and 50-μL samples added to 900 μL of reaction buffer containing 274 mM K-phosphate buffer, pH 7.2, and 46 μM reduced Cyt c. The initial rate of oxidation was followed by changes in A_{550}.

Single Channel Measurements

Synthetic lipids were obtained from Avanti Polar Lipids (Alabaster, AL). The composition of the bilayer for most experiments was 8 mM phosphatidylethanolamine (PE), 6 mM phosphatidylserine (PS), 3 mM phosphatidylcholine (PC), and 15 mM cholesterol. Cholesterol was generally included as a cheap alternative to plant sterols because it improved the electrical stability of the bilayer.

Planar lipid bilayers were painted across a 0.3-mm hole separating the cis chamber (500-μL solution in a styrene copolymer cup) from the trans chamber (1.5 mL, in a Perspex block). The bilayer was painted in identical NaCl solutions and then an osmotic gradient imposed across the membrane by perfusing the cis chamber with 20 volumes of hypertonic NaCl solution. Plasma membrane vesicles (usually 1–5 μg of protein) were added to the cis chamber and stirred with a magnetic flea. Vesicles were initially fused in NaCl solutions to facilitate identification of the channel. When fusion was detected, the reversal potential of the current was determined and the remaining vesicles perfused away from the cis chamber. Solutions were changed by perfusion of (usually) the cis chamber with 15 to 20 chamber volumes of fresh solution.

Channel activity was recorded under voltage clamp conditions using an amplifier (EPC-7, List Electronics, Damstadt, Germany). The amplifier was connected to the bilayer chambers via 3 M KCl/1% (w/v) agar salt bridges. The cis chamber was electrically grounded and all voltages are expressed as trans with respect to cis (following whole-cell electrophysiological convention). Therefore, the cis chamber corresponds to the extracellular solution, and the inward movement of cations into the cytosol is represented by a negative current and a downward deflection in single-channel current traces. Data were recorded unfiltered on a DAT recorder (Sony, Tokyo), and/or filtered at 100 Hz (to eliminate noise due to the large capacitance of the bilayer) with an 8-pole Bessel filter (Kemo, Beckenham, UK) and recorded with pCLAMP 6.03 software (Axon Instruments, Foster City, CA). pCLAMP files were sampled at 1 kHz for analysis, and Gaussian distributions were determined using the Simplex least squares method provided by pSTAT (pCLAMP6) software. Data were fitted using FigP version 2.2 (BioSoft) software. The probability of channel opening (P_{open}) data were fitted with a variant of the Boltzmann equation: $P_{open} = P_{max}/[1 + \exp(E_{0.5} - E_m)/N]$, where $n =$ RT/Fz, $P_{max} =$ maximum channel open probability, $E_m =$ membrane potential, $E_{0.5} = E_m$ at which $P_{open} = 0.5 P_{max}$, and $z =$ gating charge. Selectivity ratios were calculated using Goldman-Hodgkin-Katz or Fatt-Ginsborg equations (Hille, 1992). A variant of the Michaelis-Menten equation was used to describe conductance: $G = G_{max}([Na^+])/(K_m + [Na^+])$, where $G =$ conductance through the channel, measured between defined voltages, $G_{max} =$ maximum conductance, and $K_m = [Na^+]$ at which $G = 0.5 G_{max}$. Conductance was calculated from the linear regions of current-voltage curves (see figure legends) and was used instead of current to avoid the problem of differences in rectification at different voltages in different solutions.

Solutions

Solutions were filtered through 0.2-μm filters. Bilayer solutions were adjusted with HCl to pH 5.5 unless otherwise stated. Stocks of hydrophobic inhibitors were made up in ethanol or DMSO, and the final concentration of solvent in bilayer solutions was kept below 1%. Activities of ions in solutions were calculated using GEOCHEM-PC, version 2.1 (Parker et al., 1995).

RESULTS

Weakly Voltage-Dependent, Nonselective Cation Channel Was the Main Na^+-Permeable Channel in the Bilayer

Plasma membrane vesicle preparations were obtained from wheat roots by aqueous two-phase partitioning. Analysis of membrane marker activities indicated that preparations were highly enriched in plasma membrane relative to other membranes (Table I). The main contaminants were tonoplast and ER vesicles. The distribution of channel types in the microsomal and endomembrane preparations relative to PM could not be assessed, because the addition of even very small amounts of these vesicles generally caused rupturing of the bilayer before channel activity could be resolved. Studies of plasma membrane vesicles obtained by this method indicate that most of the vesicles are right-side-out (Larsson et al., 1987), and fuse with planar bilayers in a defined orientation (Cohen, 1986). We did not observe channels of identical conductance but opposite voltage dependence of gating or current rectification, sug-

Table I. *Assay of plasma membrane (PM) enrichment in the upper phase obtained by aqueous two-phase partitioning*

Enzymic Marker	Activity	Activity Recovered[a]	Relative Enrichment[b]
	μmol mg protein^{-1} h^{-1}	%	
Glucan synthase (PM)	2.2	15	
K$^+$, Mg^{2+}-stimulated pyrophosphatase (tonoplast)	3.9	1.0	15.0
NADH-dependent Cyt *c* reductase (ER)	5.9	1.0	15.1
Cyt *c* oxidase (mitochondria)	7.3	0.5	38.6

[a] Recovery of marker in the upper phase from the microsomal fraction. [b] Enrichment of plasma membrane relative to endomembranes, calculated as [(glucan synthase activity in upper phase)/(endomembrane marker activity in upper phase)]/[(glucan synthase activity in microsomal fraction)/(endomembrane marker activity in microsomal fraction)].

gesting that fused vesicles were of uniform orientation (cytosolic face to trans chamber).

