

H⁺ and K⁺ Electrogenic Exchanges in Corn Roots

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ABSTRACT

The membrane potential difference, the net H⁺ exchange rate, the K⁺ net flux, and the K⁺ (⁸⁶Rb⁺) influx were measured in excised corn roots as functions of the K⁺ concentration in the medium at various pH values, in the presence of poorly permeant anions. The roots behaved as a K⁺/H⁺ exchange system. By comparing the results in normal or hypoxic conditions, or in the presence of vanadate, it was possible to distinguish the active components of membrane potential and transports from the passive ones. The magnitude of the electrogenic potential was not related to the active H⁺ extrusion rate. At pH 6, the variations of the electrogenic potential resulted from variations of the stoichiometry of the active H⁺/K⁺ exchange. The same relationship between this stoichiometry and the K⁺ concentration was observed in conditions ensuring different membrane polarizations (pH 6, pH 4, or pH 6 with fusicoccin). Both metabolic and Mg-ATPase specific inhibitors stopped the active H⁺ transport and the net K⁺ influx. Nevertheless, the tracer influx in the presence of vanadate remained higher than the passive influx calculated from the permeability coefficient determined in hypoxia. It is proposed that vanadate uncouples the K⁺ moiety of the H⁺/K⁺ antiport and allows it to mediate isotopic exchanges.

It is widely accepted that plant tissues are endowed with transport systems which extrude H⁺, hyperpolarize the membrane, and ensure the energetic coupling of K⁺ uptake (12). These systems are probably driven by a vanadate sensitive Mg-ATPase at the plasma membrane level, as has been indicated using biochemical (21), and physiological approaches (8, 13, 17, 19). The coupling between K⁺ and H⁺ transports may be either direct or indirect. Direct coupling has been proposed, based on indications of a net uphill K⁺ transport in some conditions (5, 7). Indirect coupling or electrically driven K⁺ uniport, was suggested by the finding that the stimulation of H⁺ extrusion by the fungal toxin FC¹ needed the presence of an electrical shunt, which possibly could be obtained by other means than a K⁺ influx (1, 2, 15).

The electrogenic effect of a proton pump may, in theory, be regulated via the activity of the pump itself, or via the control of the K⁺ shunt (20). One approach to the question of the H⁺/K⁺ coupling in corn roots is the analysis of the effect of K⁺ on the

electrogenic component of the membrane potential of corn roots. On the basis of a mathematical analysis of the effects of K⁺ concentration on K⁺ influx and membrane potential, Cheeseman *et al.* (3, 4, 6) concluded that the concentration dependent hyperpolarization results from a progressive replacement of an active H⁺-K⁺ antiport with variable stoichiometry by an active H⁺ uniport associated with a passive K⁺ uniport, *i.e.*, that control is via the rates of H⁺ and K⁺ pumping. This conclusion implies that part of the K⁺ influx is active on thermodynamic grounds, as indicated by Cheeseman and Hanson (5) for the 9 to 17 μM K⁺ concentration range. It also implies that the active K⁺ channel would close at high K⁺ concentration, since it does not seem to mediate the exergonic influx.

The increase of the K⁺ concentration or the membrane depolarization may be the signal which activates the control of the electrogenicity. Cheeseman *et al.* (4) assumed a direct kinetic control by the K⁺ concentration. On another hand, the evidences for indirect (electrical) coupling between H⁺ and K⁺ transports (1, 2, 15) favors the hypothesis of an electrical signal (13).

In this paper, we reconsider the nature of the system which controls the electrogenicity, by comparing the active H⁺ and K⁺ transports. The nature of the signal which activates this system is also studied by measuring K⁺-H⁺ exchanges in various ionic conditions, which allow a distinction to be made between the two putative signals. We also present evidence for maintenance of the K⁺ channel in an open state at high K⁺ concentration when vanadate is inhibiting the H⁺ transport function and the electrogenicity of the pump.

MATERIAL AND METHODS

Material Preparation. Maize (*Zea mays* L., cv INRA 508) seedlings were surface sterilized for 30 min in 3% Ca hypochloride, then germinated between paper sheets moistened with deionized H₂O and grown on air-bubbled 0.2 mM CaSO₄ for 2 d. All these operations were carried out at 25°C in the dark. Excised roots were rinsed with deionized H₂O for 5 min and then washed for 1.5 h in air-bubbled 0.2 mM CaSO₄ and rinsed again for 5 min in deionized H₂O before use. It was verified that this washing allowed for full recovery of H⁺ and K⁺ transport capacities.

Membrane Potential. Cell potentials (E_m) were measured in the 20 to 25 mm region from the apex by inserting a glass micropipet in a cell of one of the three first layers of cortical cells. The external medium was continuously renewed. The pH of the flowing solution was monitored using a combined glass pH electrode located downstream in the perfusion chamber. Vanadate was added in media when indicated, as the Na⁺ salt. Media saturated with CO were contained in closed medical perfusion flasks, each connected to a rubber blister, initially filled with CO. It was progressively emptied during percolation. In this way CO was maintained at atmospheric pressure in the flasks over the media. A cover, sealed with vacuum grease and pierced with a hole for the micropipet, was set on the flowing chamber

¹ Abbreviations: FC, fusicoccin; E_m, measured membrane potential; E_{hyp}, calculated electrogenic component of E_m; P, permeability coefficients; J^{net}, net flux; Jⁱⁿ, tracer unidirectional influx; J^{act}, J^{pass}, active, respectively passive, components of the unidirectional influx; BTP, 1,3-bis(tris(hydroxymethyl)-methylamino)propane; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; KIDA, potassium iminodiacetate; PD, electropotential difference.

over the root. This procedure made it possible to obtain, in the dark, a rapid and stable depolarization without adding any chemical to the experimental medium. The external K^+ concentration was progressively increased from the lowest concentration to the highest, each bathing the material for a 10 min period. This time interval allowed stable potentials to be recorded. When studying the effect of vanadate, the pre-equilibration period was 5 min. Only five K^+ concentrations were used in each run, so that its total duration did not exceed 50 min. It was verified that the results for a given K^+ concentration did not depend on the concentration used for the previous measurement.

