

Role of the Plasmalemma H⁺-ATPase in *Pseudomonas syringae*-Induced K⁺/H⁺ Exchange in Suspension-Cultured Tobacco Cells¹

Merelee M. Atkinson*² and C. Jacyn Baker

Microbiology and Plant Pathology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT

Activation of a host plasma membrane K⁺ efflux/net H⁺ uptake exchange by pathogenic pseudomonads plays an important role in the development of hypersensitivity in tobacco (*Nicotiana tabacum*). Involvement of the plasmalemma H⁺-pumping ATPase in this response was investigated. The exchange response of suspension-cultured tobacco cells to *Pseudomonas syringae* pv *syringae* was reduced 90% or more by ATPase inhibitors including vanadate, *N*-ethylmaleimide, and *N,N'*-dicyclohexylcarbodiimide. The exchange was also strongly inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone and by slightly alkaline external pH. Respiratory inhibitors such as oligomycin and sodium azide reduced the exchange by 50% to 75%, while glycolysis inhibitors such as sodium arsenite and sodium iodoacetate decreased exchange by approximately 90%. These results suggest that plasmalemma H⁺-ATPase activity is required for the exchange response and that this may reflect a requirement for a plasmalemma pH and/or electrical potential gradient.

The HR³ is characterized by rapid and localized death of plant cells that come into contact with incompatible pathogens and is associated with disease resistance (14). The molecular basis for hypersensitivity and its association with resistance is not well understood. Activation of a plasmalemma K⁺ efflux/net H⁺ uptake exchange in tobacco is an early indicator of HR induction by incompatible *Pseudomonas syringae* pathovars (5, 6). The rapid loss of intracellular K⁺ along with the disruption of plasmalemma H⁺ gradients and intracellular pH regulation are believed to contribute to host cell death (5), although other factors such as lipid peroxidation and active oxygen production also appear to be important (11–13). Induction of the exchange response also occurs in the compatible interaction of *P. syringae* pv *syringae* with bean leaf tissue and may promote bacterial multiplication by increasing the availability of nutrients in host intercellular

spaces (1, 2). Bacterial ability to induce the exchange is controlled by a 31-kilobase pair chromosomal fragment that also controls HR induction and pathogenicity (8). However, the inducing factor has not been identified.

The molecular basis for the exchange response has not been determined, although the involvement of a plasmalemma transport protein has been suggested (5). The plasmalemma H⁺-pumping ATPase plays a major role in the regulation of plasma membrane pH and electrical potential gradients in plant cells (19). Therefore, we are investigating whether it is involved (directly or indirectly) in the exchange response. Here we report the effect of ATPase and metabolic inhibitors and external pH on the exchange response in suspension-cultured tobacco cells. Our results suggest that plasmalemma H⁺-ATPase activity is indirectly required for this response.

MATERIALS AND METHODS

Bacteria

Pseudomonas syringae pv *syringae* strain 61 was isolated from wheat by M. Sasser, University of Delaware. This bacterium is incompatible on tobacco and induces HR and the exchange response in this host (6). For controls, tobacco cells were inoculated with strain B7, which was created by Tn5 mutagenesis of the wild-type strain and does not induce the HR or exchange response in tobacco (6). Bacterial inocula for experiments were prepared from 16- to 20-h cultures grown on Kings B agar at 28°C. Bacteria were suspended in H₂O, washed twice by centrifugation at 8000g_{max} for 10 min at 20 to 25°C, and resuspended in assay buffer prior to addition to tobacco cell suspensions.

Net K⁺ and H⁺ Transport

Suspension-cultured tobacco cells were derived from *Nicotiana tabacum* cv Hicks and maintained as previously described (4). Inoculation of tobacco cells with bacteria and subsequent assay of the exchange response was as previously described (5) with minor variations. The assay medium contained 2 mM Mes-Tris (pH 5.75), 0.175 M mannitol, 0.5 mM K₂SO₄, and 0.5 mM CaCl₂, except for experiments dealing with external pH. For these experiments, Mes-Tris concentration was initially 0.5 mM. Where indicated, external pH was altered by the addition of a small volume of 100 mM Mes-Tris (pH 5.5 or 6.5) or 100 mM HEPES-Tris (pH 7.5 or 8.5) to

¹ Supported in part by U.S. Department of Agriculture Competitive Research Grants Office grants 85-CRCR-1-1779 and 87-CRCR-1-2304.

² Present address: Department of Plant Pathology, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706.

