J. Membrane Biol. 143, 37–49 (1995)

The Journal of
Membrane
Biology


# The $K^+$ Channel in the Plasma Membrane of Rye Roots Has a Multiple Ion Residency Pore

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Received: 22 June 1994/Revised: 12 September 1994

**Abstract.** The permeation of $K^+$ and $Na^+$ through the pore of a $K^+$ channel from the plasma membrane of rye roots was studied in planar 1-palmitoyl-2-oleoyl phosphatidylethanolamine bilayers. The pore contains at least two ion-binding sites which can be occupied simultaneously. This was indicated by: (i) biphasic relationships with increasing cation concentration of both channel conductance at the zero-current (reversal) potential of the channel ($E_{rev}$) and unitary-current at a specified voltage and (ii) a decline in $E_{rev}$ in the presence of equimolar $Na^+$ (*cis*):$K^+$ (*trans*) as the cation concentration was increased. To determine the spatial characteristics and energy profiles for $K^+$ and $Na^+$ permeation, unitary-current/voltage data for the channel were fitted to a three energy-barrier, two ion-binding site (3B2S) model. The model allowed for simultaneous occupancy of binding sites and ionic repulsion within the pore, as well as surface potential effects. Results suggested that energy peaks and energy wells (ion binding sites) were situated asymmetrically within the electrical distance of the pore, the *trans* energy-well being closer to the center of the pore than its *cis* counterpart; that the energy profile for $K^+$ permeation differed significantly from that of $Na^+$ in having a higher *cis* energy peak and a deeper *cis* energy well; that cations repelled each other within the pore and that vestibule surface charge was negligible. The model successfully simulated various aspects of $K^+$ and $Na^+$ permeation including: (i) the complexities in current rectification over a wide range of contrasting ionic conditions; (ii) the biphasic relationships with increasing cation concentration of both channel conductance at $E_{rev}$ and unitary-current at a specified voltage; (iii) the decline in $E_{rev}$ in equimolar $Na^+$ (*cis*):$K^+$ (*trans*) as cation concentrations were increased and (iv) the complex relationships between mole fraction and $E_{rev}$ at total cation concentrations of 100 and 300 mM.

**Key words:** $K^+$ channel — Model — Permeation — Planar lipid bilayer — Plasma membrane — Rye (*Secale cereale* L.)

## Introduction

Ion channels are integral membrane proteins which span the phospholipid bilayer, forming aqueous pores through which ions can pass rapidly down their electrochemical gradient (Tester, 1990). Ionic fluxes through a channel are governed by two separate processes, the formation of the conducting channel conformation (the gating process) and the movement of ions through the channel pore (the permeation process). We have previously described the gating process of a $K^+$ channel present in the plasma membrane of rye roots (White & Tester, 1992*a*) and in this paper we investigate the permeation of monovalent cations through this channel. This can be effected by studying how the magnitude and shape of the unitary-current *vs.* voltage (*I/V*) relationship for the channel changes in response to the concentration and identity of the permeant cations (Sanders, 1990; Alvarez, Villaroel & Eisenman, 1992; Hille, 1992). These relationships hold much cryptic information about the possible structure of the channel. In particular, they yield information regarding the spacing, the heights and depths of energy peaks and energy wells within the electrical profile of the pore, the magnitude of interactions between ions within the pore and the presence of fixed ionic charges within the channel structure (Sanders, 1990; Alvarez et al., 1992; Hille, 1992).

The $K^+$ channel from the plasma membrane of rye roots has been characterized following the incorporation

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of purified plasma-membrane vesicles into planar 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PE) bilayers (White & Tester, 1992*a,b*). Under these conditions, it exhibits complex, asymmetrical conductance properties (White & Tester, 1992*a,b*). When identical KCl concentrations are present on either side of the channel, it exhibits nonohmic *I/V* relationships. The unitary-current is greater at positive voltages than at corresponding negative voltages and the unitary-conductance increases as more extreme positive or negative voltages are applied. These phenomena are most marked at low KCl concentrations. These observations imply an asymmetry in pore structure. In addition, two lines of evidence suggest that the pore of this channel can be occupied by more than one cation simultaneously. First, the relationship between the unitary-conductance and $K^+$ concentration of the channel does not exhibit simple Michaelis-Menten kinetics (White & Tester, 1992*a*) and second, the apparent permeability of $Na^+$ relative to $K^+$ changes with concentration when assayed under bi-ionic, equimolar $Na^+$ (*cis*):$K^+$ (*trans*) conditions (results presented in the present paper). Thus, the channel exhibits flux properties consistent with a multiple ion-residency pore (Hille, 1992).

In this paper we have used a quantitative modeling approach to investigate the structure inherent in the detail of the *I/V* relationships of the $K^+$ channel obtained in the presence of varying symmetrical and asymmetrical KCl and NaCl concentrations and under bi-ionic conditions. We develop a model with three energy barriers and two ion-binding sites (a 3B2S model), which allows for both double cation occupancy and ionic interactions within the pore, to explain several aspects of the permeation process including: (i) the rectification in unitary-conductance under symmetrical ionic conditions; (ii) the biphasic relationships with increasing monovalent cation concentration of both channel conductance at $E_{rev}$ and unitary-current at a specified voltage and (iii) the changes in channel selectivity with cation concentration. Energy profiles for $K^+$ and $Na^+$ were determined from electrical recordings obtained both in the Department of Botany, University of Cambridge (some of which have been published previously, White & Tester, 1992*a,b*) and from more recent experiments performed at Horticulture Research International, East Malling. Although the data differed slightly between laboratories, possibly due to different methods of channel extraction and/or the chemicals or electrical equipment used, the energy profiles derived were consistent.

## Materials and Methods

### Plant Material

Rye (*Secale cereale* L.) was grown hydroponically in a complete nutrient medium containing (μM): $K^+$ 400, $NH_4^+$ 240, $Na^+$ 100, $Ca^{2+}$ 100, $Mg^{2+}$ 75, $Mn^{2+}$ 1.0, $Cu^{2+}$ 0.1, $Zn^{2+}$ 0.1, ferric ethylenediaminetetraacetate (FeHEDTA) 500, $NO_3^-$ 440, $Cl^-$ 100, $H_2PO_4^{2-}$ 100, $SO_4^{2-}$ 76, $BO_3^{3-}$ 2.2 and $Mo_7O_{24}^{6-}$ 0.003 (pH 6.5), under the environmental conditions described by White and Tester (1992*a*) at the Botany Department, University of Cambridge, or as described by White (1993) at Horticulture Research International, East Malling. Plants were harvested 14 days after sowing.

### Isolation of Plasma-Membrane Vesicles

Plasma-membrane vesicles were obtained by aqueous-polymer two-phase partitioning of a microsomal fraction derived from seminal roots. The whole root was used to prepare membrane fractions. Although the bulk material will represent cortical cells, membranes from other cell types may be present also. It is possible that cells in the stele may differ in function, and channel complement, from those of the cortex. At the University of Cambridge the procedure followed White and Tester (1992*a*). At East Malling the homogenization medium additionally included as protectants 2 mM phenylmethylsulfonylfluoride (PMSF), 4 mM dithioerythritol (DTE) and 0.5% (w/v) polyvinylpyrrolidone (PVP). Microsomal membranes were partitioned three times, with successive replacement of the lower, dextran phase. After the third partitioning, the upper, polyethyleneglycol phase was diluted fivefold with 5 mM N-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES), titrated to pH 7.5 with *N*-methyl-D-glucamine (NMDG), and centrifuged at 100,000 × *g* for 60 min. The resulting pellet was resuspended at a concentration of 1 mg protein $ml^{-1}$ in 5 mM TES, buffered to pH 7.5 using NMDG. All preparative steps were performed at 4°C. Membrane fractions were stored at −20°C without loss of channel forming activity.