Potassium ($^{86}Rb^+$) Influx. $^{86}Rb^+$ was used as a tracer for K^+ influx determination in the 2.5 μM to 50 mM range. Samples (approximately 1 g fresh weight) of excised roots were incubated for 8 min with the tracer and then rinsed four times in 200 ml of ice-cold 2 mM $CaSO_4$ (15 s then 45, 90 and 150 s). Counting (Cerenkov effect) was done with a liquid scintillation counter (Packard). The labeling was such that there were about 2 MBq in the sample. The $^{86}Rb^+$ influx experiments were performed with the same medium sequences that were used in E_m measurements. During the $^{86}Rb^+$ influx experiments, the media were bubbled with air or with CO_2 . In the latter case, the experiments were carried out in the dark and the media were contained in closed flasks fitted with inlet and outlet gas circuitry. The tracer was injected through the rubber cover of the flask 2 min after the material had been introduced in the CO_2 -saturated medium.

Net H^+ and K^+ Exchange Rates. Net H^+ fluxes were measured at constant pH using a pH-stat device (Metrohm) (9). Batches of 30 roots each, were mounted in devices ensuring that the xylem exudate could not flow back to the medium. Double junction reference electrodes were used to avoid KCl contamination of the media. Either 10 mM KOH or $Ca(OH)_2$ or H_2SO_4 was used to maintain a constant pH. In these experiments it was not possible to use a CO_2 bubbling; N_2 bubbling which acted over a few min period was substituted. The K^+ concentration in the medium was periodically assayed by flame photometry. The net K^+ flux was calculated from the variations of the K^+ concentration in the medium, corrected for the additions of KOH when this base was used.

Vanadate. A 50 mM stock solution (pH 8) was prepared at room temperature with Na meta-vanadate (Merck).

RESULTS

Effects of K^+ Concentration and pH on E_m . The effects of K^+ concentration and of pH on E_m in aerated media and in media saturated with CO_2 are shown in Figures 1 and 2A (no Ca^{2+} in the media). Figure 3A shows the results obtained at pH 6 in the presence of Ca^{2+} . The hyperpolarizing effect E_{hyp} of the proton pump was estimated from the magnitude of the depolarization induced by CO_2 . It first decreased as the K^+ concentration increased from 2.5 to 25 μM , and then increased in the 25 μM to 25 mM range. The same behavior was observed using 100 μM (Fig. 2B) or 200 μM (Fig. 3B) orthovanadate in place of CO_2 , or using KIDA in place of K_2SO_4 (data not shown). In the presence of Ca^{2+} (Fig. 3B), E_{hyp} reached larger values at high K^+ concentrations than it did in the absence of Ca^{2+} (Fig. 1). In both cases, it attenuated the membrane depolarization induced by high K^+ concentrations.

E_{hyp} increased with pH (Fig. 2B). At pH 4, it was nearly insensitive to the K^+ concentration up to 10 mM.

Potassium Fluxes. The effect of the K^+ concentration on $^{86}Rb^+$ influx at pH 4, pH 6, and pH 8 are shown in Figure 4A. The observed kinetics are typical of the so-called dual mechanism (10). In media saturated with CO_2 , mechanism I was absent (Fig. 5A). These results are similar to the ones obtained by Cheeseman and Hanson (4) at pH 6 with FCCP or anoxia. The kinetics at pH 6 and pH 8 were the same, both in aerated media and in the

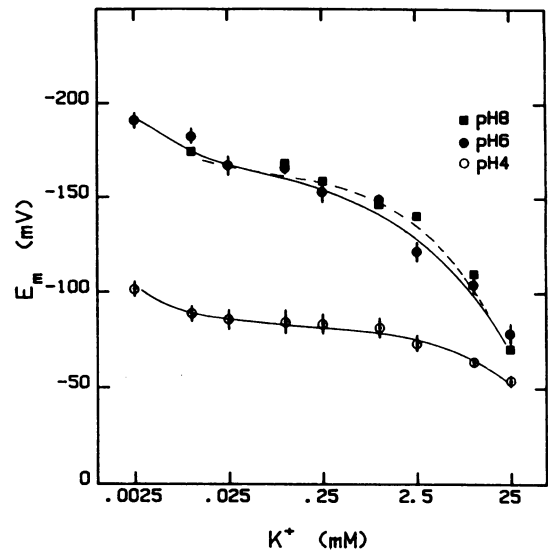


FIG. 1. Effect of the K^+ concentration in the medium on the membrane PD at various pH values. The media contained K_2SO_4 , and 2 mM BTP/ H_2SO_4 (pH 8), or 2 mM Mes/Tris (pH 6). The curves are polynomial adjustments by least square fitting. The bars are the SE (95%).