³ Abbreviations: HR, hypersensitive response; NEM, *N*-ethylmaleimide; DES, diethylstilbestrol; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

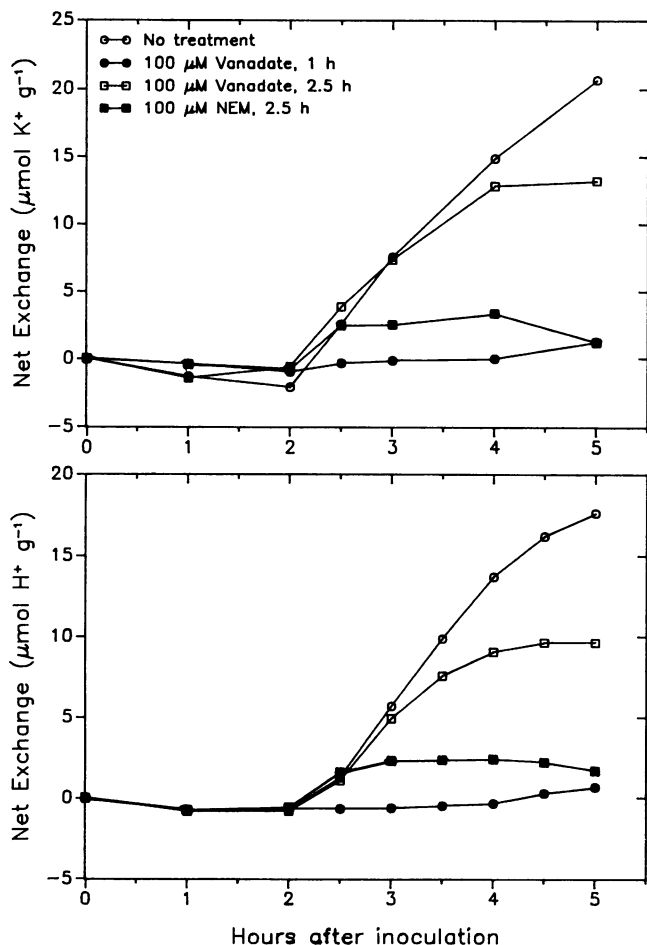


Figure 1. Effects of plasma membrane ATPase inhibitors on K⁺/H⁺ exchange in hypersensitive tobacco cells. Cell suspensions were inoculated at 0 h with wild-type (hypersensitive) or HR-negative (control) strains of *P. syringae* pv *syringae*. Inhibitors were added to cell suspensions at 1 or 2.5 h after inoculation. Data represent differences in net K⁺ and H⁺ transport between hypersensitive and control tobacco cells. Positive values indicate K⁺ loss and H⁺ uptake in hypersensitive cells relative to controls.

yield a final buffer concentration of 5 mM. Cell suspensions (0.5 g/14.5 mL assay medium) were inoculated with 0.5 mL of bacterial suspension in assay medium to achieve a final inoculum density of 10⁸ colony-forming units/mL. Tobacco cells inoculated with the wild-type and mutant bacterial strains are referred to herein as hypersensitive (or HR) and control cells, respectively. Cell suspensions were incubated in a rotary shaker at 27°C with rotation at 160 rpm. Net H⁺ transport was determined by intermittent acid-base titration of tobacco cell suspensions with 24 mM NaOH and HCl. Net K⁺ transport was determined from K⁺ concentrations of assay medium aliquots. Cell suspensions were removed from the shaker and cells allowed to settle for about 1 min, after which 0.3-mL aliquots were removed from the miniscus. Aliquots were filtered through Miracloth to remove any remaining tobacco cells and were centrifuged 5 min in a microcentrifuge to pellet bacteria. Supernatants were assayed for K⁺ content by atomic absorption spectroscopy.

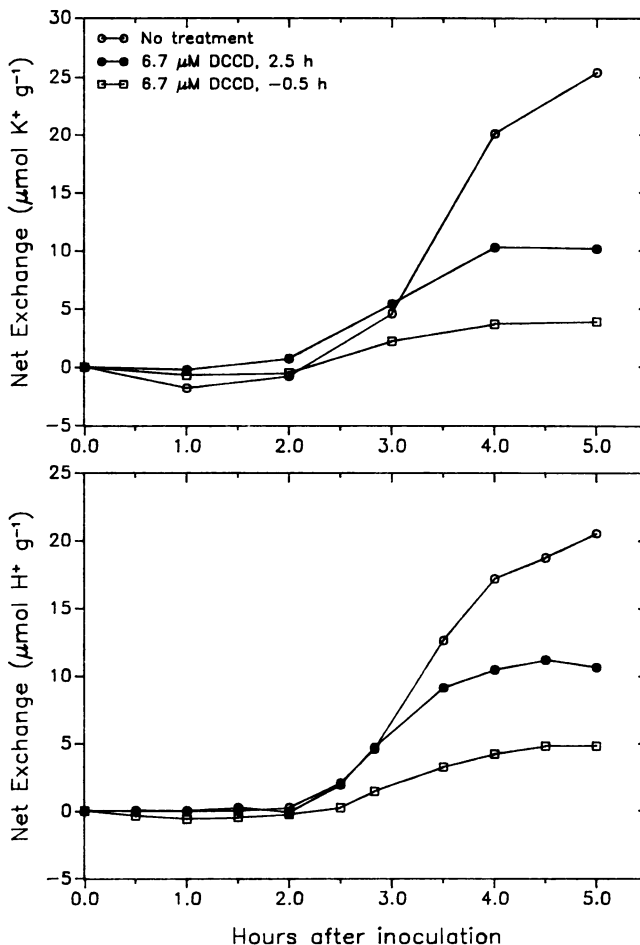


Figure 2. Effects of DCCD on K⁺/H⁺ exchange in hypersensitive tobacco cells. DCCD was added to cell suspensions at -0.5 or 2.5 h after inoculation. Other details are as in the legend to Figure 1.