### Channel Recordings

Electrical recordings were obtained following the incorporation of plasma-membrane vesicles containing ion channels into planar lipid-bilayers composed of 30 mM synthetic 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PE) dispersed in *n*-decane (White & Tester, 1992*a*). The bilayer (0.2 mm in diameter) separated solutions of 300 or 800 μl contained within a styrene copolymer cup (*cis* side) and 5 ml in an outer Perspex chamber (*trans* side). Plasma-membrane vesicles were added to the *cis* chamber in the presence of a (*cis:trans*) 300:100 mM KCl gradient. When channel activity was detected, unfused vesicles were removed by perfusing the *cis* chamber with 100 mM KCl. Further changes in solution composition were effected by perfusing a chamber with 30 chamber-volumes of the required solution. Aqueous solutions were buffered with 5 mM TES, titrated to pH 7.5 using NMDG. Experiments were performed at room temperature (20 to 23°C).

Current was monitored under voltage clamp conditions using a low noise operational amplifier with frequency compensation, connected to the bilayer chambers by calomel electrodes and 3 M KCl salt bridges. Data were stored on video tape after digitizing by a Sony audio to digital converter (PCM-701ES; 22 kHz per channel; Sony, Japan) and simultaneously displayed on a digital storage oscilloscope (Gould 1602; Gould Electronics, Hainault, Essex, UK). Membrane potentials were recorded *cis* with respect to *trans*, which was held at ground. Since the cytoplasmic side of the plasma membrane faces the vesicle lumen and vesicles fuse with a planar bilayer such that the inside becomes exposed to the *trans* chamber, plasma-membrane ion channels become oriented with their cytoplasmic face exposed to the *trans* chamber (White & Tester, 1992*a,b*). Thus, the sign of the membrane potential was opposite to that conventionally used in electrophysiological experiments of plant cells in vivo. The amplitude of single

channel currents was determined directly from channel recordings filtered at 100 Hz using an 8-pole low-pass Bessel filter (902LPF, Frequency Devices, Haverhill, MA). Movement of $K^+$ from the *cis* to the *trans* chamber is indicated by a positive current and appears as an upward deflection in current traces.

Relative ionic permeabilities were estimated using the Goldman-Hodgkin-Katz (GHK) equation from the zero-current (reversal) potential ($E_{rev}$), determined by linear interpolation, when contrasting salt concentrations or ionic species were present in the *cis* and *trans* chambers. All ionic species were corrected for activities using published activity coefficients (Robinson & Stokes, 1959). Graphical data represent the mean values of single channel current determined in at least two separate bilayers.

## Mathematical Modeling

Estimates of energy profiles for permeant monovalent cations and channel spatial characteristics were obtained using the FORTRAN computer program AJUSTE as described by Alvarez et al. (1992). Parameters were estimated by unweighted least squares. The chosen model (3B2S) had energy profiles consisting of three energy barriers and two ion-binding sites (energy wells), and allowed for single file permeation, double cation occupancy, ion-ion repulsion and surface potential effects. The energies of the unoccupied channel at zero voltage (in terms of multiples of the thermal energy, RT) were defined by three peaks G1, G2 and G3, and two wells, U1 and U2, with the postscript referring to their position relative to the *cis* compartment (Fig. 1). The distances D1 to D5 refer to the position of successive peaks and wells in the electrical field and there is no reason to assume that the electrical distance corresponds to a physical position. The effects of ion-ion interactions (electrostatic and/or allosteric) were simulated by the addition of an energy factor to the peaks and wells adjacent to an occupied well. This varied inversely with the electrical distance (*d*) from the occupied well to mimic a coulombic interaction, and was calculated as *A/d,* where *A* is the ionic-repulsion energy parameter. The effect of applied voltage was modeled by addition of the zero-voltage energy to an electrical work term proportional to the valence of the ion and electrical distance. Rate constants for transitions between permissible states were formulated by the standard Eyring rate theory expression equal to the product of a pre-exponential term, *kT/h,* (where *k/h* is Boltzmann's constant divided by Planck's constant) and an exponential function of the energy difference, $\exp(\Delta G/RT)$. A similar expression was used for bimolecular rate constants describing the entry of ions from the internal or external solutions, except that the pre-exponential factor was also multiplied by the molar activity of the ion in solution divided by the molar concentration of water (55.5 M). Thus, the reference energy state of our model corresponds to 55.5 M solution. To compare our energy values to models that use a 1 M reference state, 4.02 RT units must be added to our values.

Surface potential can have profound effects on both channel activation, via effects on the voltage sensor, and on channel conductance, by influencing ionic concentrations at the mouth of the pore (Latorre, Labarca & Naranjo, 1992). Although PE is essentially uncharged under the conditions of our experiments, surface potentials can arise from both charged amino acid side chains in the channel forming protein itself and/or nonprotein domains forming part of the molecular structure of the channel, such as acidic carbohydrate domains containing sialic acid (Latorre et al., 1992). The electrical field, which is the algebraic sum of the electrical fields induced by the applied voltage and the asymmetry of the surface potential, drops linearly through the energy profile and is added point by point to compute the free energy of each peak and well. Two parameters ($R_{scis}$ and $R_{strans}$) are included in the model to describe surface charge effects. These parameters correspond to the radii of circles (in Å) containing one electron charge in the *cis* and *trans* vestibules of the pore, respectively, from which the fixed surface charge densities at the vestibule of the pore can be calculated ($\sigma = e/\pi R^2$). They are calculated according to the Gouy-Chapman double-layer theory and therefore represent charged planar surfaces.

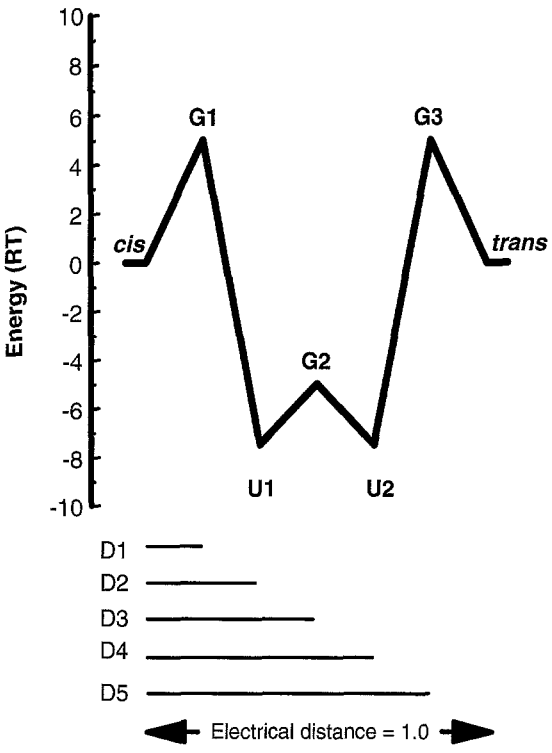



**Fig. 1.** Schematic diagram defining the energy-profile parameters of the three-barrier, two-binding-site (3B2S) model. The height of energy peaks, *G1, G2* and *G3,* and the depths of ion-binding sites (energy wells), *U1* and *U2,* are defined in terms of thermal energy, *RT,* units. Their positions are defined as D1–D5 in fractions of the electrical distance across the channel selectivity filter, with respect to the *cis* and *trans* solutions. The solution reference state is 55.5 M.