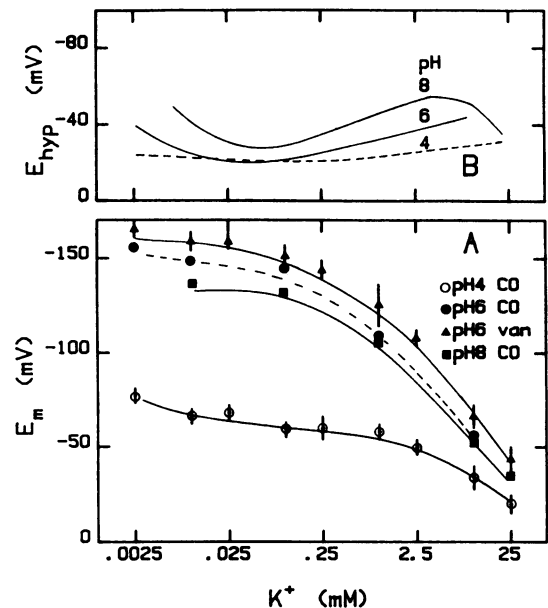


FIG. 2. Effect of the K^+ concentration in the medium on the membrane PD at various pH values in the presence of inhibitors. Same media as in Figure 1. A, Values of E_m in the presence of 100 μM vanadate (van) or in media saturated with CO_2 ; B, E_{hyp} calculated from the difference of the curves in Figures 1 and 2A. The curves are polynomial adjustments by least square fitting. The bars are the SE (95%).

presence of CO_2 . The influxes were lowered at pH 4. Vanadate (100 μM) at pH 6 was ineffective in inhibiting the influx at low K^+ concentrations. Similar results were obtained at pH 6 in the presence of Ca^{2+} (Fig. 6A). The K^+ net fluxes were measured at pH 6 and various K^+ concentrations, in aerated media, with or without 100 μM vanadate, and in media bubbled with N_2 , as bubbling with CO_2 was not possible in these experiments. It was verified that N_2 gave the same results as CO_2 in $^{86}Rb^+$ influx and membrane potential measurements (in which CO_2 was preferentially used because its effect was quicker than that of N_2). In aerated medium, and in the absence of vanadate, there was a net influx in the low concentration range, whose kinetics is shown

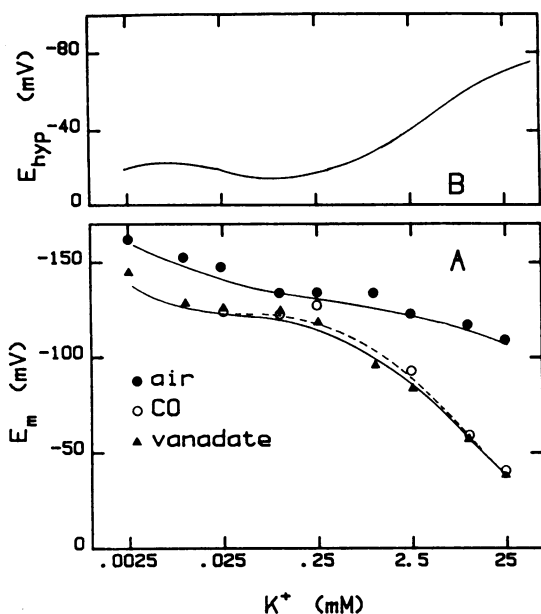


FIG. 3. Effect of the K⁺ concentration in the medium on the membrane PD at pH 6 in the presence of calcium. The media contained K₂SO₄, 2 mM Mes/Tris and 0.2 mM CaSO₄. A, Values of E_m in normal conditions or in the presence of 200 μM vanadate (van) or in media saturated with CO; B, E_{hyp} calculated from the difference of the data obtained in the absence and in the presence of 200 μM vanadate (part A). The curves are polynomial adjustments by least square fitting.

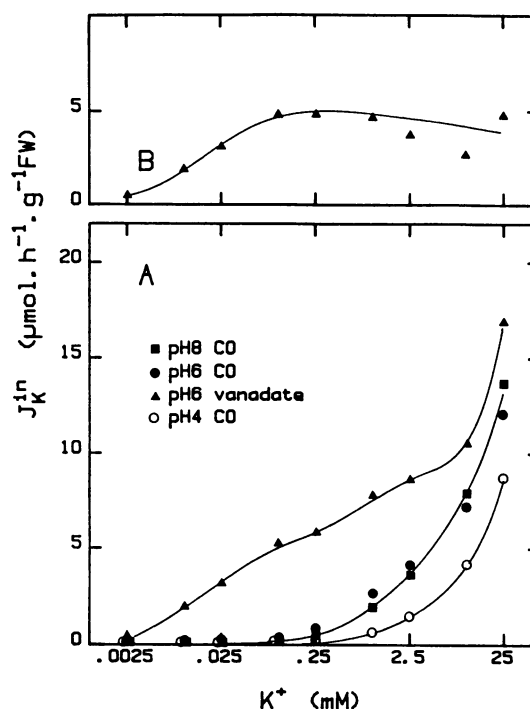


FIG. 5. Effect of the pH on the K⁺ influx kinetics in the presence of inhibitors. A, ⁸⁶Rb⁺ influx measured in the conditions of Figure 2A; B, vanadate insensitive, CO sensitive component of the influx. It was calculated by correcting the influx (vanadate pH 6, part A) for the diffusive, passive influx. The latter was calculated from the Goldman relation applied to the data of part A and Figure 2A (vanadate and CO treatments at pH 6) (see text). The curves are polynomial adjustments by least square fitting.