K⁺/H⁺ exchange data were obtained by subtracting net transport data (positive values represent K⁺ loss and H⁺ uptake) for controls from those of hypersensitive cells. This method overestimates exchange-dependent K⁺ loss slightly (about 15%) because it does not correct for the reduced K⁺ uptake that is associated with the exchange response (5).

All inhibitors were obtained from Sigma.⁴ Inhibitors were dissolved in 95% EtOH or dH₂O and added to cell suspensions in 15- to 30- μ L aliquots. Equivalent volumes of solvent were added to untreated cells. To avoid possible impairment of bacterial capacity to elicit the exchange response, most inhibitors were added to cell suspensions after initiation of the exchange (approximately 2.5 h after inoculation). Acid-base titration was performed immediately after addition of inhibitor to each cell suspension, whereas K⁺ sampling did not begin until titrations were completed for all samples. Thus, K⁺ measurements were made approximately 5 min after inhibitor addition.

All data are representative of two to four separate experiments.

⁴ Mention of a specific product or vendor does not constitute an endorsement by the USDA and does not imply a recommendation over other suitable products or vendors.

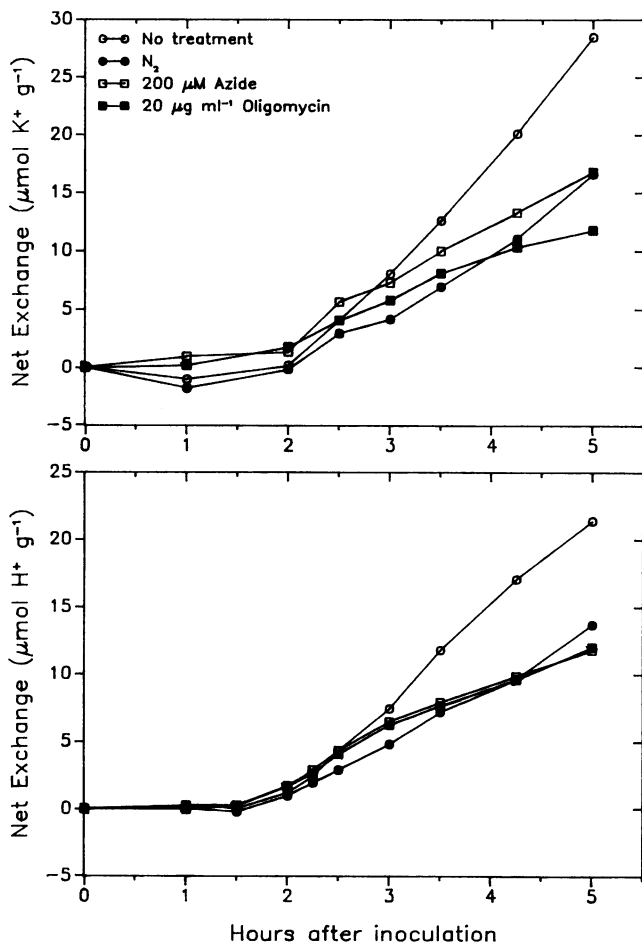


Figure 3. Effects of respiratory inhibitors and anaerobiosis on K^+/H^+ exchange in hypersensitive tobacco cells. Inhibitors or N_2 were introduced at 2.5 h after inoculation. Other details are as in the legend to Figure 1.

RESULTS

Effects of ATPase Inhibitors on K^+/H^+ Exchange

Vanadate, NEM, DCCD, and DES, inhibitors of the plasmalemma H^+ -ATPase (10, 16, 17, 19), were used to assess the role of this enzyme in the exchange response. Inhibition of exchange by NEM occurred in less than 30 min, whereas inhibition by vanadate required approximately 1.5 h (Fig. 1). When added to cell suspensions at 1 h after bacterial inoculation, vanadate inhibited initiation of the exchange. Under these conditions, an inhibitory effect on bacterial cells cannot be ruled out, although 200 μM vanadate did not alter bacterial growth rate in minimal medium (data not shown). Like NEM, DES inhibited the exchange rapidly after its addition to cell suspensions. In a representative experiment, 50 μM DES inhibited the exchange by 96.8% (H^+) and 93.3% (K^+). However, DES also caused massive K^+ loss in control cells. Lower concentrations of DES caused less K^+ loss in controls but were also less effective in inhibiting the exchange. As discussed below, other inhibitors caused less severe K^+ loss in controls.