Assays for Na$^+$-permeable channels from plasma membrane preparations using the planar lipid bilayer system revealed four channel types with Na$^+$ transport activity (assessed by current reversal potentials in simple asymmetrical NaCl solutions). The channels were initially classified according to their conductances in symmetrical 100 mM NaCl solutions. The most abundant channel type was a 43.6 ± 0.1 pS (SE, *n* = 14) channel (94% of bilayers in which any channel activity was present: 5/8 membrane preparations showed channel activity). Other channels present were approximately 160 pS (11%), 26 pS (7%), and 460 pS (4%). The less-abundant channel types were almost always observed in conjunction with the 44 pS channel, but were considered to be distinct channels rather than rare substates of the 44 pS channel, because transitions between different conductance levels were always independent. In the light of results described below, the 44 pS channel was dubbed a NSC channel, in accordance with the nomenclature for similar channels in animal and fungal systems (e.g. Siemen and Hescheler, 1993; Bihler et al., 1998), and was selected for further analysis.

The NSC channel always appeared in multiples of at least two (but up to 25) in the bilayer. Channels were sometimes observed to appear spontaneously and simultaneously in the bilayer, suggesting that they had incorporated at the same time, and sonication of vesicles did not reduce the number of channels appearing in the bilayer. This suggests that the channels were in some way co-localized in small areas of membrane. A second striking feature of the NSC channel was its long open and closed times, evident when only a few channels were present in the bilayer (Fig. 1a). However, this contrasted with the appearance of bilayer traces when large numbers of channels incorporated, where traces appeared noisy and gating events were too rapid to be easily resolved (Fig. 1b). Under these circumstances the bilayer resembled closely the noisy currents attributed to the instantaneous current in excised patches from wheat cortical protoplasts (see Fig. 9 in Tyerman et al., 1997). Current through the open single channel increased with voltage in a nonlinear manner at extreme voltages, and was larger at negative versus positive membrane voltages. These current rectifications were more pronounced in lower concentrations of NaCl (Fig. 1c).

The NSC channel was poorly selective among monovalent cations. The selectivity relative to Na$^+$, determined from reversal potentials in 100 mM bi-ionic conditions (100 mM NaCl in the trans chamber, with 100 mM monovalent chloride salt or 50 mM CaCl$_2$ in the cis chamber), was NH$_4^+$ (2.06 ± 0.05, *n* = 3) > Rb$^+$ (1.38, *n* = 1) > K$^+$ (1.23 ± 0.01,
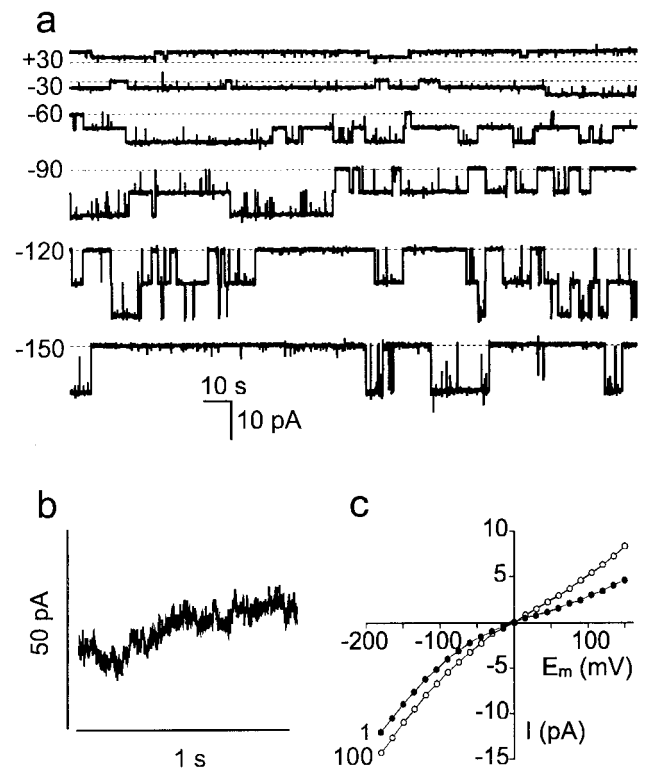

Figure 1. Characterization of the NSC channel in planar lipid bilayers. a, Single-channel traces showing two NSC channels in the bilayer at holding potentials (indicated in mV) to the left of each trace. Dashed lines represent the fully closed state for two channels. Traces were recorded in symmetrical 100 mM NaCl and filtered at 100 Hz. b, Single-channel trace showing a large number of NSC channels in the bilayer, closely resembling traces from excised patches in wheat root protoplasts (Tyerman et al., 1997) recorded at a holding potential of −30 mV, filtered at 500 Hz, and digitized at 2 kHz in symmetrical 100 mM NaCl. c, Current-voltage relations of a single NSC channel in symmetrical 1 or 100 mM NaCl solutions, demonstrating rectification of unitary current at negative voltages.