The energy profile for $K^+$ permeation and channel spatial characteristics were estimated from *I/V* curves obtained both at Cambridge, covering the range ± 200 mV in contrasting symmetrical and asymmetrical KCl concentrations, and at East Malling, covering the range ±100 mV under both single-species and bi-ionic equimolar $Na^+$ (*cis*):$K^+$ (*trans*) conditions. The energy profile for $Na^+$ permeation was estimated from *I/V* curves obtained at East Malling covering the range ±100 mV under both single-species and bi-ionic conditions. The symmetrical $K^+$ concentrations used were 1, 3, 10, 25, 50, 100, 194, 292, 500 and 1,000 mM KCl at Cambridge and 1, 3, 10, 30, 50, 100, 200, 300, 400, 500, 600, 700 and 1,000 mM KCl at East Malling. The symmetrical $Na^+$ concentrations used were 1, 3, 10, 30, 50, 100, 200, 300, 400, 500, 600, 626 and 1,000 mM NaCl. The asymmetrical $K^+$ conditions at Cambridge were: when 100 mM KCl was present on the *cis* side 1, 3, 10, 25, 50, 100, 300, 500 or 1,000 mM KCl was present on the *trans* side; when 100 mM KCl was present on the *trans* side 1, 3, 5, 10, 25, 50, 100, 280, 390, 500 or 1,000 mM KCl was present on the *cis* side. When the channel was assayed in asymmetrical $K^+$ conditions in East Malling there was 100 mM KCl on the *trans* side and 10, 50, 100, 300 or 500 mM KCl present on the *cis* side. Bi-ionic conditions had equimolar $Na^+$ (*cis*):$K^+$ (*trans*) at concentrations of 1, 3, 10, 30, 50, 100, 200, 300, 400, 500, 700 and 1,000 mM. Although ion

activities are used exclusively for all calculations, for ease of discussion the concentrations given in the text and figures are absolute concentrations unless indicated.

ABBREVIATIONS

| | |
|---|---|
| $E_{rev}$ | zero-current (reversal) potential of the channel |
| *I/V* | unitary-current/voltage |
| $K_d$ | dissociation binding constant |
| PE | 1-palmitoyl-2-oleoyl phosphatidylethanolamine |
| 3B2S | three energy-barrier, two ion-binding site |
| *A* | ionic repulsion energy |
| G1 | *cis* energy peak |
| G2 | central energy peak |
| G3 | *trans* energy peak |
| U1 | *cis* energy well |
| U2 | *trans* energy well |
| D1 | electrical distance to G1 |
| D2 | electrical distance to U1 |
| D3 | electrical distance to G2 |
| D4 | electrical distance to U2 |
| D5 | electrical distance to G3 |
| *d* | electrical distance between wells |
| $R_{scis}$ | radius of a circle containing one electron charge in the *cis* pore vestibule |
| $R_{strans}$ | radius of a circle containing one electron charge in the *trans* pore vestibule |

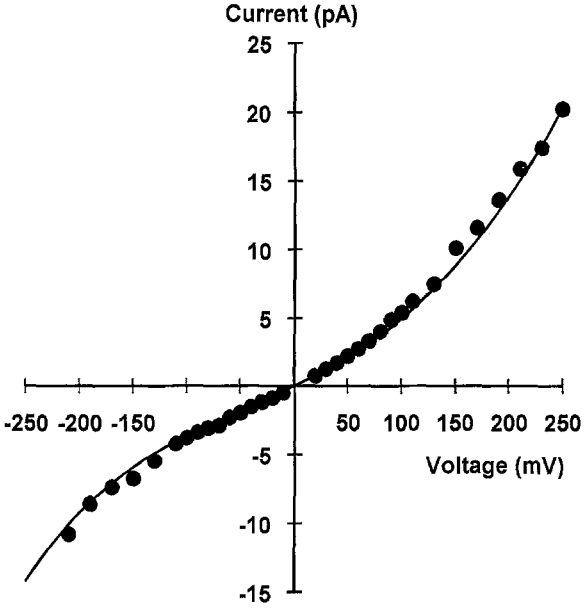



**Fig. 2.** Unitary-current/voltage relationship for the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer assayed in symmetrical 100 mM KCl. The curve is derived from a theoretical 3B2S model with the parameters shown in the Table (Cambridge).

## Results

### $K^+$ PERMEATION OF THE CHANNEL

When assayed in symmetrical KCl concentrations, the *I/V* relationship of the channel was nonohmic (Figs. 2,3; White & Tester, 1992*a,b*). The unitary-conductance was greater at more extreme positive and negative voltages and was also greater at positive voltages than at corresponding negative voltages. Both these effects were especially marked at low $K^+$ concentrations. Thus, the concentration dependencies of single channel current differed with polarity at equivalent absolute voltages (Fig. 4). Furthermore, the relationships between $K^+$ activity and both the unitary-conductance of the channel at $E_{rev}$ (White & Tester, 1992*a*) and the unitary-current of the channel at a specified voltage (Fig. 4) did not obey simple Michaelis-Menten kinetics. These observations suggest that the channel not only has an asymmetrical pore structure but also that it contains multiple binding sites for $K^+$ and can be occupied by more than one $K^+$ ion simultaneously. Indeed, the relationships between $K^+$ activity and both the unitary-conductance of the channel at $E_{rev}$ and unitary-current of the channel at a specified voltage resemble those of a two-site ion pore, for which the internal energy barrier is lower than, or equal to, the lowest entry energy barrier and within which ions repel each other (Hille & Schwarz, 1978).

On the assumption that the pore of the channel contained a minimum of two binding sites, and that the internal energy barrier was lowest, we fitted the energy profile of the channel for monovalent cations to a three energy-barrier, two binding-site (3B2S) model. Parameters were estimated by unweighted least squares. The program sometimes converged to different estimates depending upon the initial parameter values, particularly when many parameters were being estimated. Models were therefore fitted from several different starting values to try to ensure that the best possible fit was obtained. In all four data sets we obtained (Table), the height of the central peak (G2) could not be estimated with any precision because the residual sum of squares was essentially unchanged for values less than about −3.00 RT, though it increased sharply for values greater than this. The height of this peak was therefore fixed at −5.00 RT. Also, preliminary studies indicated that fits were not greatly responsive to changes in the electrical distances D1, D2 and D3 from their initial values of 0.00, 0.25 and 0.50. These parameters were therefore fixed at these values. Subsequently allowing these parameters to vary reduced the residual sum of squares by 2% in one data set and by less than 1% in the other three. However, in the absence of suitable, independent structural data for the $K^+$ channel the values for electrical distances of energy barriers and energy wells must be regarded as somewhat arbitrary.

For our initial studies we used *I/V* data reported by White and Tester (1992*a,b*) obtained under conditions where (i) $K^+$ concentrations on both sides of the channel were increased symmetrically from 1 mM to 1 M and (ii) the *cis* $K^+$ concentration was increased from 1 mM to 1 M and the *trans* $K^+$ concentration was fixed at 100 mM.