Treatment of tobacco cells with 6.7 μM DCCD prior to bacterial inoculation reduced the exchange response by ap-

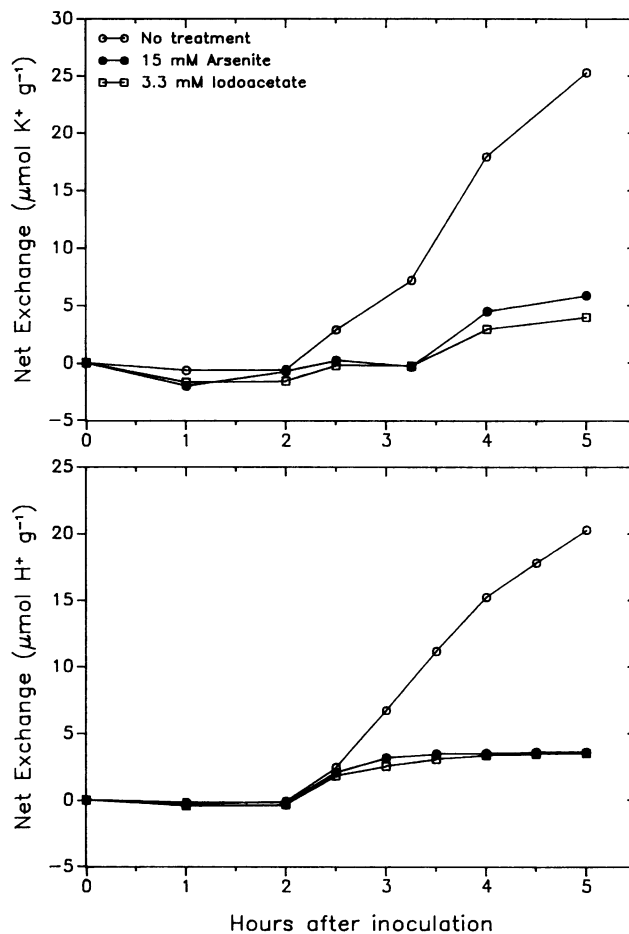


Figure 4. Effects of glycolysis inhibitors on K^+/H^+ , K^+/H^+ exchange in hypersensitive tobacco cells. Inhibitors were added to cell suspensions at 2.5 h after inoculation. Other details are as in the legend to Figure 1.

proximately 80% (Fig. 2). For these experiments, tobacco cells were incubated for 30 min in assay medium containing DCCD, after which cells were collected by filtration, washed thoroughly, and resuspended in fresh assay medium. Cell suspensions were then immediately inoculated with bacteria. Two separate dose-response experiments indicated IC_{50} (concentration giving 50% inhibition) values of 2.7 and 3.4 μM . Complete inhibition of the exchange required DCCD concentrations greater than 20 μM . Since DCCD was washed from cells prior to bacterial inoculation, it is unlikely that this treatment affected the bacterial HR induction process. However, treatment of bacterial cells with 10 μM DCCD for 30 min prior to inoculation of tobacco cell suspensions did not reduce subsequent induction of the exchange (data not shown). DCCD also inhibited the exchange when added to cell suspensions at 2.5 h after bacterial inoculation. Under these conditions, inhibition was detected within 30 min after DCCD addition.

Effects of Other Inhibitors and External pH on K^+/H^+ Exchange

The addition of respiratory inhibitors azide and oligomycin to cell suspensions at 2.5 h decreased subsequent expression