$n = 3$) $\approx$ Cs$^+$ (1.18 $\pm$ 0.09, $n = 3$) > Na$^+$ > Li$^+$ (0.83 $\pm$ 0.06, $n = 3$) > TEA$^+$ (0.21, $n = 1$) $\approx$ Ca^{2+} (0.21 $\pm$ 0.01, $n = 3$). The channel was distinct from the majority of animal NSC channels in its higher selectivity for monovalent cations over Ca^{2+} (Siemen and Hescheler, 1993). P_{Na}:P_K was 0.8 in 100 mM bi-ionic solutions, but appeared to increase as external [NaCl] was lowered against a constant concentration of KCl in the cytosolic (trans) compartment. This is illustrated in Figure 2A, where the current reversal potentials for inward movement of Na$^+$ against a K$^+$ concentration of 100 mM shifted in a less than Nernstian manner as the cis NaCl concentration was decreased. Thus, the reversal potentials for Na$^+$ current through the channel were
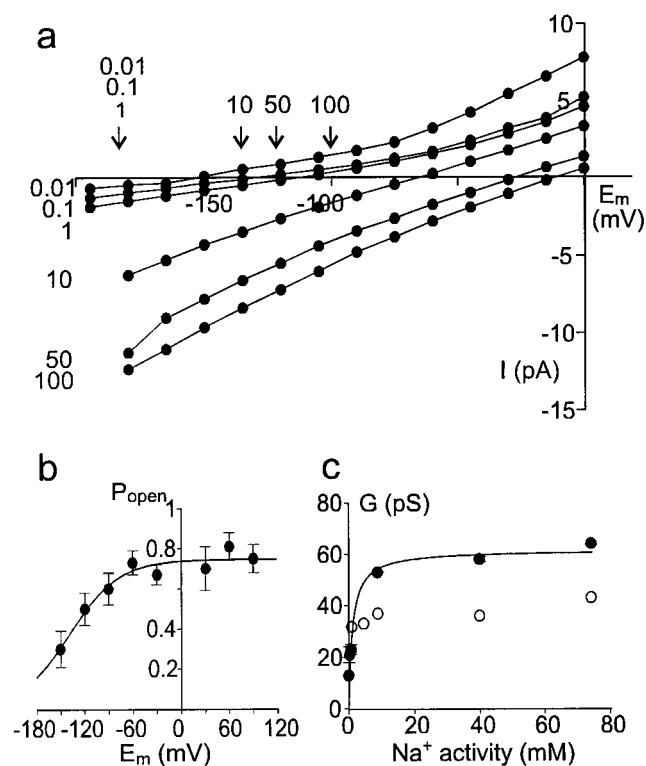
always positive of physiological membrane potentials measured by microelectrode impalement in intact roots in the same extracellular solutions, even in 0.01 mM NaCl. These data indicate that the channel would catalyze inward movement of Na$^+$ into the root over the range 0.01 to 100 mM NaCl. The effect of Na$^+$ influx on the E_m of root cells may be balanced by efflux of other cations in low-salt solutions or by Cl$^-$ influx in conditions of high external Cl$^-$ (Skerrett and Tyerman, 1994; Tyerman et al., 1997).

P_{open} of the NSC channel was fitted with a Boltzmann equation, giving a gating charge of 0.82 (indicating that the channel was only weakly voltage-gated) and an $E_{0.5}$ of -139 ± 6 mV ($r^2 = 0.94$) (Fig. 2b). Thus, the NSC channel would be open at least part of the time at all physiological membrane potentials. This suggested that channel gating might be regulated by other factors in addition to voltage. However, a range of intracellular effectors known to modulate gating by direct interactions with cation channels of various categories had no effect on the activity of the NSC channel in the bilayer when applied to the trans chamber. However, two reservations pertain. First, assessment of subtle modulations of P_{open} was impossible, because of the high numbers of NSC channels generally present in the bilayer and the slow gating of the channel. Thus only strong inhibitors or activators of channel activity would have been identified by this method. Secondly, the already high P_{open} of the channel in the bilayer made it problematic to assess the effect of putative activators of channel opening. Nevertheless, it was expected that any effects on channel activity should be pronounced at physiological membrane voltages, where P_{open} was relatively low.

Rises in cytosolic Ca^{2+} activity activate Ca^{2+}-activated nonspecific channels in animal cells, but neither increasing (up to 0.5 mM) nor effectively eliminating Ca^{2+} (by addition of up to 10 mM EGTA) at the cytosolic face of the channel affected its activity. Mg^{2+}-ATP is used routinely in patch-clamp intracellular solutions to stimulate channel activity; however, the addition of 4 mM Mg^{2+}-ATP to the trans face did not affect the NSC channel nor activate other conductances within the bilayer. Cyclic nucleotides (0.1 mM cAMP or cGMP) gate a number of voltage-independent cation channels, but did not strongly affect the NSC channel. Cytosolic polyamines (0.5 mM spermine) (Lopatin et al., 1994) and Mg^{2+} (4 mM) (Matsuda et al., 1987; Vandenberg, 1987) cause rectification in some inward-rectifying K$^+$ channels, but had no effect on this channel. However, the addition of 0.1 to 0.5 mM flufenamate to the intracellular (but not the extracellular) chamber caused a flickery block of the channel. This resembled the effect of cytosolic flufenamate on Ca^{2+}-activated nonspecific cation (CAN) channels in rat exocrine cells (Gögelein et al., 1990), although the affinity of the NSC channel for flufenamate appeared to be lower.

Cytosolic pH had a small effect on the conductance of the NSC channel, evident in the higher rate of influx of Na$^+$ with 100 mM KCl, pH 7.4, in the cytosolic chamber ($V_{max} = 61.4 \pm 7.4$ pS), compared with influx in symmetrical NaCl, pH 5.5 (V_{max} approximately 46 pS) (Fig. 2c). This increase in conductance was not due to an effect of cytosolic K$^+$, since conductance remained 44 pS with 100 mM KCl, pH