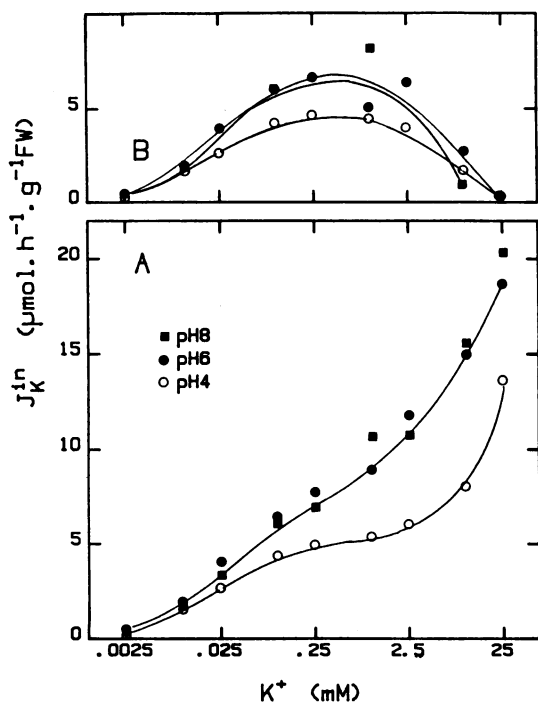


FIG. 4. Effect of pH on the K⁺ influx kinetics. A, ⁸⁶Rb⁺ influx measured in the conditions of Figure 1; B, active component of the influx, obtained by correcting the data of part A for the passive influx. The latter was calculated from the Goldman relation applied to the data of Figures 2A and 5A (CO treatments) (see text). The curves are polynomial adjustments by least square fitting.

on Figure 7. Unidirectional efflux was estimated by subtracting the net K⁺ flux from the K⁺ (⁸⁶Rb⁺) influx. It was negligible in the low concentration range, and increased less with the K⁺ concentration than did the influx. The net influx was totally suppressed by hypoxia as well as by vanadate; thus, the efflux was equal to the influx. In the first case, this corresponded to very low exchange rates across the membrane. By contrast, the low net flux in the presence of vanadate corresponded to relatively high exchange rates (Figs. 5A and 6A).

Proton Exchanges. The H⁺ net exchange rates were measured at pH 4, 6, and 8, with 0.1 mM K⁺ or 10 mM K⁺ (K₂SO₄). A net H⁺ extrusion was observed in all these conditions (Table I). It was not dependent on the K⁺ concentration, nor on the presence of Ca²⁺ (at pH 6), but was greatly increased with pH. Adding 100 μM vanadate resulted in a decrease of the rate of H⁺ appearance in the medium, within 2 min or less, leading to an apparent H⁺ net influx at pH 4. This influx attained 5 μmol·h⁻¹·g⁻¹ fresh weight in 0.1 mM K⁺ and 2.5 μmol·h⁻¹·g⁻¹ fresh weight in 10 mM K⁺ (these values refer to the steady state observed for 30 min or more). Similar results were obtained using N₂ bubbling in place of vanadate. It was verified that vanadate did not inhibit the respiration rate (O₂ consumption measured with a Clark electrode in the presence of 200 μM vanadate was 96% of the control). Control experiments without roots proved that the observed effects of vanadate and N₂ were not due to artefactual reactions of the pH electrode. The magnitude of the vanadate or N₂ effects on the net H⁺ transport, was used as an estimation of the true H⁺ extrusion rate by the pump, which appeared to be far less sensitive to the pH than was the net H⁺ transport (Table I). Since the H⁺ net transport was virtually zero in the presence of vanadate at pH 6, the rate of H⁺ appearance in the medium could be directly used as an estimation of the pump activity at

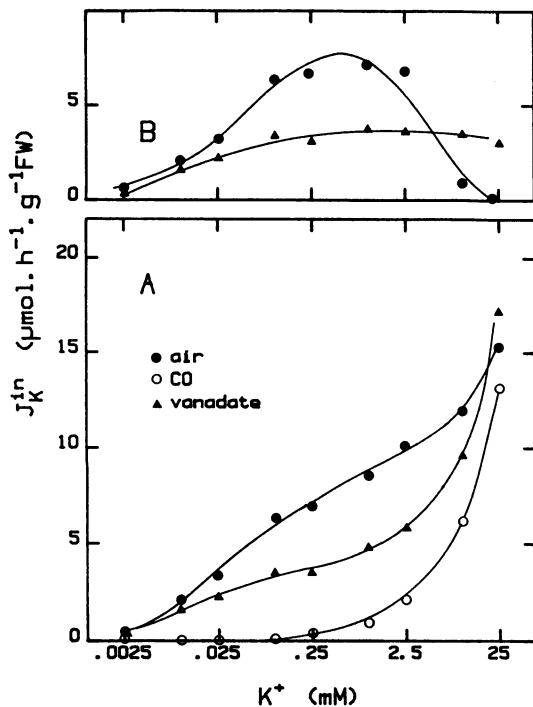


FIG. 6. K^+ influx kinetics at pH 6 in the presence of calcium, in normal conditions or in the presence of inhibitors. A, $^{86}Rb^+$ influx measured in the conditions of Figure 3; B, active component (\bullet) of the influx. The active component was calculated by correcting the measured influx (part A, air) for the passive diffusive influx. The latter was calculated from the Goldman relation applied to the data of Figures 3A and 6A (CO treatments) (see text). The vanadate insensitive-, CO sensitive-component of the influx (\blacktriangle) was obtained by applying the same correction to the influx measured in the presence of vanadate. The curves are polynomial adjustments by least square fitting.