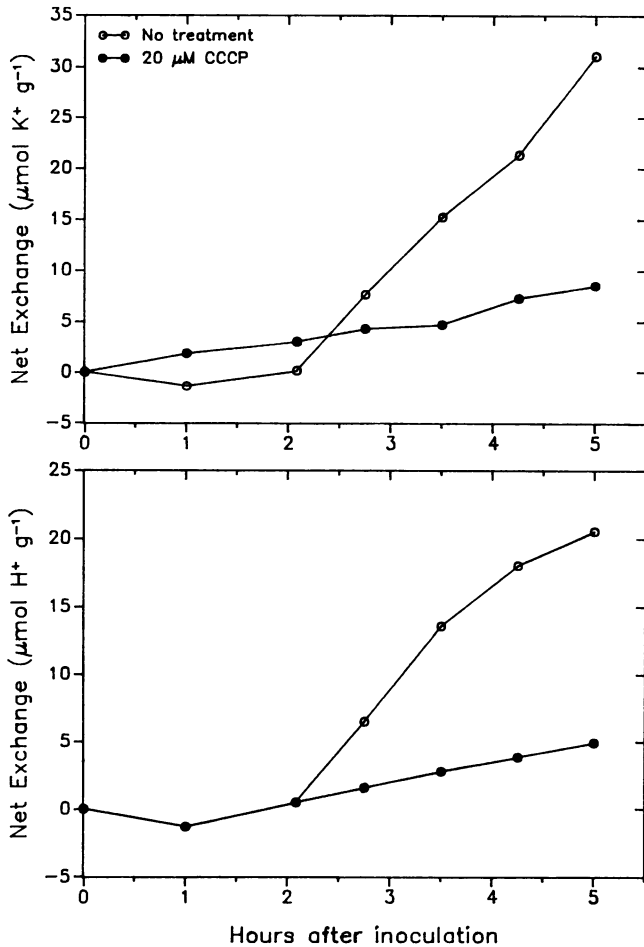


Figure 5. Effects of CCCP on K⁺/H⁺ exchange in hypersensitive tobacco cells. CCCP was added to cell suspensions at 2 h after inoculation. Other details are as in the legend to Figure 1.

of the exchange (Fig. 3). Inhibition ranged from 50% to 75% in individual experiments. To achieve near-anaerobic conditions, cell suspensions were exposed continuously to bubbled nitrogen without shaking. This treatment also inhibited the exchange but was slightly less effective than azide or oligomycin. The addition of 75 μM salicylhydroxamic acid to inhibit cyanide-resistant respiration had little effect on the exchange response. In a representative experiment, azide alone blocked 65.6% of exchange-associated H⁺ uptake, while azide + salicylhydroxamic acid blocked 67.7%. Sodium arsenite and sodium iodoacetate, glycolysis inhibitors, inhibited the exchange response by approximately 90% (Fig. 4).

CCCP (20 μM) reduced exchange rates by about 90% (Fig. 5). Although CCCP is a respiratory uncoupler, it inhibited the exchange more effectively than respiratory inhibitors. Dissipation of the plasmalemma H⁺ gradient by CCCP may thus account for its strong inhibitory effect. Exchange rates were insensitive to external pH over the range of 5.5 to 6.5, but decreased sharply above pH 7 (Fig. 6). Maximum inhibition was not observed until 30 to 45 min after medium pH was adjusted upward.

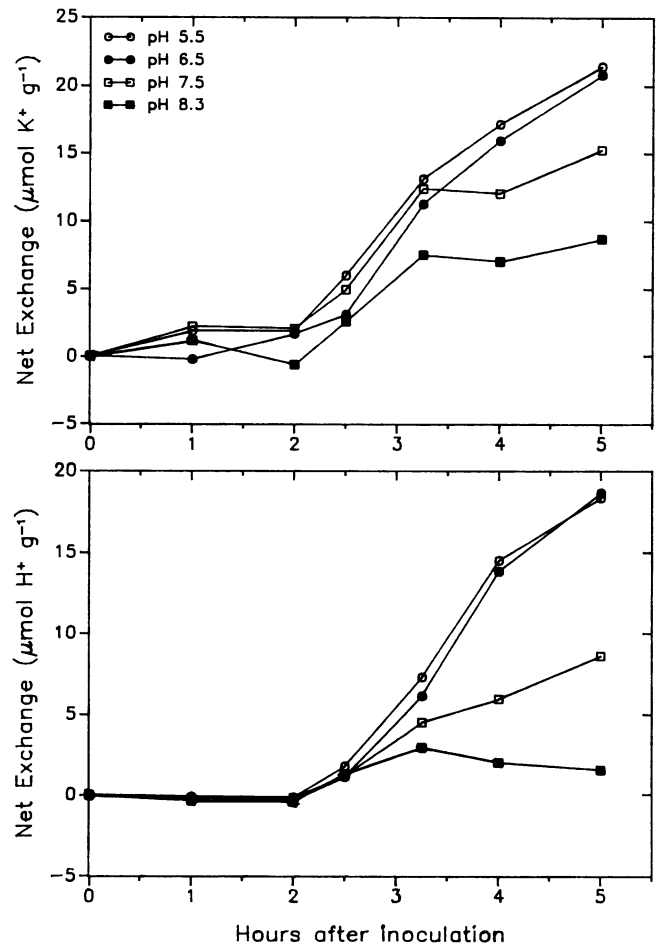


Figure 6. Effects of external pH on K⁺/H⁺ exchange in hypersensitive tobacco cells. External pH was initially maintained at 5.75 for all treatments but was adjusted to 5.5, 6.5, 7.5, or 8.3 at 2.5 h after inoculation. Other details are as in the legend to Figure 1.

Effects of Inhibitors and External pH on Net K⁺ and H⁺ Transport

Representative transport data from which exchange data were derived are shown in Figures 7 and 8. Control tobacco cells exhibited either a net K⁺ uptake or negligible net transport, whereas hypersensitive cells rapidly lost K⁺ beginning 2 to 2.5 h after inoculation (Fig. 7). All treatments decreased net K⁺ loss from hypersensitive cells except for DES, which caused extensive K⁺ loss in both hypersensitive and control cells. ATPase and metabolic inhibitors and CCCP caused moderate K⁺ loss from control cells, whereas high external pH stimulated net K⁺ uptake in controls.