Figure 2. Na$^+$ transport characteristics of the NSC channel. a, Current-voltage relations of the NSC channel in cis solutions of various NaCl concentrations (indicated in mM), with a constant trans solution of 100 mM KCl plus 5 mM N-Tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES)-BTP, pH 7.4. E_m values measured by microelectrode impalement of intact cortical cells in the same NaCl solutions are indicated by arrows. Points represent the mean of two to three bilayers. Error bars are omitted for clarity. b, Open probability of the NSC channel measured in symmetrical 100 mM NaCl. Recordings were made over 20-min intervals at each holding potential. Points represent means $\pm$ SE, $n = 4$ bilayers, and are fitted with a variant of the Boltzmann equation. c, Inward Na$^+$ conductance through the NSC channel in symmetrical NaCl solutions (pH 5.5; $\bigcirc$) or bi-ionic conditions: trans = 100 mM KCl, buffered to pH 7.4 with 5 mM TES-BTP; cis = varied NaCl, pH 5.5 ($\bullet$). Conductance was measured as the slope of current between E_{rev} and 30 to 90 mV negative of E_{rev} (depending on available data points). Data obtained in bi-ionic conditions are fitted with a Michaelis-Menten equation, with $K_m = 1.2 \pm 0.9$ mM Na$^+$ activity, and $V_{max} = 61.4 \pm 7.4$ pS ($r^2 = 0.93$). Data represent mean $\pm$ SE, $n = 3$ (bi-ionic conditions) or the mean of two experiments (symmetrical NaCl).

5.5, in the cytosolic chamber. The increased conductance is likely to be due to some allosteric effect of cytosolic pH, rather than to increased net influx of H$^+$, since the current reversal potential was unaffected by the pH of the KCl solution. The channel was unaffected by 0.5 mM extracellular Glu (an activator of cation nonselective Glu receptors) and extracellular pH (which gates acid-sensing cation channels). The insensitivity of the NSC channel to intra- and extracellular gating agents might indicate that its gating mechanism is either simply voltage dependent or involves cytosolic interactions absent from the bilayer environment. The latter is more likely, given the kinetics of transport of Na$^+$ via the channel (Fig. 2c). Na$^+$ influx through the channel saturated at relatively low external Na$^+$ concentrations, with a K_m of 1.2 ± 0.9 mM Na$^+$ (Fig. 2c). However, Na$^+$ influx into wheat roots was linear over the range 5 to 100 mM NaCl (Davenport, 1998). Thus, while a low-millimolar K_m would be appropriate for a channel normally involved in cation uptake from dilute soil solutions, it is likely that channel activity is higher in high-salt solutions if the channel does play a role in toxic Na$^+$ uptake.

Single-Channel Characteristics of Na$^+$ Influx via the NSC Channel Resemble Na$^+$ Influx into Wheat Root Segments

Characteristics of Na$^+$ influx into root segments and via the NSC channel were compared by application of organic and inorganic inhibitors to root segments and to the extracellular face of the channel. TEA$^+$ is an inhibitor of all known plant K$^+$-selective channels, verapamil is an inhibitor of voltage-gated L-type Ca^{2+} channels, and quinine, amiloride, and flufenamate inhibit various types of nonselective cation channels identified in animal systems. Flufenamate, amiloride, and TEA$^+$ had no effect on the activity of the NSC channel when added to the cis chamber. Verapamil and quinine had no effect on the inward Na$^+$ current (and would therefore have no effect on Na$^+$ influx into roots via the NSC channel), although both caused a rapid block of the outward (K$^+$) current (Fig. 3). Verapamil apparently bound with high affinity to the channel (Fig. 3a), and its effect could not be fully reversed by prolonged perfusion with control solution. Verapamil also trans-inhibits outward-rectifying K$^+$ currents in some plant systems (Terry et al., 1992; Thomine et al., 1994; Tyerman et al., 1997), so these data may indicate some structural homology between the NSC channel and plant KOR channels. Tetrodotoxin (100 μM), a potent blocker of animal voltage-gated Na$^+$ channels, and Cs$^+$ (1–5 mM), a K$^+$ channel blocker, also had no effect on Na$^+$ transport via the NSC channel.

Unidirectional Na$^+$ influx into root segments from plants grown in low-salt, NaCl, and high-nutrient conditions was generally insensitive to organic cation channel inhibitors (Table II). Verapamil caused a variable increase of Na$^+$ influx in low-salt-grown plants, which was not due to an effect of verapamil on membrane potential (data not shown), and remains unexplained. This stimulation by verapamil of Na$^+$ influx in low-salt-grown plants but not in NaCl- or high-nutrient-grown plants was the only pharma-
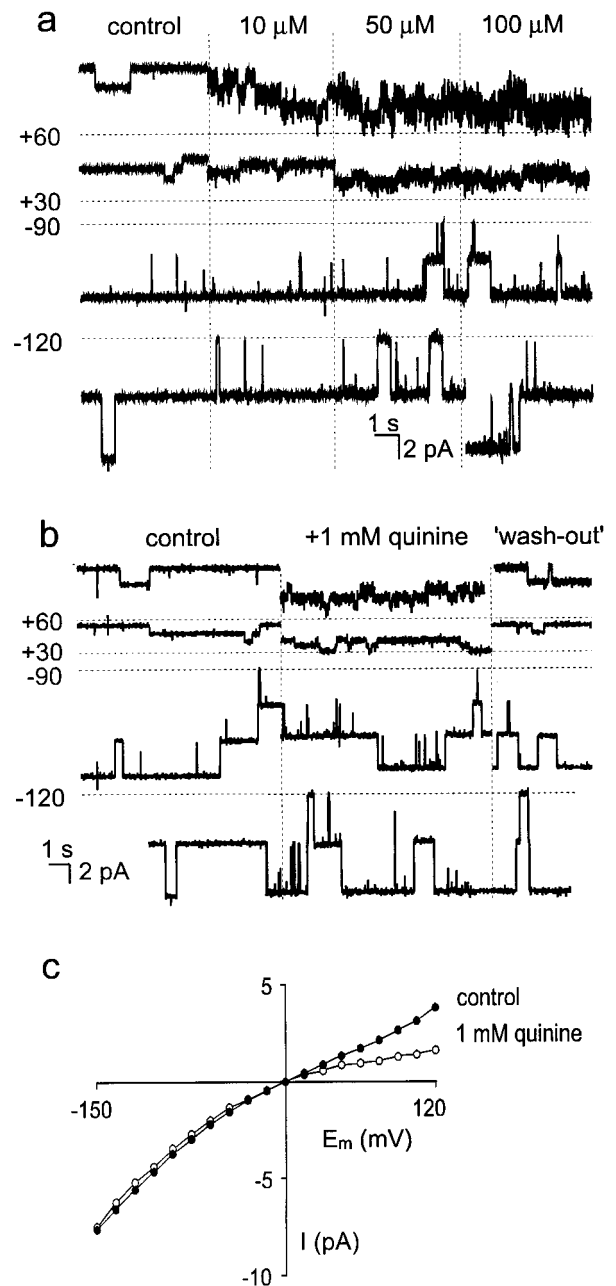