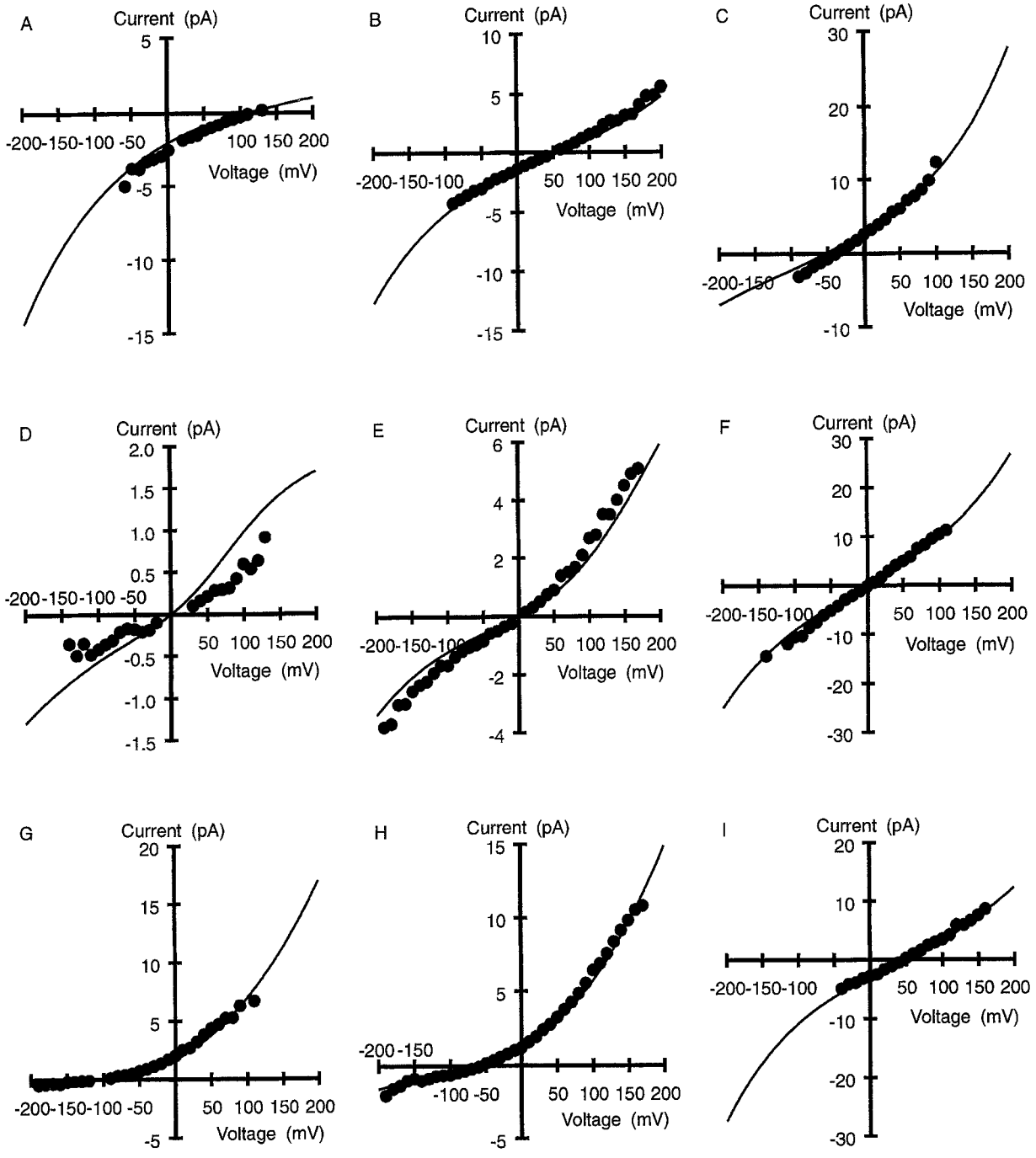
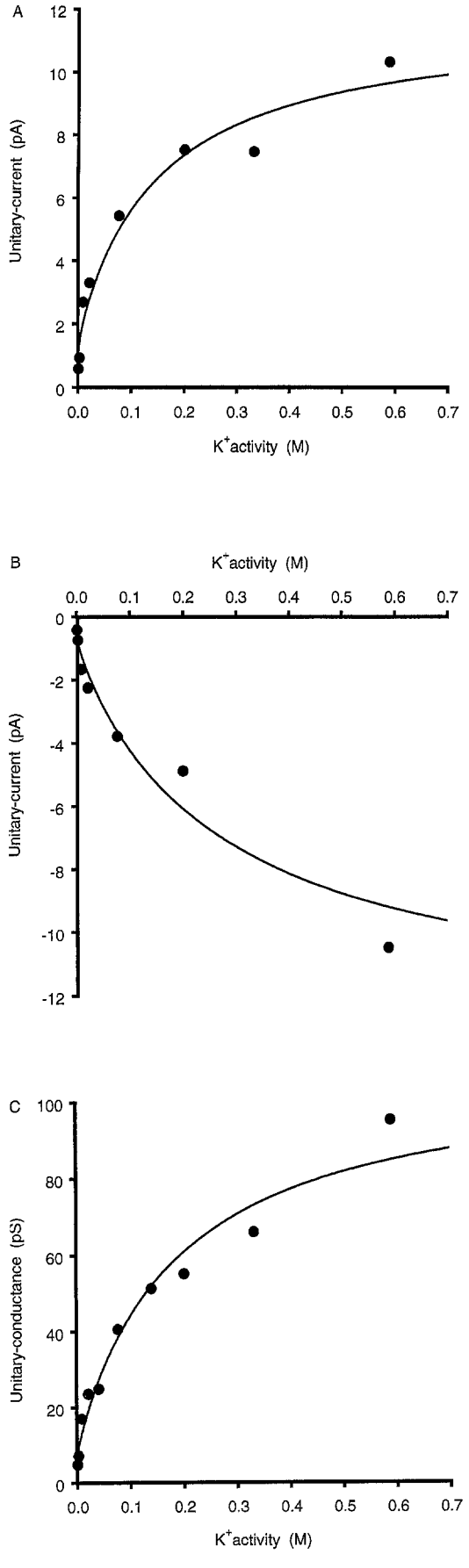



**Fig. 3.** Unitary-current/voltage relationships for the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer. Solutions contained (*cis:trans*) (*A*) 1:100, (*B*) 10:100, (*C*) 1,000:100, (*D*) 1:1, (*E*) 10:10, (*F*) 1,000:1,000, (*G*) 100:1, (*H*) 100:10 and (*I*) 100:1,000 mM KCl. The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (Cambridge).

This was supplemented by data obtained under these conditions, and at the same time, for voltages higher than ±100 mV and also for conditions in which the *trans* $K^+$ concentration was increased from 1 mM to 1 M while the *cis* $K^+$ concentration was maintained at 100 mM. The energy profile for $K^+$ permeation, channel spatial characteristics, surface potentials and ion-ion interactions within the pore were estimated by fitting the 3B2S model to all the data (Table). The generated model simulated the complex rectifying *I/V* relationships of the channel over a wide range of symmetrical and asymmetrical KCl concentrations (Figs. 2,3). The model fitted best at high KCl concentrations and worst at low KCl concentrations. In addition, the model simulated the biphasic relation-




ships between $K^+$ activity and both the unitary-conductance of the channel at $E_{rev}$ and the unitary-current of the channel at a specified voltage (Fig. 4).

In the model of the channel the *cis* energy peak (G1) was lower than the *trans* energy peak (G3) and the center energy peak (G2) was lowest by definition. Thus, maximum conductance, which is proportional to the heights of energy barriers (Hille, 1992), will be determined by the extreme energy peaks, G1 and G3. The model predicts that at $E_{rev}$ the unitary-conductance of the channel increases with increasing $K^+$ activity to a maximum of 103.6 pS at $K^+$ activities between 5.0 and 6.5 M then declines at higher $K^+$ activities. However, these must remain theoretical predictions since these $K^+$ activities exceed $K^+$ solubility. The *cis* energy-well (U1) was deeper than the *trans* energy-well (U2) leading to differences in the relationship between unitary-current and $K^+$ concentration dependent upon polarity (Fig. 4). The saturating KCl concentration is proportional to the depth of the energy well and a deeper energy well implies stronger binding and consequently a lower saturating ionic concentration (Hille, 1992). However, both the polarity of the asymmetrical *I/V* relationship and the strong rectification at high negative voltages in the presence of symmetrical 100 mM KCl (Fig. 2) were accounted for mainly by bringing the *trans* energy well (D4) and the *trans* energy peak (D5) into the electrical distance of the pore (*cf.* Levitt, 1986). Although some rectification in unitary-conductance at low cation concentrations might be generated by surface potential effects, constrained models in which energy peaks and energy wells were symmetrically placed yielded unsatisfactory fits. In the asymmetrical pore model, the vestibule contained negligible surface charge ($3.82 \cdot 10^{-5}$ e/&$^2$ *cis* and $1.22 \cdot 10^{-4}$ e/&$^2$ *trans*), a conclusion which is independently supported by the observation that the conductance of the channel was unaffected by addition of the impermeant cation $Ca^{2+}$ at concentrations up to 1 mM (White & Tester, 1992*a*).