Control cells normally exhibited a net H⁺ extrusion, whereas hypersensitive cells exhibited net H⁺ uptake, which began at 2 to 2.5 h after bacterial inoculation (Fig. 8). Inhibitors blocked H⁺ extrusion and caused a slight net H⁺ uptake in controls, whereas net H⁺ uptake in hypersensitive cells was decreased. Since all inhibitors gave similar results, only those for NEM are shown. Net H⁺ extrusion increased dramatically in both control and hypersensitive cells as external pH increased.

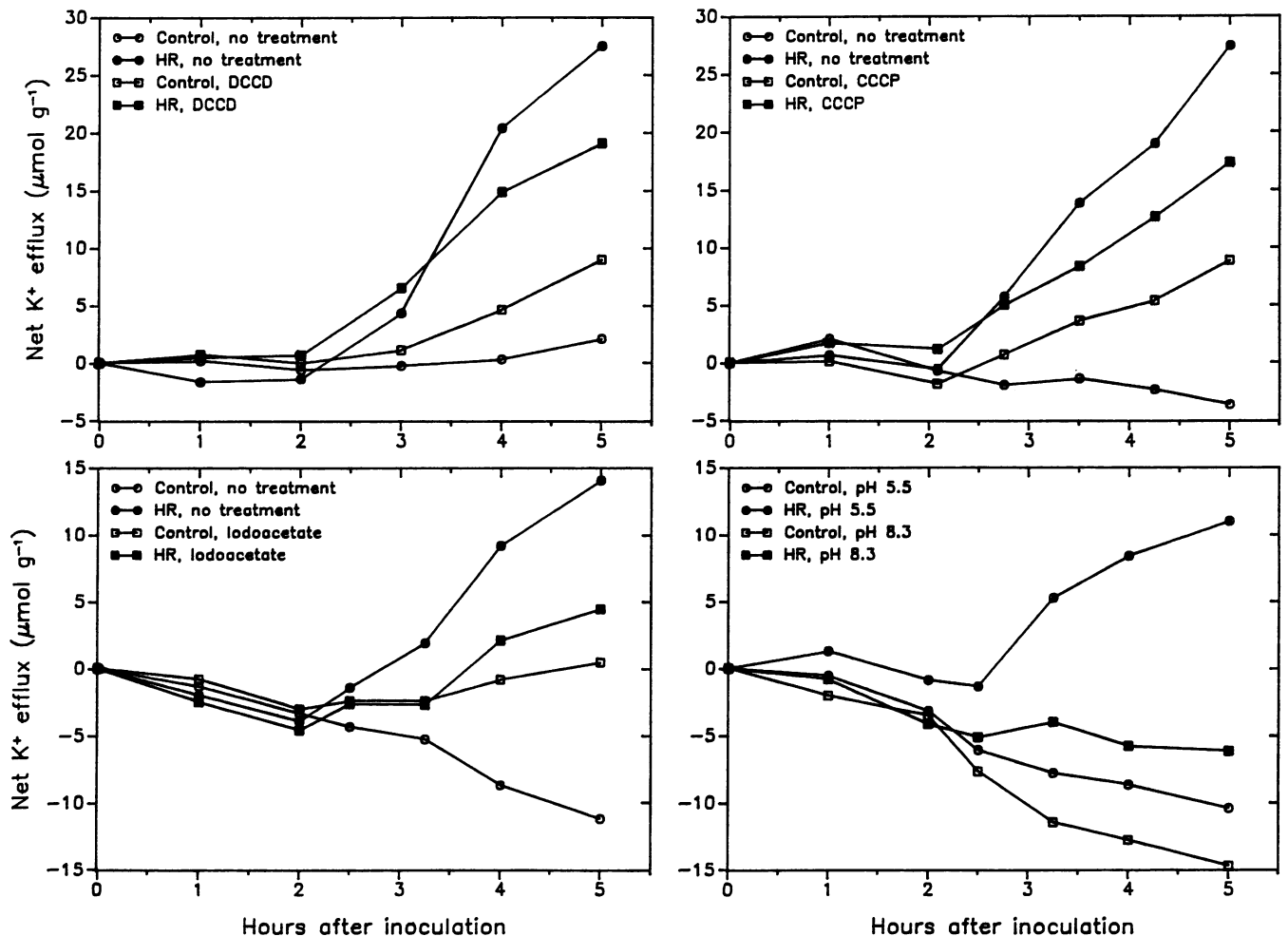


Figure 7. Effects of metabolic and ATPase inhibitors and external pH on net K⁺ transport in hypersensitive and control tobacco cells. Cell suspensions were inoculated at 0 h with wild-type (HR) or HR-negative (control) strains of *P. syringae* pv *syringae*. Inhibitor or pH treatments were initiated at 2.5 h except for CCCP, which was added at 2 h. Increasing values indicate net K⁺ loss, decreasing values indicate uptake.