Figure 3. Extracellular verapamil and quinine inhibited only the outward current. a, Single-channel trace of four NSC channels in the bilayer, showing the effect of verapamil at a range of membrane voltages (indicated in mV on the left). b, 1 mM quinine caused reversible blockade of the outward current. c, Rapid block of the NSC channel by 1 mM quinine produced an apparent decrease in unitary conductance of the single channel. All data were recorded in 100 mM NaCl:100 mM KCl plus 5 mM TES-BTP, pH 7.4, cis/trans.

cological difference between plants of different growth regimes. Na$^+$ influx from 100 mM NaCl was slightly higher in NaCl-grown compared with low-salt-grown plants, and was lowest in high-nutrient-grown plants (Table II). Cortical cell membrane potentials in 100 mM NaCl solutions were similar in all growth conditions (-100 ± 9 mV, $n = 4$

Table II. *Effects of cation channel inhibitors on Na⁺ influx into roots of wheat plants grown in low-salt, 100 mM NaCl, and high nutrient growth solutions, measured in 100 mM NaCl*

Numbers represent mean ± SE, $n = 6$.

Treatment	Low Salt	NaCl 100 mM	High Nutrient
	nmol g fresh wt⁻¹ min⁻¹		
Control	1,390 ± 82	1,533 ± 28	1,095 ± 25
10 mM TEA-Cl	1,433 ± 57	1,496 ± 28	1,056 ± 48
1 mM Quinine	1,409 ± 39	1,579 ± 50	1,085 ± 23
0.1 mM Verapamil	2,420 ± 365	1,336 ± 63	1,154 ± 44
0.1 mM Amiloride	1,202 ± 193	1,411 ± 25	1,136 ± 52
0.1 mM Flufenamate	1,340 ± 30		
1 mM GdCl₃	250 ± 6		

and -110 ± 15 mV, $n = 4$, -95 ± 5 mV, $n = 4$ in low-salt-, NaCl-, and high-nutrient-grown plants, respectively).

Na⁺ influx through the NSC channel and into root segments was partially inhibited by divalent cations (Fig. 4). Both Ca^{2+} and Mg^{2+} caused inhibition of Na⁺ influx through the channel by a voltage-independent reduction in unitary conductance through the open channel, with no apparent effect on gating. In striking accord with data for Ca^{2+} inhibition of $^{22}Na^+$ influx into root segments, the inhibitory effect of Ca^{2+} in 100 mM NaCl saturated at

around 3 mM Ca^{2+} activity, with a K_i value of 0.65 ± 0.25 mM Ca^{2+} (versus $K_i = 0.61 \pm 0.04$ mM in root segments) (Fig. 4, a and b). Outward current through the channel was not inhibited and the reversal potential did not change, indicating that Ca^{2+} was not permeating. Ca^{2+} partially inhibited Na⁺ influx into low-salt-grown roots (Fig. 4b) in a manner analogous to that observed previously in a range of cereal roots (Rains and Epstein, 1967; Leigh and Wyn Jones, 1973; Zidan et al., 1991; Davenport et al., 1997). Microelectrode impalement of cortical cells showed that the addition and removal of Ca^{2+} from the bathing solution did not alter membrane potentials in 100 mM NaCl solutions (data not shown).

Comparative values for Ca^{2+} inhibition of Na⁺ influx at lower concentrations of NaCl could not be obtained in the bilayer, because Ca^{2+} was permeant at higher Ca^{2+}:Na⁺ ratios, and so the component of current attributable to Na⁺ could not be distinguished. However, the channel was less permeable to Mg^{2+}, so data for Mg^{2+} inhibition of Na⁺ inward current from 10 mM extracellular NaCl are presented with comparable data for $^{22}Na^+$ influx into root segments (Fig. 4, c and d). The kinetics of Mg^{2+} inhibition of Na⁺ influx were almost identical in both systems and resembled the effect of Ca^{2+}.