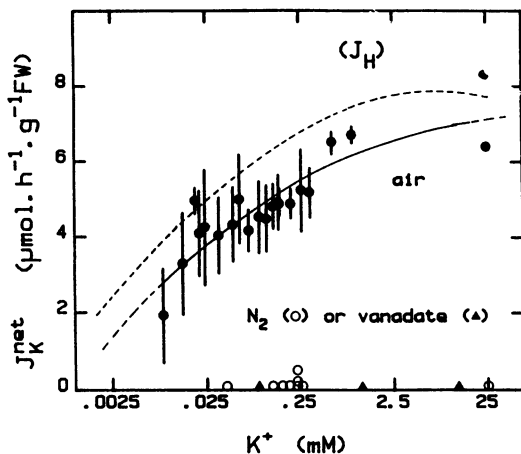


FIG. 7. Effect of the K^+ concentration of the net K^+ flux at pH 6. The media contained K_2SO_4 , and the pH was maintained by automatic titration with a pH-stat system. Net K^+ influx in aerated media, and in the presence of $100 \mu M$ vanadate or in media saturated with N_2 . The curve for aerated media is a polynomial adjustment by least square fitting. The dashed line is the polynomial adjustment for the net H^+ efflux measured in the same conditions (Fig. 8A).

this pH. Figure 8A shows the effect of the K^+ concentration on this activity in the absence of Ca^{2+} .

Effect of Fusicoccin. The effects of $10 \mu M$ FC on the $^{86}Rb^+$ influx, the membrane polarization and the net H^+ efflux are shown in Table II. Parallel runs were conducted in aerated

conditions and in hypoxia. In $100 \mu M K^+$ as in $10 mM K^+$, the classical stimulation of the K^+ and H^+ fluxes and hyperpolarization was obtained. A clear hyperpolarization and a slight H^+ extrusion remained observable in hypoxia, suggesting that FC allowed the pump to tap some source of energy independent of aerobic metabolism. It was verified that these CO-resistant, FC-induced hyperpolarization and H^+ extrusion disappeared when $100 \mu M$ vanadate was added to the medium.

DISCUSSION

In the following discussion, it is assumed that the membranes seal perfectly, and that tonoplast potential is constant and negligible, thus the potential measured with microelectrodes is identified with the plasmalemma potential. The electrogenic system opposes the depolarization of the membrane induced by high external K^+ concentrations, with maximum efficiency in the presence of Ca^{2+} . As shown in Figure 2 B, E_{hyp} increases with pH.

Inspection of Table I reveals that the true H^+ extrusion is poorly dependent on the pH when K^+ is present in the medium. In this case, the main origin of the expelled H^+ is probably the synthesis of K^+ salt of carboxylates. In the absence of K^+ at pH 4, the pump probably extrudes the H^+ which passively entered the cells. The very low extrusion rate observed in the absence of K^+ at pH 6 is in accordance with this hypothesis. Figures 7 and 8A show that the net fluxes of H^+ and K^+ are similar at pH 6. Since the H^+ passive influx is negligible at this pH, one may consider that the charges extruded by the H^+ pump reenter the cells as K^+ influx. In the case of such a $1 H^+/1 K^+$ exchange, the variations of the electrogenicity may result either from variations of the permeability of the passive pathway which shunts the pump, or from variations of the net charge transported by the latter.

Control of the K^+ Permeability. The permeability coefficients of K^+ were estimated with the assumption that they were not modified in the presence of CO. The data of Figures 2, 3, 5, and 6 (E_m and tracer influxes in the presence of CO) were used with the Goldman relation for calculating P_K (Table III). From $25 \mu M K^+$ to $10 mM K^+$, E_{hyp} increased at pH 6 from about $-20 mV$ to about $-45 mV$ (no Ca^{2+} , Fig. 2B) or $-70 mV$ ($0.2 mM Ca^{2+}$, Fig. 3B). In the same concentration range, P_K was found to decrease from $2.6 \cdot 10^{-9} m \cdot s^{-1}$ to $0.7 \cdot 10^{-9} m \cdot s^{-1}$ (no Ca^{2+}), or from $1.3 \cdot 10^{-9} m \cdot s^{-1}$ to $0.6 \cdot 10^{-9} m \cdot s^{-1}$ ($0.2 mM Ca^{2+}$). The lower values of P_K in the presence of Ca^{2+} may be due to its depolarizing effect on the membrane surface charge as known for the squid giant axon (11). The Nernst criterion applied to the experimental conditions of Figure 7, assuming that the cytoplasm contained $100 mM K^+$, predicted that the K^+ equilibrium potentials were more negative than the observed E_m values (Fig. 1) from 2.5 to $250 \mu M K^+$ in the medium. Thus, the net K^+ influx measured in this range seems to be active. For higher concentrations, the effect of the decrease of P_K on E_m was evaluated, assuming that the net K^+ influx (Fig. 7) is via the passive channel. Applying the Goldman relation showed that the variations of P_K with the K^+ concentration in Table III were too small to account for the observed variations of E_{hyp} . For instance, a 13-fold decrease of P_K should be necessary, instead of the observed 3-fold decrease from $1 mM K^+$ to $25 mM K^+$. This conclusion was strengthened by the fact that the hyperpolarizing effect of the P_K decrease was exaggerated in these calculations, since the passive channel probably conducted only a part of the net K^+ influx (see below). Similarly, the increase of the electrogenic component of the potential with pH (Fig. 2B) could not be attributed to a regulation of the passive K^+ permeability, since there was no clear decrease of P_K with pH (Table III).

