Plant Physiol. (1979) 63, 191-194
0032-0889/79/63/0191/04/$00.50/0

Ion Transport in Isolated Protoplasts from Tobacco Suspension Cells

II. SELECTIVITY AND KINETICS[1]

Received for publication May 15, 1978 and in revised form August, 24, 1978

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ABSTRACT

Protoplasts were enzymically isolated from suspension cultured cells of *Nicotiana glutinosa* L. and aspects of transport selectivity and kinetics were studied. In the presence of Ca^{2+}, transport was selective for K^+ (^{86}Rb) over Na^+. $^{36}Cl^-$ transport was inhibited by Br^- or I^- but not by $H_2PO_4^-$. The kinetic data for short term (30 minutes) K^+ influx over the range of 0.05 to 100 millimolar KCl were complex but similar to those observed in other plant tissues. In contrast, the kinetic data for Cl^- and $H_2{}^{32}PO_4^-$ over the same concentration range were different from those observed for K^+, and could be accounted for by a single isotherm in the range of 0.05 to 4 millimolar and by an almost linear increase in influx rate above 4 millimolar. The kinetic data for Cl^- transport into intact cultured cells were identical in character to those observed for isolated protoplasts. The results support the view that enzymic removal of the cell wall produced no significant alteration in the transport properties of the protoplast.

In the preceding paper (7), we showed that it is feasible to use protoplasts isolated from cultured tobacco cells to study ion transport. Transport of K^+ (^{86}Rb), $^{36}Cl^-$, and $H_2{}^{32}PO_4^-$ into isolated tobacco protoplasts was tightly coupled to energy production by aerobic respiration. Influx of these ions responded to changes in pH or to substances such as Ca^{2+} or fusicoccin, in a similar manner as has been reported for intact plant cells and tissues. There was no indication that the transport properties of the protoplast was significantly altered by enzymic removal of the cell wall.

In this paper we characterize further the transport properties of isolated tobacco protoplasts by considering aspects of the selectivity and kinetics of transport in these cells.

MATERIALS AND METHODS

Cell Culture and Protoplast Isolation. Experiments were conducted with protoplasts isolated from cultured cells of *Nicotiana glutinosa* L. as described in the accompanying paper (7). Briefly, cells were mixed with 1% (w/v) Cellulysin and 0.2% Macerase in 0.7 M mannitol and incubated for 4 hr to digest the cell wall and release the protoplast. The protoplasts were filtered, and then collected and washed by repeated centrifugation in 0.7 M mannitol. The final protoplast pellet (2–3 ml packed volume from 20 g fresh weight of cultured cells) was suspended in 5 volumes of 0.7 M mannitol.

Ion Influx Measurement. Short term influx was determined by following labeled ion transport into isolated protoplasts or cultured cells over a 30-min period as described in the preceding paper (7). The assay medium contained 0.7 M mannitol (protoplasts only), 2 mM Tris-MES buffer (desired pH), K(^{86}Rb)Cl; K^{36}Cl, or $KH_2{}^{32}PO_4$ (at desired concentrations), 1 mM $CaSO_4$ (unless indicated otherwise), and other additions (see tables and figure legends) to a final volume of 5 ml. The influx measurement was initiated by addition of radioactive tracer and the cells were incubated at 30 C with gentle agitation. Influx values were determined by linear regression analysis of tracer uptake over the initial linear absorption period for K^+ and $H_2PO_4^-$ and after a 15-min pretreatment period for Cl^-. Influx of Cl^- into cultured cells was determined after a 3-hr pretreatment in H_2O (7).

RESULTS AND DISCUSSION

Selectivity. The addition of Ca^{2+} to the medium reduced K^+ (^{86}Rb) influx into the isolated protoplasts (Fig. 1 and ref. 7). In the presence of Ca^{2+}, Na^+ did not interfere with K^+ influx suggesting specificity for K^+ over Na^+ (Fig. 1). This observation is in agreement with the conclusion that adequate Ca^{2+} is essential for specific K^+-Rb^+ transport in the presence of Na^+ (2, 3).

The influx of Cl^- was strongly inhibited by Br^- or I^- (Fig. 2) suggesting that these ions share a common transport system (2, 3). Part of the inhibition observed with I^- may be because of the inherent toxicity of I^-. Phosphate did not inhibit Cl^- influx (not shown).