Trivalent cations also partially inhibited Na⁺ influx through the NSC channel, but appeared to act via two distinct mechanisms: a voltage-independent reduction in
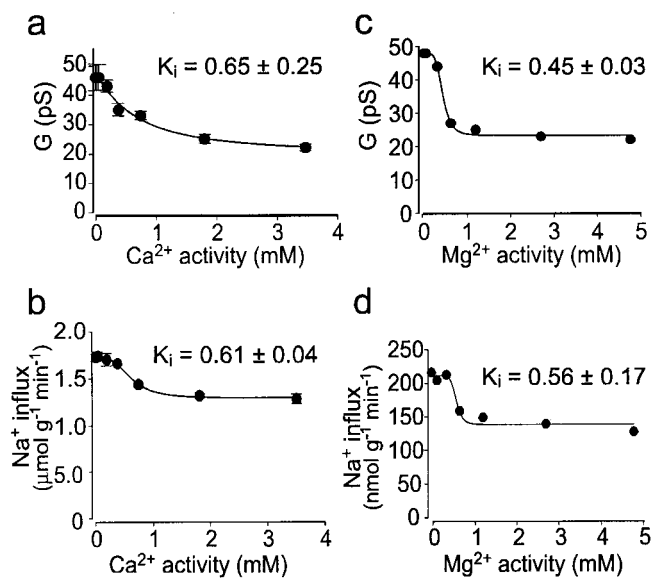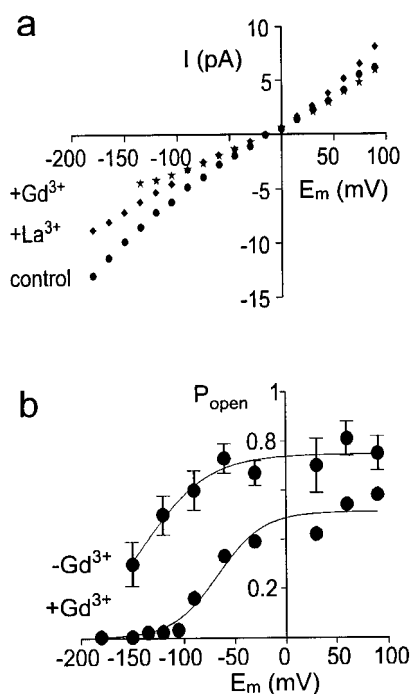
Figure 4. Extracellular divalent cations partially inhibited Na⁺ influx by reducing the unitary conductance of the NSC channel. a, Ca^{2+} reduced unitary conductance of the NSC channel, measured between -60 and 0 mV in 100 mM NaCl. Points represent the means ± SE of three bilayers, and are fitted with a Hill dose-response curve ($r^2 = 0.98$). b, Inhibition by Ca^{2+} of $^{22}Na^+$ influx into low-salt-grown seedlings in 100 mM NaCl. Data represent mean ± SE, $n = 3$, and are fitted with a Hill dose-response curve ($r^2 = 1.00$). c, Mg^{2+} reduced unitary conductance of the NSC channel, measured between -135 and -75 mV in 10 mM NaCl:100 mM KCl plus 5 mM TES-BTP, pH 7.4, cis/trans. Data are from a single bilayer, and are fitted with a Hill dose-response curve ($r^2 = 1.00$, Hill coefficient = 4.83 ± 0.79). d, Inhibition by Mg^{2+} of $^{22}Na^+$ influx into low-salt-grown seedlings in 10 mM NaCl. Data represent mean ± SE, $n = 3$, and are fitted with a Hill dose-response curve ($r^2 = 0.96$). K_i values are given in mM.

Figure 5. Extracellular trivalent cations reduce unitary conductance and open probability of the NSC channel. a, Effects of 1 mM GdCl₃ or 1 mM LaCl₃, applied to the cis face, on single channel current, in 100 mM NaCl:100 mM KCl + 5 mM TES-BTP, pH 7.4, cis/trans. b, Inclusion of 1 mM Gd^{3+} in the cis solution reduced P_{max} and shifted the voltage dependence of channel opening from $E_{0.5} = -139$ mV in the control to $E_{0.5} = -67$ mV (data are fitted with modified Boltzmann equations: gating charge was 0.82 and 1.17, respectively).

unitary conductance (Fig. 5a) and a voltage-dependent reduction in P_{open} (Fig. 5b). The interaction of these two mechanisms would account for the large inhibition of Na$^+$ influx by Gd^{3+} observed in root segments (80%; Table II). Gd^{3+} appeared to inhibit the channel via a mechanism common to trivalent cations and was not inhibitory at the low micromolar concentrations at which it specifically blocks mechanosensitive channels.

DISCUSSION

We have identified the NSC channel, a nonselective cation channel from wheat root plasma membrane that corresponds very closely in its single-channel characteristics to the properties of Na$^+$ transport at the protoplast (Tyerman et al., 1997) and root levels. The channel was able to account at the molecular level almost completely for the characteristics of Na$^+$ influx into roots in 100 mM NaCl, and so could account for the bulk of toxic Na$^+$ influx in wheat.

Na$^+$ influx was insensitive to a range of cation channel inhibitors, indicating that Na$^+$ was not entering the roots via a TEA$^+$-sensitive K$^+$ channel nor a verapamil-sensitive Ca^{2+} channel or K$^+$-selective outward rectifier. The only positive diagnostic trait was the partial inhibition of Na$^+$ influx by divalent and trivalent cations, which is typical of Na$^+$ influx in cereal roots (Figs. 4 and 5; Table II). The Na$^+$ transporter was likely to be constitutively expressed, since Na$^+$ influx appeared almost identical in low-salt- and NaCl-grown plants, and was also similar in plants grown in high nutrient solution (Table II).

A screen of wheat root plasma membrane for Na$^+$-permeable channels identified four candidates, of which the most abundant was a 44-pS channel (the NSC channel) with the characteristics of Na$^+$ transport predicted by patch-clamp studies. The other three channels occurred at much lower frequencies in the bilayer and are still being characterized. The plasma membrane origin of the NSC channel was assumed on the basis of its high frequency of appearance, the relative purity of the membrane preparations (Table I), and arguments presented in earlier papers (Piñeros and Tester, 1995; White, 1995). The NSC channel was nonselective among monovalent cations, but was selective for monovalent over divalent cations ($P_{Ca}:P_{Na} = 0.21 \pm 0.01$). Thus, it would catalyze relatively indiscriminate uptake of monovalent cations in vivo, and would also allow some influx of divalent cations, depending on the ratio and composition of the soil solution. The channel was weakly voltage dependent, and so could appear voltage insensitive over the voltage ranges often employed in patch-clamp studies (e.g. −130 to +30 mV: Tyerman et al., 1997) (Fig. 2b). Channels tended to appear in multiples in the bilayer, but were few enough to be easily resolved as single channels (as opposed to the denser clustering typical of patches from wheat cortical protoplasts).