DISCUSSION

We have shown that bacterium-induced K⁺/H⁺ exchange in suspension-cultured tobacco cells can be inhibited by ATPase and metabolic inhibitors, the protonophore CCCP, and slightly alkaline external pH. These results suggest that the exchange is dependent upon plasmalemma H⁺-ATPase activity. The effects of inhibitors on whole cell systems must always be interpreted with caution. However, the use of many different treatments and the consistency of the data support our interpretation. A second concern is whether the effects of various treatments on the exchange can be attributed to their effects on plant rather than bacterial cells. We believe this is true for several reasons. First, some treatments such as vanadate, DCCD, and slightly alkaline external pH (2) did not appear to be harmful to bacteria. Second, we generally added inhibitors to cell suspensions after initiation of the exchange. In tobacco leaves, a brief period of bacterial protein synthesis is required for HR induction, after which bacterial protein synthesis, and presumably viability, is no longer required (14, 15). This period is believed to reflect the time required for

bacterial synthesis of an HR elicitor. In suspension-cultured tobacco cells, the induction period ends at the onset of the exchange (L.D. Keppler, I. Yucel, and S. W. Hutcheson, personal communications). Thus, the subsequent use of inhibitors should not affect bacterial production of the putative elicitor. Finally, we have previously shown (3) that induction of a similar exchange response in tobacco cells by a purified bacterial pectate lyase is inhibited by CCCP.

It has been previously suggested (5) that the exchange response is mediated by a plasma membrane transport protein, and the results presented here are consistent with this view. Although our data suggest that ATPase activity is required, this requirement may be indirect. For example, mediation of the exchange by another transporter may require a plasmalemma pH or electrical potential gradient generated by the ATPase. Dependence on pH gradients is characteristic of certain known or putative H⁺/cation antiporters (7, 9), and many ion channels are voltage-gated (18). The involvement of a plasmalemma NADH or NADPH oxidase in the exchange response has also been suggested (11, 13). However, at present, we have no direct evidence for any of these

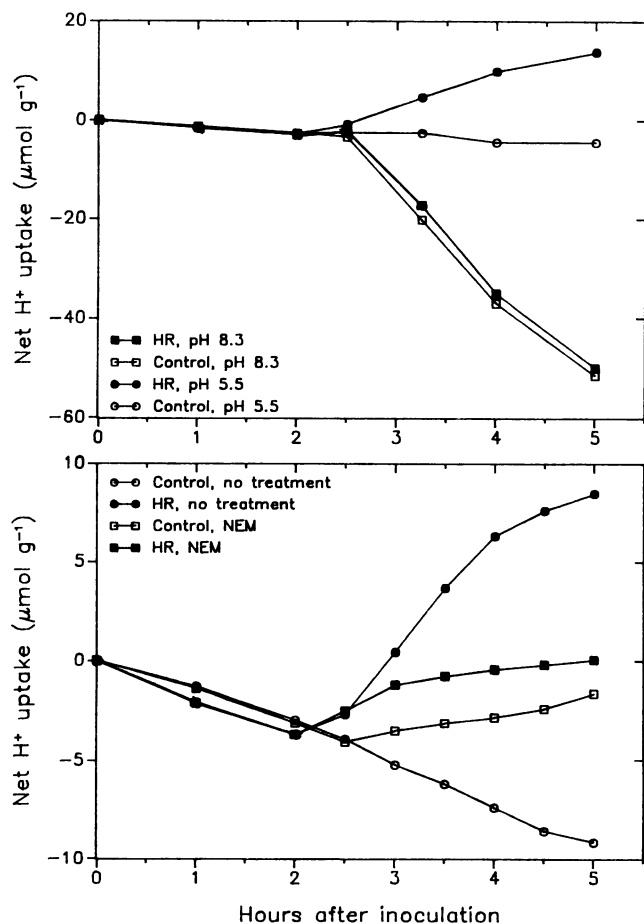


Figure 8. Effects of NEM and external pH on net H⁺ transport in hypersensitive and control tobacco cells. Inhibitor or pH treatments were initiated at 2.5 h after inoculation. Increasing values indicate net H⁺ uptake, decreasing values indicate extrusion.

possibilities. Future investigations will focus on identification of plasma membrane components that initiate and mediate the exchange response.