Control of the Net Charge Transported by the Pump. The electrogenic effect depends on the net current carried by the

Table I. Effects of pH and K⁺ Concentration on the H⁺ Extrusion Rate

The net H⁺ exchange rate was measured at the indicated pH values, with an automatic pH-stat system, in 2 mM CaSO₄, or 0.05 mM K₂SO₄ + 0.2 mM CaSO₄ or 5 mM K₂SO₄ + 0.2 mM CaSO₄. The true H⁺ extrusion rate was estimated from the sudden variation of the net exchange rate following the addition of 100 μM vanadate (type A experiments) or bubbling N₂ (type B).

| pH | Net and True H ⁺ Extrusion Rates in the Following Conditions | | | | | | | | | |
|-----|---|------|-----------------------|------|--------|------|----------------------|------|--------|------|
| | 2 mM CaSO ₄ type A | | 0.1 mM K ⁺ | | | | 10 mM K ⁺ | | | |
| | | | Type A | | Type B | | Type A | | Type B | |
| | Net | True | Net | True | Net | True | Net | True | Net | True |
| | $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ | | | | | | | | | |
| 4.0 | 0.0 | 5.4 | 0.0 | 8.0 | 2.3 | 6.4 | 1.7 | 4.8 | 1.7 | 5.1 |
| | | | 2.3 | 6.2 | | | 0.9 | 7.8 | 3.0 | 5.2 |
| 6.0 | 0.9 | 0.3 | 4.9 | 4.9 | 5.8 | 5.2 | 5.1 | 4.6 | 6.5 | 5.7 |
| | | | 6.6 | 5.7 | 5.8 | 5.6 | 6.4 | 5.9 | 6.4 | 6.1 |
| | | | 6.4 | 6.4 | | | | | | |
| | | | 5.9 | 5.9 | | | | | | |
| 8.0 | | | 8.1 | 5.1 | 6.5 | 6.5 | 7.5 | 4.9 | 7.5 | 7.5 |
| | | | 10.7 | 5.6 | | | 10.2 | 4.8 | | |

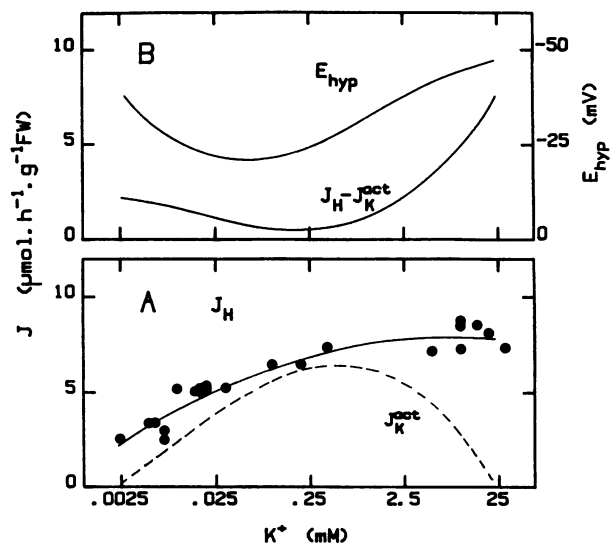


FIG. 8. Effect of the K⁺ concentration on the net H⁺ flux at pH 6. The media contained K₂SO₄, and the pH was maintained by automatic titration with a pH-stat system. The net H⁺ efflux was calculated from the base delivery. A, Net H⁺ influx in aerated media. The full line is a polynomial adjustment by least square fitting. The dashed line is the polynomial adjustment for the active component of the K⁺ influx measured in the same ionic conditions (Fig. 4B, pH 6). B, Comparison of E_{hyp} (Fig. 2B) and the estimated net charge transport by the pump. The latter is calculated as the difference between the curves of the flux of K⁺ and H⁺ shown in panel A.

pump. Both the rate of the H⁺ pumping and the net charge transferred per H⁺ determine the value of this current. From 10 μM K⁺ to 25 mM K⁺, the rate of the H⁺ extrusion increased at pH 6 from about 4 μmol·h⁻¹·g⁻¹ fresh weight to 7 μmol·h⁻¹·g⁻¹ fresh weight (Fig. 8A), and the net K⁺ influx showed a similar behavior (Fig. 7). As discussed above, this variation of the pumping rate alone cannot explain the increasing hyperpolarization in Figures 2B and 3B. In the same way, the results of Table I eliminate the acceleration of the H⁺ pumping as the cause of the increase of the electrogenic effect of the pump between pH 4 and pH 8 (Fig. 2B). Rather, the control of the electrogenicity must depend on the net charge transported per elementary cycle of the pump, *i.e.* on the presence of a direct