K^+ Influx Kinetics. The kinetic data for ^{86}Rb-labeled K^+ influx into isolated tobacco protoplasts over the range of 0.05 to 100 mM were complex and did not fit the Michaelis-Menten equation (Fig. 3). The Eadie, Hofstee plot (1) was not linear, but rather was representative of a curved line indicating that the apparent K_m increased with increasing K^+ concentration (Fig. 3). Analysis of the data from 0.05 to 0.8 mM K^+ (Fig. 3) showed good agreement with Michaelis-Menten kinetics. These results are consistent with kinetic analysis of K^+ transport in a variety of plant cells and tissues (2–4, 8, and references therein).

Cl^- Influx Kinetics. The data for $^{36}Cl^-$ influx into tobacco protoplasts, over the range of 0.05 to 100 mM, did not follow simple Michaelis-Menten kinetics (Fig. 4). This was especially apparent when the data were transformed according to the Eadie, Hofstee equation (Fig. 4). At lower concentrations of Cl^-, 0.05 to 3.2 mM, the rate of transport responded as predicted by the Michaelis-Menten equation (Fig. 4).

The kinetic data of Cl^- influx over the range of 0.05 to 100 mM were different than usually observed for higher plant tissues where the kinetic data for Cl^- influx are similar to those for K^+ influx (2, 3). For tobacco protoplasts, Cl^- influx showed the expected saturation at low Cl^- concentration, but with increases in Cl^- concentration above about 3 mM, Cl^- influx increased in a linear

[1] This research was supported in part by Grant PCM 7680295 from the National Science Foundation to R. T. L.

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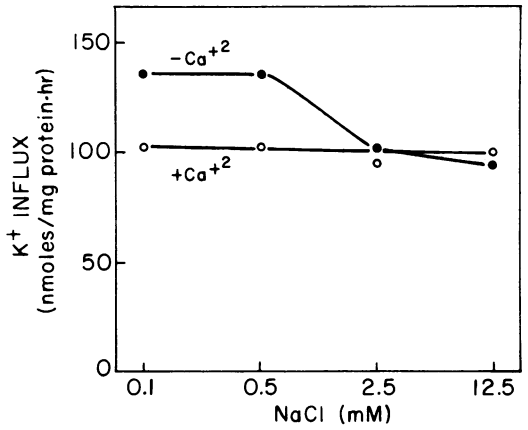

FIG. 1. Rate of K^+ (^{86}Rb) influx into protoplasts isolated from cultured tobacco cells as a function of increasing NaCl in the absence or presence of $CaSO_4$. Influx values were measured in 1 mM KCl, 2 mM Tris-MES (pH 6.5), 0.7 M mannitol, 1 mM $CaSO_4$ (where indicated), and NaCl as indicated.

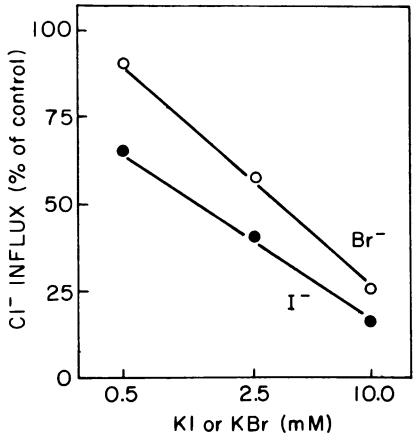

FIG. 2. Rate of $^{36}Cl^-$ influx into tobacco protoplasts as a function of increasing KBr or KI. Influx values were measured in 1 mM KCl, 2 mM Tris-MES (pH 6.0), 1 mM $CaSO_4$, 0.7 M mannitol, and KBr or KI as indicated. Rates presented relative to control rates with no KI or KBr.

fashion suggesting diffusive entry rather than carrier-linked transport. Nonsaturating Cl^- influx of a diffusive nature has also been observed in corn root tips (11) and in potato tissue (6).

The kinetic data of Cl^- influx were determined for intact tobacco cells to test the possibility that the Cl^- kinetic data for protoplasts were an artifact produced by the enzymic removal of the cell wall. The kinetic data for Cl^- influx into cultured tobacco cells were similar to those for the isolated protoplasts (Fig. 5).

Phosphate Influx Kinetics. The kinetic data for $H_2{}^{32}PO_4^-$ influx, as a function of increasing concentration, were similar to those observed for Cl^-. Transport of $H_2PO_4^-$ at high concentrations (6.4–100 mM) increased without any suggestion of saturation (Fig. 6). The Eadie, Hofstee plot of the $H_2PO_4^-$ data showed the same type of sharp break as observed for the kinetic data for Cl^-. $H_2PO_4^-$ influx over the range of 0.05 to 3.2 mM showed excellent agreement with the Michaelis-Menten equation (Fig. 6).