The distinctive biphasic dependence of both unitary-conductance at $E_{rev}$ and unitary-current at a specified voltage on $K^+$ activity is generated by ion-ion repulsion within the pore, and the elevation of conductance by repulsion is more significant in pores having high terminal barriers, when exit from the pore is rate limiting (Hille & Schwarz, 1978). The energy parameter (*A*) that raises the energy of the peaks and wells adjacent to the occupied well by the factor *A/d*, was 1.22, which would effectively lower the affinity for $K^+$ binding by a factor

**Fig. 4.** The relationships between (*A*) unitary-current at +100 mV, (*B*) unitary-current at −100 mV and (*C*) unitary-conductance at $E_{rev}$ (determined between $E_{rev} \pm 30$ mV) and $K^+$ activity of the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer. The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (Cambridge).

**Table.** Estimated parameters for the 3B2S double-residency model for $K^+$ and $Na^+$ permeation of the $K^+$ channel in the plasma membrane of rye roots

| | Single ionic species | | | Bi-ionic conditions | |
|---|---|---|---|---|---|
| | Cambridge | East Malling | | East Malling | |
| | $K^+$ | $K^+$ | $Na^+$ | $K^+$ | $Na^+$ |
| G1 | 2.49 ± 0.15 | 4.43 ± 0.16 | 3.98 ± 0.26 | 1.36 ± 0.16 | 3.55 ± 0.09 |
| G2 | −5.00 | −5.00 | −5.00 | −5.00 | −5.00 |
| G3 | 4.34 ± 0.13 | 4.12 ± 0.15 | 4.65 ± 0.49 | 4.42 ± 0.12 | 4.47 ± 0.19 |
| U1 | −10.86 ± 0.21 | −8.46 ± 0.19 | −9.16 ± 0.43 | −11.48 ± 0.23 | −7.99 ± 0.39 |
| U2 | −9.06 ± 0.20 | −7.86 ± 0.18 | −7.72 ± 0.44 | −6.86 ± 0.15 | −8.39 ± 0.29 |
| A | 1.22 ± 0.07 | 1.81 ± 0.11 | 1.75 ± 0.18 | 1.85 ± 0.09 | |
| D1 | 0.00 | 0.00 | 0.00 | 0.00 | |
| D2 | 0.25 | 0.25 | 0.25 | 0.25 | |
| D3 | 0.50 | 0.50 | 0.50 | 0.50 | |
| D4 | 0.62 ± 0.01 | 0.64 ± 0.01 | 0.66 ± 0.02 | 0.60 ± 0.02 | |
| D5 | 0.86 ± 0.01 | 0.98 ± 0.02 | 0.86 ± 0.03 | 0.93 ± 0.03 | |
| $R_{scis}$ | 91.3 ± 25.0 | 42.9 ± 6.5 | 48.8 ± 9.3 | 1,000[a] | |
| $R_{strans}$ | 51.0 ± 2.8 | 1,000[a] | 96.2 ± 114.5 | 68.6 ± 15.4 | |
| $N$ | 480 | 345 | 257 | 264 | |
| SUMSQ | 174.58 | 68.89 | 15.10 | 7.47 | |

Parameters were estimated from *I/V* relationships obtained in the presence of either a single permeant cationic species or bi-ionic, equimolar $Na^+$ (*cis*):$K^+$ (*trans*). Experiments were performed either at the University of Cambridge or at H.R.I. East Malling, as indicated. Standard errors are given for parameters that were free to vary. SUMSQ is the residual sum of squares and $N$ is the number of experimental points. The solution reference state is 55.5 M.

[a]Parameter estimates are on the boundary of the parameter space.

of 27.0. Thus, if the well depth energies are converted to dissociation binding constants ($K_d$) for $K^+$ at the more conventional, 1 M solution reference state, using the expression $K_d = \exp(4.02 + \text{energy}_{well}/RT)$, then $K^+$ binding to U1 and U2 in an unoccupied channel at zero volts has $K_d$s of 1.1 and 6.5 mM which are increased to 29.0 and 175.5 mM in an occupied channel. In this context it is worth noting that in channels which hold more than one ion simultaneously, the destabilization which results from ion-ion repulsion is thought to enable ions to pass through rapidly despite high-affinity binding. At high concentrations, transport through multiply occupied pores is restricted and may decline, since the number of vacant binding sites declines and the new ones forming at the extremes of the pore are more rapidly filled by an ion in the bathing solution than by an ion crossing from the adjacent binding site (Hille & Schwarz, 1978). No self-block was observed in our experiments, probably because almost all channels were singly occupied at the KCl concentrations tested. However, the parameters of the model do predict a decline in conductance at $E_{rev}$ at (unobtainable) $K^+$ activities above 6.5 M.

These experiments were repeated at East Malling and, while the *I/V* relationships obtained for the $K^+$ channel were qualitatively similar to those obtained in Cambridge, they differed quantitatively. For example, in the presence of symmetrical 100 mM cation chloride estimates (determined between ±30 mV; mean ± SE from $n$ determinations) of unitary-conductances for $K^+$ and $Na^+$ at $E_{rev}$ were 40.2 ± 0.5 pS ($n$ = 147) and 23.6 ± 0.9 pS ($n$ = 12) in Cambridge, but 47.6 ± 0.9 pS ($n$ = 62) and 31.9 pS ($n$ = 2) in East Malling. The relationship between unitary-conductance and KCl concentration differed also (*data not shown*). The reason for these discrepancies is not known. Consequently, estimates of the energy profiles for $K^+$ permeation and ionic repulsion differed between data sets (Table).

Fitting the *I/V* curves obtained at East Malling when $K^+$ was the only permeant cation, suggested a higher *cis* energy peak and shallower energy wells (although U2 was still deeper than U1), with a lower energy difference between the external peaks and adjacent wells than data obtained at Cambridge. Later we argue that this may be an inappropriate energy profile, since it was unable to predict the observed $E_{rev}$ under bi-ionic $Na^+$ (*cis*):$K^+$ (*trans*) conditions or in mixtures of monovalent cations. In addition, a higher estimate for ion-ion interactions within the pore was obtained from the East Malling data. However, $A$ was always greater than unity, which implies that ions repel each other within the pore, and is consistent with model predictions from other pores that multiple ion occupancy increases the maximal channel unitary-conductance (Hille & Schwarz, 1978; Hille, 1992). By contrast, similar estimates of the spatial characteristics of the channel were obtained from both Cambridge and East Malling data, both indicating that the

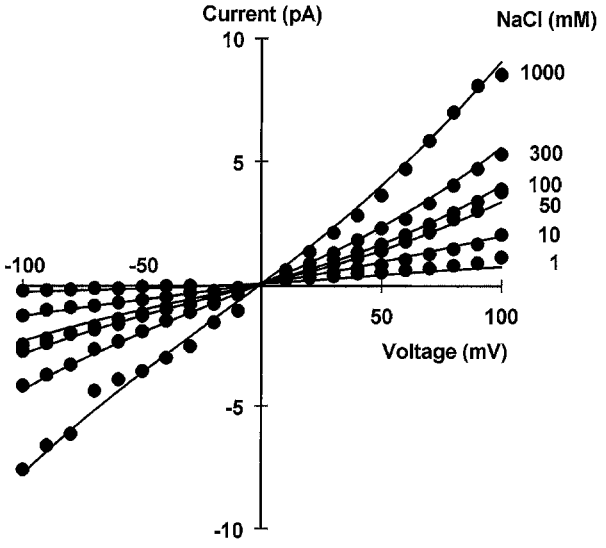



**Fig. 5.** Unitary-current/voltage relationships for the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer assayed in symmetrical 1,000, 300, 100, 50, 10 and 1 mM NaCl. The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (Single ion species, East Malling).