K⁺/H⁺ exchange with variable stoichiometry, as proposed by Cheeseman and Hanson (4). Using the passive permeability coefficients determined in anoxia or with uncoupler for calculating the passive component of the influx in the normal situation, these authors obtained complex kinetics for the active component. They hypothesized two antagonistic effects of K⁺, namely an activation of the H⁺ extrusion system and a progressive inhibition of its K⁺-transporting moiety. The theoretical curves in Figure 4B were calculated in the same way, using the P_K values (Table III) derived from the data on Figure 5A. At pH 4.0, 6.0, or 8.0, the influx was mostly active in the micromolar concentration range. It is progressively replaced by the passive influx as the K⁺ concentration increases. The increasing difference between the active K⁺ influx (Fig. 4, pH 6.0) and the active H⁺ efflux (Fig. 8A) measures the net charge transport responsible for the electrogenic potential (Fig. 8B). The appearance of this transport at about 250 μM K⁺ coincides with the reversal of the sign of the K⁺ electrochemical potential gradient as estimated above.

In the high K⁺ concentration range, Ca²⁺ greatly increased E_{hyp} (Figs. 2B and 3B). The above analysis gave similar values of the active K⁺ influx both in the absence and in the presence of Ca²⁺ (Figs. 4B and 6B), but lower diffusive K⁺ influx in the presence of Ca²⁺, in spite of more negative E_m (Figs. 1 and 3A). This was due to the observed lower values of P_K (Table III). Thus, the increase of E_{hyp} as a function of the external K⁺ concentration in the presence of Ca²⁺ was due to the progressive inhibition of the active K⁺ transport, as in the absence of Ca²⁺. Ca²⁺ increased the efficiency of this system to oppose the membrane depolarization at high K⁺ concentrations by reducing the permeability of the passive K⁺ shunt.

The data of Table II indicate that FC accelerated the net H⁺ extrusion and the ⁸⁶Rb⁺ influx, without modifying the stoichiometry of the pump, as judged from the J_H^{net}/J_K^{act} ratios both in 100 μM K⁺ and in 10 mM K⁺. Furthermore, it did not seem to modify the passive K⁺ permeability. Thus, the hyperpolarizing effect of FC simply results from the acceleration of the pump, while the transferred net charge remains fixed at the value necessary for E_m control in the absence of the toxin. The hyperpolarizing effect of FC was stronger in 100 μM K⁺ than in 10 mM K⁺, in spite of a lesser acceleration of H⁺ extrusion. This was due to the fact that the conductance of the passive K⁺ shunt increased with K⁺ concentration, as predicted by the Goldman relation. The hyperpolarizations induced by FC in hypoxia seem

Table II. Effects of Fusaricocin (FC) on Membrane Polarization and Transports

E_m is the membrane potential (mV), J_H^{net} , the net H^+ flux, and J_K^{in} the K^+ ($^{86}Rb^+$) influx. J_K^{pass} and J_K^{act} are the passive (respectively active) components of the K^+ influx. J_H/J_K^{act} is the stoichiometric ratio of the fluxes in the pump. The fluxes are expressed as $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ fresh weight. P_K is the K^+ permeability coefficient ($\text{m}\cdot\text{s}^{-1}$) calculated assuming a specific surface area of $0.1\text{ m}^2\cdot\text{g}^{-1}$ fresh weight. The media contained 0.2 mM CaSO_4 , 2 mM Mes-Tris (pH 6) and K_2SO_4 and FC as indicated. Parallel runs were performed in normal aerated conditions and in CO -saturated media in the dark.

| | E_m , Fluxes, and Permeability Coefficients Determined in the Following Conditions | | | | | | | |
|-----------------------|--|---------------------|---------|---------------------|-------------|---------------------|---------|---------------------|
| | 0.1 mM K^+ | | | | 10 mM K^+ | | | |
| | Air | | Hypoxia | | Air | | Hypoxia | |
| | 0 FC | 10 μM FC | 0 FC | 10 μM FC | 0 FC | 10 μM FC | 0 FC | 10 μM FC |
| E_m^a | -144 | -185 | -123 | -163 | -117 | -122 | -60 | -67 |
| J_H^{net} | -7.2 | -10.7 | 0.0 | -0.6 | -7.7 | -12.0 | 0.0 | -0.5 |
| $J_K^{in}{}^b$ | 8.4 | 11.6 | 0.2 | 0.2 | 14.5 | 17.2 | 6.4 | 7.4 |
| $J_K^{pass}{}^c$ | 0.2 | 0.2 | | | 11.5 | 12.6 | | |
| J_H^{net}/J_K^{act} | 0.9 | 1.0 | | | 2.6 | 2.6 | | |
| $P_K \times 10^9$ | | | 1.0 | 1.0 | | | 0.7 | 0.7 |

^a Means of 4 to 10 values (SD less than 8% of the means). ^b Means of 2 values (relative difference less than 5%). ^c Calculated from the Goldman relation, using the P_K values determined in the presence of CO and Ca^{2+} .