Note Added in Proof

While this paper was in press, J. L. Salzwedel, M. E. Daub, and J. Huang (Plant Physiol [1989] 90: 25–32) reported that increased extracellular pH delays HR-associated death of tobacco cells inoculated with *P. syringae* pv *pisii*. These results, together with ours, support the K⁺ efflux/H⁺ influx model for the exchange response. However, alternative suggestions have been made. A. J. Huerta and T. M. Murphy (Plant Physiol [1989] 90: 749–753) proposed a K⁺/HCO₃⁻ cotransport model for a similar, but UV-induced, K⁺ efflux in rose cells that is not reduced by increased extracellular pH values of 8 and 9. The UV-induced K⁺ efflux is reduced by ATPase and respiratory inhibitors and is believed to be dependent upon intracellular ATP (Murphy and Wilson [1982] Plant Physiol 70: 709–713). Keppler *et al.* (11, 13) proposed that net H⁺ uptake during the HR may result from active oxygen production rather than from H⁺ influx. Although our results appear

consistent with H⁺ influx, we cannot exclude these alternative mechanisms.

ACKNOWLEDGEMENTS

We thank L.D. Keppler, D.R. Bush, and D.G. Luster for critical reviews of the manuscript.

LITERATURE CITED

- Atkinson MM, Baker CJ (1987) Alteration of plasmalemma sucrose transport in *Phaseolus vulgaris* by *Pseudomonas syringae* pv. *syringae* and its association with K⁺/H⁺ exchange. *Phytopathology* 77: 1573–1578
- Atkinson MM, Baker CJ (1987) Association of host plasma membrane K⁺/H⁺ exchange with multiplication of *Pseudomonas syringae* pv. *syringae* on *Phaseolus vulgaris*. *Phytopathology* 77: 1273–1279
- Atkinson MM, Baker CJ, Collmer A (1986) Transient activation of plasmalemma K⁺ efflux and H⁺ influx in tobacco by a pectate lyase isozyme from *Erwinia chrysanthemi*. *Plant Physiol* 82: 142–156
- Atkinson MM, Huang JS, Knopp JA (1985) Hypersensitivity of suspension-cultured tobacco cells to pathogenic bacteria. *Phytopathology* 75: 1270–1274
- Atkinson MM, Huang JS, Knopp JA (1985) The hypersensitive reaction of tobacco to *Pseudomonas syringae* pv. *pisii*: Activation of a plasmalemma K⁺/H⁺ exchange mechanism. *Plant Physiol* 79: 843–847
- Baker CJ, Atkinson MM, Collmer A (1987) Concurrent loss in Tn5 mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the HR and host plasma membrane K⁺/H⁺ exchange in tobacco. *Phytopathology* 77: 1268–1272
- Brierley GP, Jurkowitz M, Chávez E, Jung DW (1977) Energy-dependent contraction of swollen heart mitochondria. *J Biol Chem* 252: 7932–7939
- Huang HC, Schuurink R, Denny TP, Atkinson MM, Baker CJ, Yucel I, Hutcheson SW, Collmer A (1988) Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *J Bacteriol* 170: 4748–4756
- Isom LL, Cragoe EJ, Limbird LE (1987) α₂-Adrenergic receptors accelerate Na⁺/H⁺ exchange in neuroblastoma X glioma cells. *J Biol Chem* 262: 6750–6757
- Katz DB, Sussman MR (1987) Inhibition and labeling of the plant plasma membrane H⁺-ATPase with *N*-ethylmaleimide. *Plant Physiol* 83: 977–981
- Keppler LD, Baker CJ (1989) O₂⁻-initiated lipid peroxidation in a bacteria-induced hypersensitive reaction in tobacco cell suspensions. *Phytopathology* 79: 555–562
- Keppler LD, Novacky A (1986) Involvement of membrane lipid peroxidation in the development of a bacterially induced hypersensitive reaction. *Phytopathology* 76: 104–108
- Keppler LD, Atkinson MM, Baker CJ (1989) Active oxygen production during a bacteria-induced hypersensitive reaction in tobacco suspension cells. *Phytopathology*, in press
- Klement Z (1986) Hypersensitivity. In MS Mount, GG Lacy, eds, *Phytopathogenic Prokaryotes*, Vol 2. Academic Press, New York, pp 149–177
- Klement Z, Goodman RN (1967) The role of the living bacterial cell and induction time in the hypersensitive reaction of the tobacco plant. *Phytopathology* 57: 322–323
- Oleski NA, Bennett AB (1978) H⁺-ATPase activity from storage tissue of *Beta vulgaris*. IV. *N,N'*-Dicyclohexylcarbodiimide binding and inhibition of the plasma membrane H⁺-ATPase. *Plant Physiol* 83: 569–572
- Soliz M (1984) Dicyclohexylcarbodiimide as a probe for proton translocating enzymes. *Trends Biochem Sci* 9: 309–312
- Stevens CF (1984) Biophysical studies of ion channels. *Science* 225: 1346–1350
- Sze H (1984) H⁺-translocating ATPases of the plasma membrane and tonoplast of plant cells. *Physiol Planta* 61: 683–691