*trans* energy well was best positioned close to the center of the electrical distance of the pore. Finally, in both data sets values for the parameters describing surface charge, $R_{scis}$ and $R_{strans}$, were large, implying negligible surface charge and, therefore, no charged groups near the pore entrances.

### $Na^+$ Permeation of the Channel

When assayed in symmetrical NaCl concentrations the channel exhibited analogous rectification in unitary-conductance (Fig. 5) and biphasic relationships between $Na^+$ activity and both the unitary-conductance of the channel at $E_{rev}$ and the unitary-current of the channel at a specified voltage (Fig. 6) to those observed when $K^+$ was the permeant cation. After fitting the *I/V* curves obtained under symmetrical NaCl concentration to a 3B2S model, it simulated these phenomena.

### Dependence of Channel Selectivity on Monovalent Ion Concentration

When equimolar concentrations of $Na^+$ (*cis*) and $K^+$ (*trans*) surrounded the channel, $E_{rev}$ (and therefore the

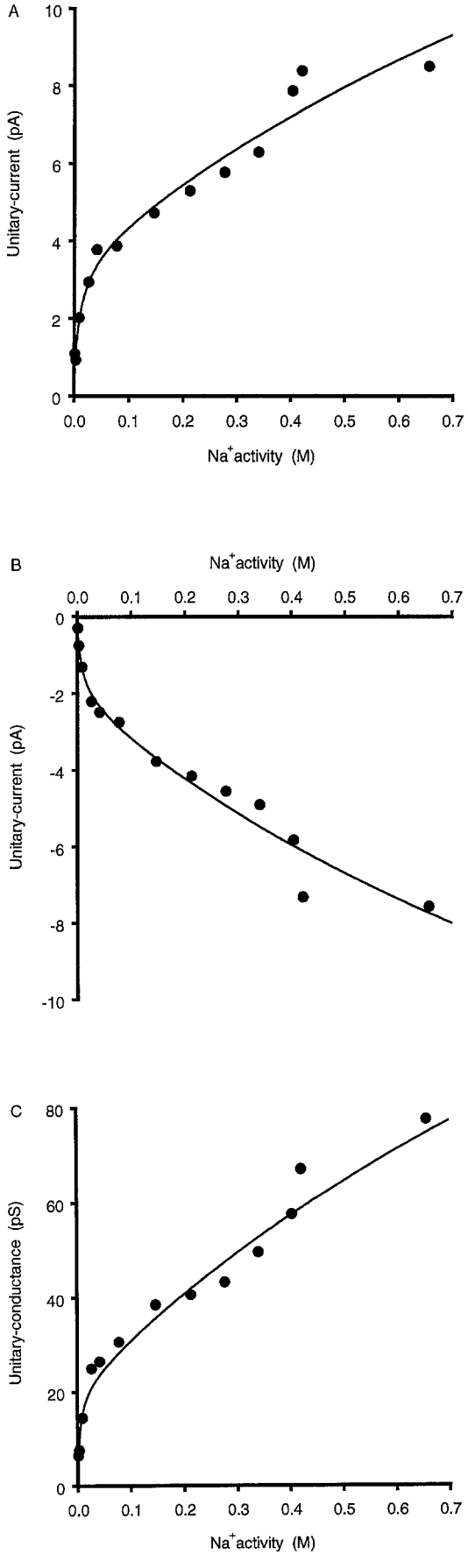



**Fig. 6.** The relationships between (*A*) unitary-current at +100 mV, (*B*) unitary-current at −100 mV and (*C*) unitary-conductance at $E_{rev}$ (determined between $E_{rev} \pm 30$ mV) and $Na^+$ activity of the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer. The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (Single ion species, East Malling).

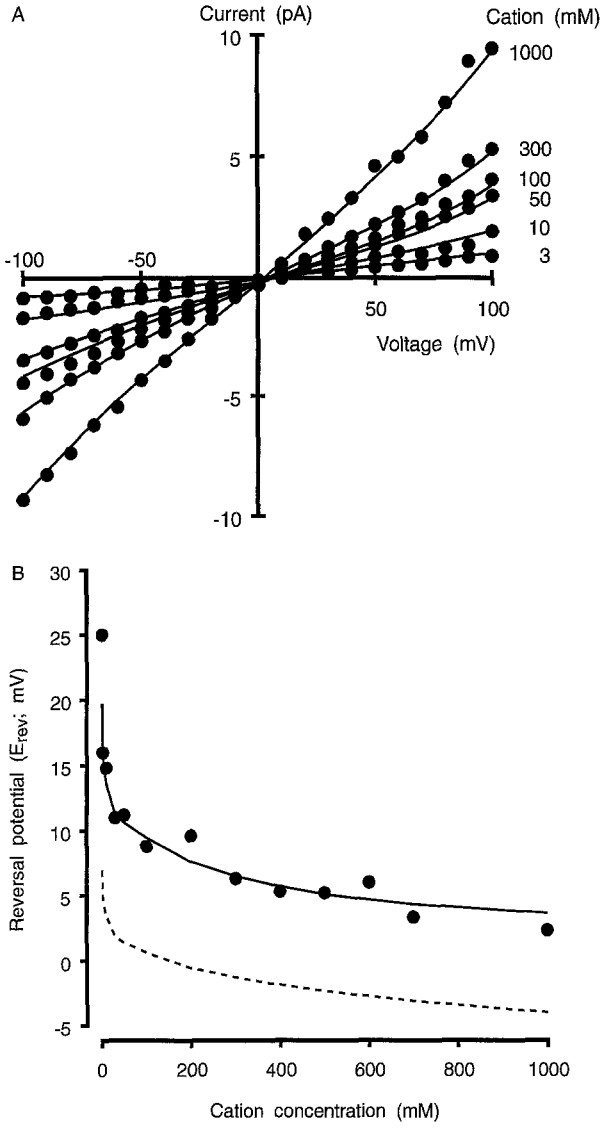



**Fig. 7.** (A) Unitary-current/voltage relationships for the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer assayed in equimolar NaCl (*cis*):KCl (*trans*) at concentrations of 1,000, 300, 100, 50, 10 and 3 mM. (*B*) The relationship between $E_{rev}$ and monovalent ion concentration for the $K^+$ channel from the plasma membrane of rye roots incorporated into a planar PE bilayer assayed in equimolar NaCl (*cis*):KCl (*trans*). The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (East Malling). The continuous lines used parameters derived from bi-ionic conditions and the broken line used parameters derived from experiments using single ionic species.

relative permeability of the channel to $K^+$ and $Na^+$) depended upon the total monovalent ion concentration present (Fig. 7*B*). At low monovalent ion concentrations the channel was appreciably more permeable to $K^+$ than to $Na^+$. However, as the monovalent ion concentration was increased, the permeability ratio $P_K/P_{Na}$ declined, from 2.68 at 1 mM ($E_{rev}$ = 25.0 mV) to 1.19 at 1 M ($E_{rev}$ = 2.3 mV). This phenomenon is considered to be diagnostic for a pore structure which can be occupied by more than one ion simultaneously, where the presence of one ion within the pore can influence the permeation of another via electrostatic and/or allosteric interactions (Hille & Schwarz, 1978; Hille, 1992), although we note, for completeness, that other mechanisms could account for this phenomenon, such as conformational changes in the channel protein induced by ion binding to a modulatory site or even a pore-binding site.