Some of the correspondence between the characteristics of Na$^+$ influx in wheat root segments, cortical protoplasts, and the NSC channel are presented in Table III. A range of cation channel inhibitors had no effect on Na$^+$ influx in any of the three systems, suggesting that influx was mediated by the same inhibitor-insensitive mechanism in each case. Na$^+$ influx through the NSC channel was partially inhibited by Ca^{2+} in a manner closely analogous to that of Na$^+$ influx into wheat roots and protoplasts (Fig. 4, a and b; Tyerman et al., 1997). Ca^{2+} inhibition saturated at similar Ca^{2+} activities and with similar K_i values in all three systems (Table III). However, the maximum inhibitory effect of Ca^{2+} was around 50% for both the single-channel and protoplast currents, but was only 25% in roots. This difference may indicate the contribution of other, Ca^{2+}-insensitive transporters to root Na$^+$ influx, or may be due to residual Ca^{2+} in the root apoplast, which would reduce the apparent maximum influx rate (in zero Ca^{2+} treatments). In both the NSC channel and the instantaneous current in wheat root protoplasts, Ca^{2+} inhibited Na$^+$ influx by a reduction in unitary conductance of the open channel, without affecting P_{open}. It is therefore unnecessary to posit separate Ca^{2+}-sensitive and -insensitive pathways

Table III. *Comparison of the NSC channel with patch-clamp recordings of instantaneous Na$^+$ currents and ^{22}Na$^+$ influx in wheat roots*

Characteristic[a]	NSC Channel	Instantaneous Current[b]	^{22}Na$^+$ Influx into Root Segments
Conductance	44 pS	≈30 pS	−
$P_{Na}: P_K$	0.76	0.8	−
Permeability sequence[c]	Rb$^+$ > K$^+$ > Cs$^+$ > Na$^+$ > Li$^+$	Rb$^+$ > K$^+$ > Cs$^+$ > Na$^+$ > Li$^+$	−
K_i for Ca^{2+} inhibition	0.65 mM	0.31 mM	0.61 mM
K_i for Mg^{2+} inhibition[d]	0.45 mM	−	0.56 mM
Voltage dependence	P_o ↓ -ve of −90 mV	None	−
"Clustering"	Yes	Yes	−
Inhibition[e]			
10 mM TEA$^+$	None	None	None
10 to 100 μM Verapamil	↓ Outward, no effect on inward	None	↑ In low-salt roots
1 mM Quinine	↓ Outward, no effect on inward	−	None
100 μM Amiloride	None	−	None
100 μM Flufenamate	None	−	None

[a] All data were obtained in solutions of approximately 100 mM extracellular NaCl, or other monovalent cation for permeability ratios, except for the K_i for Mg^{2+} inhibition. [b] Tyerman et al., 1997. [c] Determined from conductance between −90 mV and E$_{rev}$ for the NSC channel, and current magnitude at E$_{Cl}$ for the instantaneous current (Tyerman et al., 1997). [d] Measured with 10 mM extracellular NaCl. [e] All compounds added to the extracellular solution.

of Na^+ influx in roots, since a single transporter can show partial sensitivity to Ca^{2+} inhibition. Partial sensitivity of a NSC channel to inhibition by Ca^{2+} has also been observed in a nonselective cation current in guard cells of *Aster tripolium* and *Aster amellus*, although in this case Ca^{2+} both blocked and reduced P_{open} of the channel (Véry et al., 1998).

Mg^{2+} appeared to act upon the NSC channel via the same mechanism as Ca^{2+}, and caused almost identical partial inhibition of $^{22}Na^+$ influx into roots (Fig. 4, c and d). The similarity of the effect of Mg^{2+} and Ca^{2+} in root tissue and in the bilayer suggests that Ca^{2+} reduces Na^+ uptake into wheat roots by direct interaction with the NSC channel in vivo rather than via a cytosolic signal transduction pathway. This is because the effect of divalent cations persists even in the reductionist conditions of the bilayer, and because Mg^{2+} can substitute partially for Ca^{2+} in reducing Na^+ influx but cannot substitute at all for Ca^{2+} in its cytosolic signaling role (Malmendal et al., 1998). This result has implications for the role of Ca^{2+} in alleviating Na^+ toxicity. Ca^{2+} has long been known to ameliorate the symptoms of salt stress in plants when supplied in concentrations above that normally required for growth in nonsaline media (0.1 mM) (Hyder and Greenway, 1965). The ameliorative effect of Ca^{2+} is partial and saturates at around 10 mM (Maas and Grieve, 1987; Cramer et al., 1989; Ehret et al., 1990). Mg^{2+} is also effective at low concentrations, but may itself become toxic above 1 mM (Kawasaki and Mortisugo, 1978; Carvajal et al., 1999). Recently, *sos3*, a mutant of Arabidopsis that is hypersensitive to NaCl but is restored to normal phenotype by supplementation of Ca^{2+} in the growth medium, was characterized (Liu and Zhu, 1997, 1998). The SOS3 protein resembles yeast calcineurin, and has therefore been implicated in Ca^{2+} signaling pathways proposed to regulate ion transport in plants (Bressan and Hasegawa, 1998; Epstein, 1998). However, supplemental divalent cations are unlikely to relieve salt stress via a Ca^{2+}-specific signaling pathway, since Mg^{2+} would be ineffective. Therefore, it seems more likely that divalent cations act in a direct extracellular role to reduce Na^+ uptake (via NSC channels) and thereby reduce toxicity symptoms.