Table III. K^+ Permeability Coefficients Estimated from the Measurements of Membrane PD and Tracer Influx in the Presence of CO

The permeability coefficients were calculated with the Goldman equation applied to the $^{86}Rb^+$ influx measurements of Figures 4 and 6, and the measured membrane PD values. The specific cell surface was taken as $0.1\text{ m}^2\cdot\text{g}^{-1}$ fresh weight.

| pH | P_K at following K^+ Concentrations (mM) | | | | | | | | |
|------------------|--|------|-------|-----|------|-----|-----|-----|-----|
| | 0.0025 | 0.01 | 0.025 | 0.1 | 0.25 | 1 | 2.5 | 10 | 25 |
| | $\text{m}\cdot\text{s}^{-1} \times 10^9$ | | | | | | | | |
| 4.0 ^a | 4.2 | 3.2 | 1.8 | 1.2 | 1.0 | 0.8 | 0.7 | 0.6 | 0.7 |
| 6.0 ^a | 4.2 | 2.6 | 2.6 | 2.3 | 2.0 | 1.8 | 1.3 | 0.8 | 0.7 |
| 6.0 ^b | 2.1 | 1.3 | 1.3 | 1.2 | 1.1 | 1.1 | 0.9 | 0.6 | 0.6 |
| 8.0 ^a | | 2.8 | | 1.6 | | 1.3 | | 0.9 | 0.8 |

^a No calcium. ^b 0.2 mM CaSO_4 in the media.

to be at variance with the weak effects of the toxin on H^+ extrusion and K^+ influx. The calculation of P_K in hypoxia (Table II) indicated that the observed fluxes could explain the observed hyperpolarizations only if the K^+ influx was totally passive, even in the presence of FC. One explanation may be that in hypoxia, the toxin activated the sole H^+ moiety of the K^+/H^+ pump. Clearly, more experimental work is needed on this question.

The rate of the H^+ extrusion did not seem to increase with the pH (Table I). Thus, the increase of the electrogenic component of E_m with the pH (Fig. 2B) probably did not result from the acceleration of the pump. On the other hand, the comparison of the curves of the total $^{86}Rb^+$ influx and of its active component (Fig. 4) reveals that the stoichiometry of the direct H^+/K^+ exchange was not dependent on the pH. Thus, the mechanisms of the hyperpolarization in response to increasing K^+ concentration and increasing pH, are probably not the same. The permeability coefficient of H^+ was estimated with the help of the Goldman relation using the values of the passive influx and the measured E_m in the presence of vanadate or in hypoxia (Fig. 2A). The cytoplasmic pH was taken as 7.5 (18) and the exchange surface area as $0.1\text{ m}^2\cdot\text{g}^{-1}$ fresh weight (14). P_H was $5.4 \cdot 10^{-8}\text{ m}\cdot\text{s}^{-1}$ at $100\text{ }\mu\text{M K}^+$ ($J_H^{net} = 5\text{ }\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ fresh weight, $E_m = -64\text{ mV}$), and $3.5 \cdot 10^{-8}\text{ m}\cdot\text{s}^{-1}$ at 10 mM K^+ ($J_H^{net} = 2.5\text{ }\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ fresh weight; $E_m = -42\text{ mV}$). Thus, P_H was 45 to 58 times higher than P_K (Table III), which agrees with the results of Pitman *et al.* for barley roots (16). Assuming that the values of P_H were the same in the absence of inhibitors, and using the measured

values of E_m in aerated 5 mM K_2SO_4 (Fig. 3), it was possible to estimate the passive influxes of K^+ and H^+ at pH 4 and pH 6. Such a calculation indicated that 80% of the net charge extruded by the pump reentered the root as H^+ at pH 4, and 0.5% at pH 6. In spite of the considerable uncertainty about the significance and values of P_H , it may be concluded that the decline of E_{hyp} with decreasing pH (Fig. 3) resulted from a progressive shift from a low conductance shunt (K^+) to a high conductance one (H^+). This hypothesis is in accordance with the virtual insensitivity of E_{hyp} to the K^+ concentration at pH 4 (Fig. 2).

Both vanadate and hypoxia largely suppressed the active net influx (Fig. 7). Nevertheless, only the hypoxia inhibited the tracer active influx. This influx was largely maintained in the presence of $100\text{ }\mu\text{M}$ vanadate (Figs. 5A and 6A), although this treatment inhibited the K^+ net influx as well as the H^+ extrusion (Fig. 7; Table I). Thus, vanadate could be thought to induce a K^+ efflux balancing the measured influx. This could signify that the inhibition of the H^+ pump by vanadate was accompanied by the uncoupling of the pump and the K^+ active transport moiety, enabling bidirectional K^+ flux across the latter. Suppressing the energization of the H^+ pump by hypoxia without uncoupling it from the K^+ system would block both of them. Influx via the uncoupled K^+ system was estimated by subtracting the calculated passive influx from the total influx in the presence of vanadate. The K^+ dependent inhibition was no longer evident, and more classic saturation kinetics were obtained (Figs. 5B and 6B). This further suggested that the concentration-dependent progressive

inhibition of the active K⁺ uptake was not an intrinsic property of the K⁺ transport system, but rather resulted from its interaction with the H⁺ transport moiety.

Conclusion. The effects of the K⁺ concentration of H⁺/K⁺ exchange and E_m were measured in five conditions (air, hypoxia, vanadate, FC, and pH 4) ensuring various levels of membrane polarization. The conclusions are (a), the regulation of E_m is mainly exerted via variations of the stoichiometry of the direct K⁺/H⁺ exchange as proposed by Cheeseman and Hanson (4), (b) this stoichiometry is controlled by the K⁺ concentration rather than by E_m. The K⁺ channel seems to be close either when the H⁺ pump is not energized (hypoxia), or when the K⁺ concentration exceeds about 25 mM. When the H⁺ pump is specifically blocked by vanadate, the K⁺ channel remains open at high K⁺ concentrations, being unable to energize the K⁺ net influx but mediating isotopic exchanges.

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