Initially, energy profiles for monovalent cations and spatial parameters of the channel derived from experiments performed at East Malling in the presence of a single cationic species (Table) were used to predict $E_{rev}$ in the presence of equimolar $Na^+$ (*cis*) and $K^+$ (*trans*) over the concentration range 1 mM to 1 M (Fig. 7*B*). Although these parameter estimates predicted a decline in $E_{rev}$ (and, therefore, a decline in $P_K/P_{Na}$), the predicted values of $E_{rev}$ were consistently lower than those determined empirically. Further estimates of the energy profiles for monovalent cations and spatial parameters of the channel were obtained, therefore, from experiments performed under bi-ionic conditions (Table). These accurately simulated both the *I/V* relationships obtained under bi-ionic, equimolar $Na^+$ (*cis*):$K^+$ (*trans*) conditions (Fig. 7*A*) as well as the relationship between $E_{rev}$ and monovalent ion concentration, except at low concentrations (1 mM; Fig. 7*B*).

Fitting data obtained under bi-ionic conditions to the 3B2S model yielded similar conclusions about the spatial characteristics of the pore as the experiments performed in the presence of a single ionic species, namely that the *trans* energy well was best placed at an electrical distance close to the center of the pore, while the *trans* energy peak was close to the extreme electrical distance (Table). The *cis* energy peak was higher for $Na^+$ than for $K^+$, but the *trans* energy peak was similar for both monovalent cations. The *cis* energy well was substantially deeper for $K^+$ than $Na^+$, but the *trans* energy well was apparently shallower. Although the height of the central energy peak was fixed at the same value for both $K^+$ and $Na^+$ energy profiles, this is consistent with the observation that $E_{rev}$ approaches zero at high monovalent ion concentrations. The value obtained for the ion-ion interaction parameter (*A*) was consistent with the values obtained in experiments using single ionic species. It is physically meaningful to obtain a constant value for *A* for both $K^+$ and $Na^+$ if we assume that ion-ion interactions are due to electrostatic repulsion between ions of the same valence and distance of separation. Once again, there was no indication of any substantial surface charge in the vestibules to the pore.

The contrasting $K^+$ and $Na^+$ energy profiles are consistent with theoretical treatments concerning selectivity in 3B2S channels (Hille & Schwarz, 1978). Theoretically, at low ion concentrations $E_{rev}$ depends upon all high energy barriers in the energy profiles for $K^+$ and $Na^+$, which in our case are the extreme barriers. Thus, since $E_{rev}$ is positive at low ionic concentrations (Fig. 7*B*), the

external energy-barriers for $K^+$ are expected to be lower than for $Na^+$ (Table). In the range of medium ionic activities $E_{rev}$ is influenced both by the heights of the energy barriers and by the depths of the energy wells. The general decline in $E_{rev}$ to a value approaching zero indicates that although the external barriers and/or energy wells differ between $K^+$ and $Na^+$, the central barrier is similar for both $K^+$ and $Na^+$ since at high activity $E_{rev}$ depends only upon the electrochemical activity ratio and on the difference between the central energy barriers.

## Anomalous Mole Fraction Phenomena

A further test of a multiple ion-residency pore is the possession of "anomalous mole-fraction" phenomena (Hille, 1992). This term refers to the observation of a minimum in the relationship between unitary-conductance or $E_{rev}$ and the mole fraction of two permeant test ions held at a constant total ionic strength. In a pore which can be occupied by a single ion only, the conductance and $E_{rev}$ of the channel should increase monotonically as the mole fraction of the less permeant species is increased. However, in a pore which can be occupied by more than one ion simultaneously, conductance and $E_{rev}$ may exhibit a minimum upon increasing the mole fraction of the less permeant ion. These anomalous effects indicate interactions between permeating ions within the pore, and, therefore, are evidence for a multiple ion-residency pore.

When 100 mM KCl was present on the *trans* side and the mole fraction of $Na^+$ was changed relative to $K^+$ at a total monovalent ion concentration of 100 mM on the *cis* side, $E_{rev}$ increased monotonically with no hint of a minimum (Fig. 8*A*). This was equally true when the total monovalent cation (*cis*) and KCl (*trans*) were 300 mM (Fig. 8*B*). Using the values obtained under bi-ionic conditions for the spatial parameters of the channel and the energy profiles for the permeation of monovalent cations (Table), we calculated $E_{rev}$ under the conditions used to search for anomalous mole fraction effects. The predicted changes in $E_{rev}$ upon increasing the mole fraction of $Na^+$ relative to $K^+$ at total monovalent ion concentrations of both 100 and 300 mM approximated the emprical data, but revealed additional complexities (Fig. 8). At a total monovalent cation concentration of 100 mM a monotonic increase in $E_{rev}$ was both predicted and observed. However, at a total monovalent cation concentration of 300 mM a complex relationship, without a discrete minimum, between $E_{rev}$ and the mole fraction of $Na^+$ was predicted, which was not explicit in the experimental values. Changes in $E_{rev}$, as the mole fraction of $Na^+$ was altered, were fitted less well using the parameters derived from experiments in which a single ionic species was present (*data not shown*). This appears to be

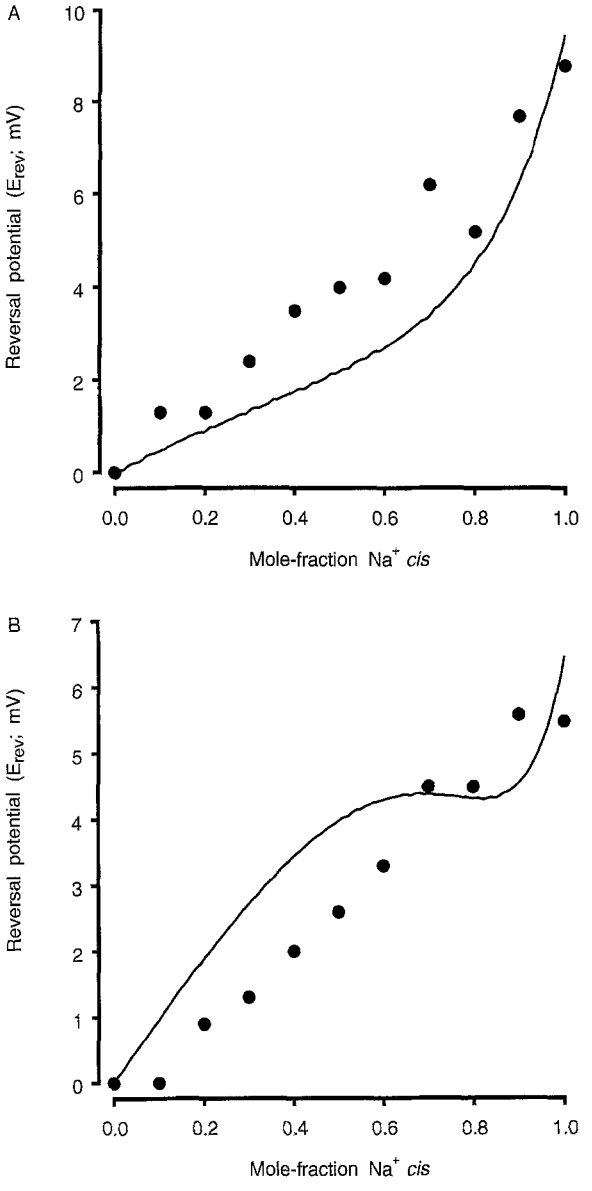



**Fig. 8.** Relationships between $E_{rev}$ and the mole fraction of $Na^+$ ($[Na^+]/[Na^+] + [K^+]$) in the *cis* compartment at total monovalent ion concentrations of (*A*) 100 mM and (*B*) 300 mM. Equimolar KCl concentrations were present in the *trans* compartment. Data are from characteristic experiments. The curves are derived from a theoretical 3B2S model with the parameters shown in the Table (Bi-ionic conditions).

a consequence of inappropriate estimates of the $K^+$ energy profile obtained in the presence of $K^+$ alone.