Kinetic Constants for Ion Influx. Analysis of the kinetic data produced the constants summarized in Table I. In general, the apparent K_m values were higher than those reported for ion transport in a variety of plant tissues (3). The close correspondence between the K_m values for Cl^- transport in tobacco protoplasts and cultured cells (pretreated for 3 hr in H_2O, see 7) suggests that the higher K_m values are probably a characteristic of the cells and not the result of an alteration produced during protoplast isolation.

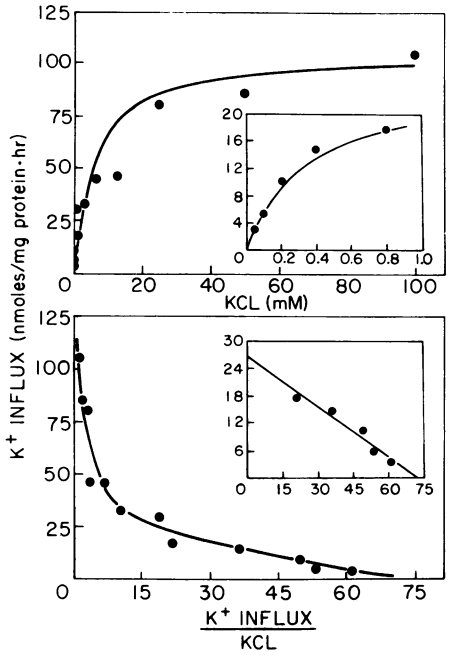

FIG. 3. Upper: K^+ (^{86}Rb) influx into tobacco protoplasts as a function of increasing KCl. Influx values were measured in 1 mM $CaSO_4$, 2 mM Tris-MES (pH 6.0), 0.7 M mannitol, and KCl as indicated (0.05–100 mM). Hyperbola drawn using the Michaelis-Menten equation and the kinetic constants as determined by best fit to the equation:

$$\frac{[S]}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}}[s]$$

Inset shows K^+ (^{86}Rb) influx as a function of low K^+ concentration (0.05–0.8 mM) with curve drawn as above. Lower: Eadie, Hofstee plots of data. Inset shows data for low K^+ concentrations with line of best fit determined by linear regression.

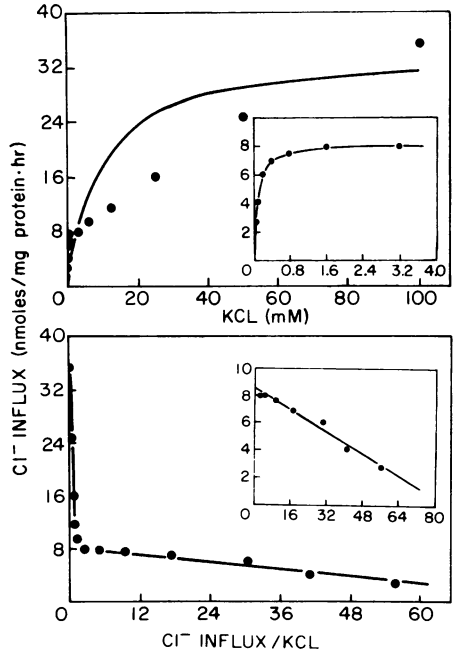

FIG. 4. Upper: $^{36}Cl^-$ influx into tobacco protoplasts as a function of increasing KCl. Influx values determined and curves drawn as in Figure 3. Inset shows $^{36}Cl^-$ influx as a function of low Cl^- concentrations (0.05–3.2 mM). Lower: Eadie, Hofstee plots of data. Inset shows data for low Cl^- concentration with line of best fit determined by linear regression.

As expected (7), the V_{max} for Cl^- transport in cultured cells was lower than that for isolated protoplasts.

The Hill coefficient for K^+ transport over the range of 0.05 to 100 mM was 0.64 (Table I) which is characteristic of K^+ transport in other tissues (3 for review) and is consistent with the view that K^+ transport is mediated by a negative cooperative transport system (4, 5). The kinetic data of Cl^- and $H_2PO_4^-$ transport were not negative cooperative suggesting that the molecular mechanism of cation and anion transport in these cells may be fundamentally different. Whatever the explanation may be for the kinetic data, the fact that such data were observed for protoplasts provides proof that the complex kinetics observed are not a function of ionic interaction with cell walls or an artifact associated with multicellular tissues.