The partial inhibition of Na^+ influx through the NSC channel by multivalent cations could result from charge-screening effects (Kinraide, 1998), but these mechanisms were shown not be involved in this case (the investigation of electrostatic interactions will be presented in a separate paper, and can be found in Davenport, 1998). Alternatively, di- and trivalent cations may interact with the channel at an allosteric binding site that alters channel conformation to reduce cation transport. Trivalent cations both reduced unitary conductance and caused a voltage-dependent reduction in P_{open} (Fig. 5), suggesting that they may act via at least two distinct inhibitory mechanisms.

No survey of Na^+- or K^+-permeable channels has been conducted before in wheat using the bilayer technique. However, a study of K^+-permeable channels from rye root plasma membrane yielded a complement of five channels, some of which were nonselective for monovalent cations (White and Tester, 1992; White, 1997). Four of these corresponded fairly closely in conductance to the four channel types identified from wheat root. The most abundant of the rye root channels in the bilayer was a 49-pS channel (conductance measured in 280:100 mM KCl, cis:trans) that was voltage independent and partially inhibited by quinine and Ca^{2+} but not by TEA^+. This channel corresponded in its single-channel characteristics to an instantaneous, nonselective cation current measured in rye root protoplasts (White and Lemtiri-Chlieh, 1995). The 49-pS channel probably represents a rye analog of the NSC channel in wheat, since both channels showed similar cation selectivity, voltage independence, high P_{open}, long open and closed times, rectification of unitary conductance, and insensitivity to TEA^+ (White and Tester, 1992; White and Ridout, 1995). Extracellular quinine inhibited both the inward and the outward K^+ current in the rye 49-pS channel, however, the outward current was more severely affected. This corresponded to the asymmetrical effect of quinine on the wheat NSC channel, where only the outward current was blocked (Fig. 3, b and c). Thus, the rye and wheat channels are highly analogous, suggesting that NSC channels may be ubiquitous in cereals and constitute a common pathway for toxic Na^+ influx in these species. NSC channels have also been partially characterized in the plasma membrane of several leaf cell types, including a TEA^+-insensitive cation channel in pea leaf epidermis (Elzenga and van Volkenburg, 1994) and a Na^+-permeable channel in guard cells of the halophytic *A. tripolium* and the glycophytic *A. amellus* (Véry et al., 1998).

The insensitivity of the NSC channel to most inhibitors (Table III) and the lack of effect of a range of intracellular modifiers of channel activity make it impossible to class the channel in any of the categories of known cation channels from plants or animals. This may reflect the novelty of the channel and its specificity to plant function. Alternatively, it may arise from the loss during vesicle isolation of some regulatory subunit that affects the gating properties of the channel. This is unlikely, at least in the case of ligand and cyclic nucleotide binding, since these phenomena have been shown to occur in cytosolic domains of the pore-forming channel subunit (Goulding et al., 1994; Montal, 1995). Mechanosensitive degenerin channels reconstituted from individual pore-forming subunits have also been demonstrated to retain sensitivity to amiloride (Waldmann et al., 1995, 1996). In its selectivity, high P_{open}, and slow gating kinetics, the NSC channel from wheat most closely resembled the CAN channels identified in a variety of animal cell types. However, the NSC channel showed no response to rises or buffering of intracellular Ca^{2+} (which have been shown to affect CAN channels via a direct interaction between the channel and Ca^{2+} without intermediate signaling steps; Partridge and Swandulla, 1988). Thus, the NSC channel probably represents a novel class of plant cation channel. However, it is likely that channel activity is regulated in planta by mechanisms not investigated in the present study, since channel activity was high in the bilayer and relatively insensitive to cis cation concentrations. This contrasts with the almost linear relationship between Na^+ influx and external Na^+ concentration in roots, and the difference could not be explained by effects

of external salt solution on transporter expression or on E_m of intact root cells (Davenport, 1998).

The physiological role of the channel in non-saline conditions remains to be determined. The relatively high permeability and selectivity of the channel for NH_4^+ (Table III) suggests that the channel could function in the low-affinity uptake of NH_4^+ for nutritional purposes (see also White, 1996). NH_4^+ currents measured in symbiosomes from *Rhizobium*-infected soybean roots were also nonselective for monovalent cations (although the single channels differed from the wheat NSC channel in having a subpicosiemen, voltage-dependent conductance), suggesting that highly NH_4^+-selective channels may not exist in plants (Tyerman et al., 1995). Alternatively, the moderate permeability of the channel to TEA$^+$ (Table III) may suggest a role in the uptake of relatively large molecules such as basic amino acids or other organic cations, the size of which preclude selectivity against smaller cations (Hille, 1992). However, the low selectivity of the NSC channel could be a functional prerequisite (rather than a necessary evil) if the primary function of the channel were nonspecific transport of cations for the regulation of osmotic potential.

In conclusion, the wheat root NSC channel identified in planar lipid bilayers demonstrated the characteristics predicted at the single-channel level to underlie Na$^+$ influx measured in root segments and cortical protoplasts (Tyerman et al., 1997), and is therefore likely to be the main mechanism of toxic Na$^+$ influx into wheat. These data constitute the first comprehensive description of Na$^+$ influx in cereals, provide the basis for future identification of the gene(s) responsible for toxic Na$^+$ influx and the design of strategies for modification of expression or selectivity of the protein to enhance whole-plant salt tolerance.

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