It is noteworthy that, although the $K^+$ channel in the plasma membrane of rye roots has a multiple ion-residency pore, it does not exhibit an anomalous mole fraction effect under the conditions used here. Indeed, in 3B2S models anomalous minima are predicted only when the energy profile for at least one of the ions has high energy barriers at both ends of the channel (Hille & Schwarz, 1978). This special requirement is apparently not exhibited by $K^+$ or $Na^+$ in the $K^+$ channel we have

studied. Thus, the absence of anomalous mole fraction effects does not preclude the existence of a multiple ion-residency pore structure.

## Discussion

### Physiological Implications of Concentration-dependent Selectivity

The $K^+$ channel studied in this paper resides in the plasma membrane of rye roots (White & Tester, 1992*a,b*), where its physiological role is determined by its tissue location, selectivity and voltage dependence. The physiological role of the $K^+$ channel studied here is equivocal since its tissue location and voltage dependence in vivo are unknown. When studied in planar PE bilayers the channel has a voltage-independent open probability ($P_o$) of about 0.8 (White & Tester, 1992*a,b*), but this is unlikely to be the case in vivo, and the mechanisms which regulate the gating of the channel are unknown. However, if we are to assume that the channel transports monovalent cations into the root cell, its cation selectivity between $K^+$ and $Na^+$ will be of physiological consequence. Although $Na^+$ can be used as a vacuolar osmoticum in plant cells, the cytoplasmic concentration of $Na^+$ must be minimized since it is antagonistic to enzymic reactions which require $K^+$ and is therefore cytotoxic. In temperate regions, the $Na^+$ concentration of soil solutions (0.1 to 1.0 mM) is similar to their $K^+$ concentration (Marschner, 1986). Under these low monovalent cation concentrations, the channel shows a high selectivity for $K^+$ over $Na^+$ and the plant will maintain appropriate tissue concentrations of $K^+$ and $Na^+$. However, in tropical soils, where $Na^+$ concentrations may reach 50 to 100 mM, the selectivity of the channel will favor excessive $Na^+$ entry into the root cell. In this environment, the activity of the channel must be restricted by either genetic or biochemical mechanisms, and/or plants must possess efficient $Na^+$ efflux mechanisms ($Na^+$-ATPase or $H^+/Na^+$ antiport) to reduce the cytoplasmic $Na^+$ loading (Jeschke, 1984; Haro et al., 1993). It would be instructive, therefore, to investigate how the activity of this channel is regulated in cultivars of rye that have contrasting salt tolerance. In this context it can be noted that although there was no difference in the cation selectivity of outward-rectifying $K^+$ channels between protoplasts from root cells of either salt-tolerant or salt-sensitive wheat genotypes (Schachtman, Tyerman & Terry, 1991) or between protoplasts from salt-unadapted or salt-adapted suspension-cultured tobacco cells (Murata et al., 1994*a,b*), the permeability of the plasma membrane to both $K^+$ and $Na^+$ was reduced in protoplasts of cells adapted to salinity.

### Structure of the Channel Pore

Several lines of evidence suggest that the pore structure of the $K^+$ channel in the plasma membrane of rye roots has multiple ion-binding sites and can be occupied by more than one monovalent ion simultaneously. The channel does not have simple Michaelis-Menten kinetics, but exhibits biphasic relationships with increasing cation concentrations in both unitary-conductance at $E_{rev}$ and in the unitary-current at a specified voltage (Figs. 4,6; White & Tester, 1992*a*). In addition, $E_{rev}$ in the presence of equimolar $Na^+$ (*cis*):$K^+$ (*trans*) declines as monovalent ion concentration is increased (Fig. 7). These relationships can be simulated by ion permeation models which are composed of three energy barriers and two ion-binding sites in which the central energy barrier is low. Cations move in single file through the pore and up to two cations, which repel each other, can reside in the pore simultaneously (Hille & Schwarz, 1978). The permeation of $K^+$ through most $K^+$ channels of animal origin similarly involves the single file movement through a pore that can be occupied simultaneously by more than one ion (Hille & Schwarz, 1978; Yellen, 1987; Hille, 1992; Pongs, 1993), and this scheme has been proposed for several $K^+$ channels of plant membranes (Tester, 1990).

We have estimated the energy profiles for $K^+$ and $Na^+$ permeation in the plasma membrane $K^+$ channel of rye roots from experiments performed under a variety of conditions (Table). The results suggest that the energy peaks and energy wells are situated asymmetrically within the electrical distance of the pore. In particular, the *trans* energy well occurs at an electrical distance close to the center of the pore, while the *trans* energy peak remains close to the extreme electrical distance. For $K^+$ the central energy peak was lowest, and the *cis* energy peak appeared to be lower than the *trans* energy peak. Also for $K^+$ the *cis* energy well was deeper than the *trans* energy well. The *cis* energy peak was lower for $K^+$ than for $Na^+$, but the *trans* energy peak was similar for both monovalent cations. The *cis* energy well appeared to be deeper for $K^+$ than $Na^+$, but the relative heights of the *trans* energy wells were ambiguous. The central energy peak was lower than the external energy peaks, and similar for both $K^+$ and $Na^+$. Cations repelled each other within the pore, and the energy parameter ($A$) that raises the energy of the barriers and wells adjacent to an occupied well by the factor $A/d$ ranged from 1.22 to 1.85 and $d$, the electrical distance between wells, ranged from 0.35 to 0.41. Thus, the energy of the unoccupied binding site in an occupied pore would be increased by between 3.30 to 5.29 RT units, and its $K_d$ by 27- to 198-fold. There was no indication of any substantial surface charge in the vestibules to the pore.

In thermodynamic terms, the selectivity of the $K^+$ channel will be determined by the balance between the

energy of removing water from the cation and the energy of allowing the partially dehydrated cation to interact with stabilizing charges and dipoles of the selectivity filter (Hille, 1992). The external energy barriers can be equated with the dehydration of a cation as it enters the selectivity filter and the energy wells with binding sites stabilizing the dehydrated cation. In structural terms the cation binding sites, and therefore the cation selectivity of the channel, may be determined by ionic interactions with either electronegative oxygen ligands projecting into the transmembrane pore, which are fixed in a rigid geometry to mimic the waters of ionic hydration (Hille, 1992), or the $\pi$ electrons at the face of aromatic side chains such as Phe, Tyr and Trp (Heginbotham & MacKinnon, 1992; Kumpf & Dougherty, 1993; Miller, 1993).

### Parameter Estimates

During our studies it was observed that parameter estimates of pore structures and energy profiles were somewhat dependent upon the range of cation concentrations and voltages tested. A truly representative fit can only be achieved using a wide range of cation concentrations to determine the depth of energy wells and heights of energy peaks, and extreme voltages (±200 mV) to distinguish structural parameters and surface potential effects on cation permeation. This has also been noted in other, similar modeling studies (Ravindran et al., 1992; Naranju & Latorre, 1993). However, with some modifications the AJUSTE program could be used to investigate how standard errors of parameter estimates in the model depend on the cation concentrations and voltages for which the data are available as well as on the true values of parameters. This would provide a valuable tool in planning future experiments.

### Perspectives

Refinements to the model developed in this paper will require further electrophysiological experiments combined with the techniques of protein biochemistry and molecular biology. For example, impermeant ion channel blockers could be used to determine the location of ion-binding sites and the use of specific protein-modifying reagents may reveal the chemical groups involved in permeation. When the amino acid sequence of this channel is known, the analysis of single channel electrical recordings can be used in combination with molecular genetic approaches, such as site-directed mutagenesis or the construction of chimeric genes, to guide molecular models in converting the amino acid sequence of the channel protein into the functional regions of the channel.

We thank Prof. O. Alvarez (Universidad de Chile, Santiago, Chile) for supplying the computer program AJUSTE and Prof. D. Sanders (University of York, UK), Prof. D. Gradmann and Dr. G. Thiel (University of Göttingen, Germany) for stimulating ideas. This work was supported by the Agricultural and Food Research Council.

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