Estimated Ion Fluxes. As reported in this and the accompanying paper (7), the rates of short term labeled ion transport (presumably across the plasma membrane) from 1 mM salt solution for K^+, Cl^-, $H_2PO_4^-$ and Ca^{2+} were about 50, 8, 6, and 20 nmol/mg protoplast protein·hr, respectively. For K^+ transport, the rate was 50 divided by 760,000 protoplasts/mg protein (7) or 6.58×10^{-5} nmol/protoplast·hr. For a protoplast with an average diameter of about 30 μM (12), the surface area is 2.83×10^{-9} m^2. The average flux of K^+, or amount of K^+ passing through a unit area of protoplast membrane per unit of time, can be calculated to be 6.5 nmol/m^2·sec. Similarly, the estimated fluxes for Cl^-, $H_2PO_4^-$, and Ca^{2+} would be 1, 0.8, and 2.6 nmol/m^2·sec, respectively. These are comparable to fluxes estimated for these ions in other plant cells (9, 10).

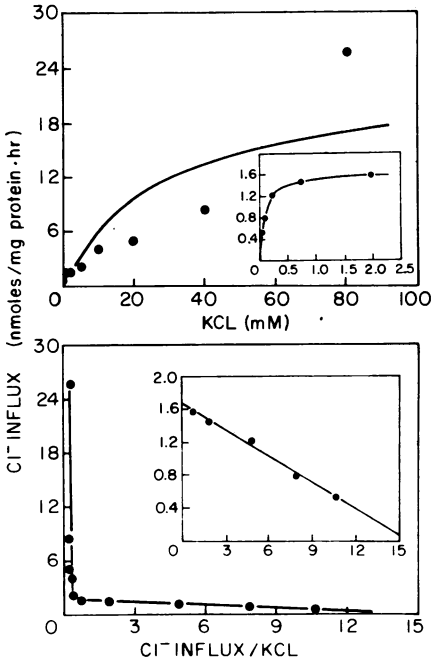

FIG. 5. Upper: $^{36}Cl^-$ influx into cultured tobacco cells as a function of increasing KCl. Influx was measured over a 50-min period in 2 mM Tris-MES (pH 6.0), 1 mM $CaSO_4$, and KCl as indicated (0.05–80 mM). Curves drawn as in Figure 3. Inset shows $^{36}Cl^-$ influx as a function of low Cl^- concentration (0.05–2.0 mM). Lower: Eadie, Hofstee plots of data. Inset shows data for low Cl^- concentration with line of best fit determined by linear regression.

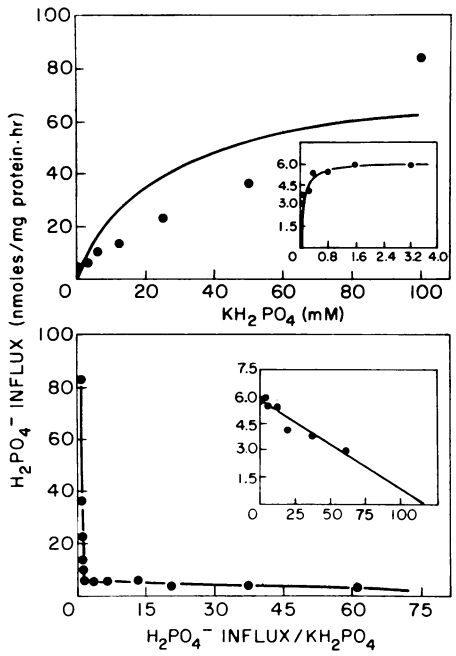

FIG. 6. Upper: $H_2{}^{32}PO_4^-$ influx into protoplasts isolated from cultured tobacco cells as a function of increasing KH_2PO_4 (0.05–100 mM). Influx (at pH 5.5) determined and curve drawn as in Figure 3. Inset shows $H_2{}^{32}PO_4^-$ influx as a function of low $H_2PO_4^-$ concentration (0.05–3.2 mM). Lower: Eadie, Hofstee plots of data. Inset shows data for low $H_2PO_4^-$ concentration with line of best fit determined by linear regression.

Table I. Kinetic constants for ion influx into protoplasts and intact cells of tobacco.

Kinetic constants were determined by the use of the third linear transformation of the kinetic data in Figures 3-6 as recommended in reference 1.

Ion	Range (mM)	Km (mM)	Vmax (nmole/mg protein·hr)	Hill Coefficient
		Isolated Protoplasts		
K^+	0.05-0.8	0.36	29	1.04
	0.05-100	*	*	0.64
$H_2PO_4^-$	0.05-3.2	0.058	5.9	0.98
	0.05-100	*	*	+
Cl^-	0.05-3.2	0.095	8.4	1.04
	0.05-100	*	*	+
		Intact Cells		
Cl^-	0.05-2.0	0.105	1.66	1.02
	0.05-80	*	*	+

* Kinetic constants could not be calculated.
\+ Hill plot gave a sigmoidal curve and the Hill coefficient could not be calculated.

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