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INHIBITION OF SERINE TRANSPORT INTO TOBACCO CELLS BY CHLORPROMAZINE AND A23187

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The transport of serine into cultured tobacco cells (*Nicotiana tabacum* L.) was inhibited 50% by 25 μM chlorpromazine or 2 μM A23187. The inhibition was rapid, being achieved within 10 to 20 min after addition of the inhibitor. Inhibition depended upon the continued presence of the inhibitor in the transport medium. Transport totally recovered within 1 h following transfer of the cells to medium lacking inhibitor. Transport did not recover after treating cells with 100 μM chlorpromazine because of a loss of cell viability. Chlorpromazine was not toxic and was less inhibitory to transport when La^{3+} replaced Ca^{2+} in the medium. The Ca^{2+} content of the cells, measured using $^{45}Ca^{2+}$, was increased more than 2-fold by chlorpromazine and A23187. The transport of sulfate into the cells was also inhibited by chlorpromazine and A23187. We propose that these two compounds inhibit transport by elevating free cytoplasmic Ca^{2+} which adversely affects the driving force for sulfate and serine transport.

Introduction

In tobacco cells, Ca^{2+} has three reported effects on transport [1–3]. First, Ca^{2+} causes a time-dependent stimulation of several transport systems, a process which is inhibited by cycloheximide [2] and La^{3+} [1]. Second, transfer of cells to Ca^{2+}-free media causes a rapid decline in the rate of amino acid uptake, this decline is prevented by the addition of La^{2+} [1]. Third, cells transferred to medium lacking Ca^{2+} lose substantial amounts of transported serine, this loss is significantly reduced by either La^{3+} or K^{+} [1]. The first of these effects was interpreted as indicating a function of Ca^{2+} in the transport process per se, possibly by the induction of physical or biochemical changes in the membrane which facilitate proton pumping [1,3]. Whereas the latter two effects were interpreted as indicating a structural role of Ca^{2+} in maintaining the functional integrity of the plasma membrane [1].

Our objective was to further characterize these calcium effects. The fact that the non-permeant ion La^{3+} can, in some respects, substitute for Ca^{2+} suggests that one site of Ca^{2+} action is superficial. In contrast, the fact that La^{3+} cannot substitute for Ca^{2+} in stimulating transport suggests either specific binding of Ca^{2+} to a membrane component or an intracellular site of action. In a variety of systems, chlorpromazine and A23187 have been useful as probes of Ca^{2+}-mediated processes. Chlorpromazine has been used as a relatively selective inhibitor of calmodulin-mediated processes [4–6] and a transport probe [7,8], whereas A23187 is a Ca^{2+}-ionophore which facilitates Ca^{2+} movement across biological membranes [9,10]. We used these compounds as probes to elucidate the

Abbreviation: bisTris propane, 1,3-bis(tris(hydroxymethyl) methylamino)propane.

role of Ca^{2+} in amino acid transport into cultured tobacco cells.

Materials and Methods

Nicotiana tabacum var. Xanthi cells were cultured in modified B5 medium [11]. L-[U-^{14}C]Serine and ^{45}Ca were purchased from Amersham, Arlington Heights, IL; A23187 from Calbiochem-Behring, La Jolla, CA; and chlorpromazine from Sigma Chemical Co., St. Louis, MO.

Transport. Cells (approx. 1 g) were harvested by vacuum filtration and washed with 50 ml of 5 mM Bistris propane (pH 6.0) containing 1% sucrose; care was taken not to dry the cells, i.e. allow the liquid level to drop below the level of the cells. The washed cells were sectored into quarters with a spatula and placed in 36 ml of 5 mM bisTris propane (pH 6.0) containing 1% sucrose and 0.5 mM $CaCl_2$. Transport was initiated by the addition of 4 ml of 5 mM [^{14}C]serine (0.25 μCi) and cells incubated for 20 min at 22°C. Cells were reharvested by vacuum filtration, washed with 30 ml of transport medium minus serine, weighed accurately on a Mettler balance and placed in 1 ml water and 10 ml liquid scintillation fluid.

Calcium distribution. Cells were harvested as above and incubated for 3 h in transport medium containing 0.5 mM $^{45}CaCl_2$ (1 Ci/mol). The cells were filtered and placed in 0.8 cm internal diameter columns (Polypropylene Econo-columns, Bio-Rad Laboratories, Richmond, CA) with 5 ml of unlabeled transport medium and the column stoppered. After 5 min the stopper was removed, the filtrate collected in a scintillation vial, 5 ml of transport medium was added to the cells and the column stoppered. This procedure was repeated at 10, 15, 20, 30, 40, 50, 60, 90, 120, 180 min. 10 ml liquid scintillation fluid was added to each sample and radioactivity counted.

Viability. The ability of cells to exclude Trypan blue was used as a measure of viability. Filtered cells were placed in 0.5% (w/v) Trypan blue in water and examined microscopically. Cells stained blue were considered to be non-viable.

Results and Discussion

The effect of culture age, pH and divalent cations on serine transport into tobacco cells has been reported [1]. Transport was concentration-dependent and saturable with half maximal rates of transport at $2 \cdot 10^{-5}$ M (Fig. 1).

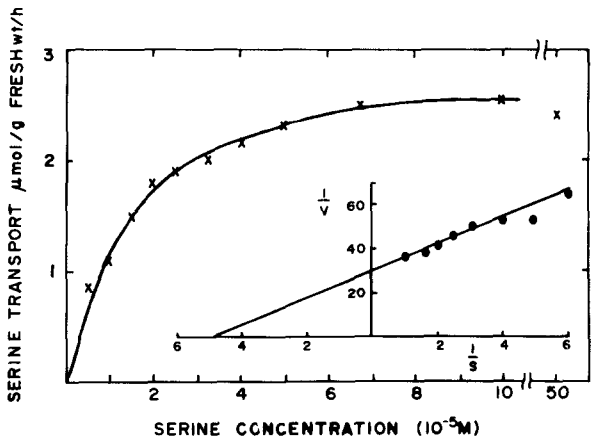

Fig. 1. Effect of serine concentration on serine transport into cultured tobacco cells. Cells were preincubated in 1% sucrose, 5 mM bisTris propane (pH 6.0) and 0.5 mM $CaCl_2$ for 4 h before addition of [^{14}C]serine. Insert: a Lineweaver-Burk plot of data, where $1/S$ is given in M^{-1} ($\times 10^{-4}$) and $1/v$ is given in g fresh weight·h·μmol^{-1} ($\times 10^{-4}$).

Inhibition of serine transport by chlorpromazine

Transport was inhibited greater than 50% by 25 μM chlorpromazine (Fig. 2) and inhibition was rapid, i.e. greater than 90% of the inhibition occurred within 10 min (Fig. 3). Transport recovered without a lag following transfer of cells into new medium lacking chlorpromazine. The time-course of recovery was independent of the period the cells had been exposed to chlorpromazine, cells placed in chlorpromazine for 10 min or 2 h recovered at the same rate (Fig. 3). The rapidity of the inhibition and the absence of a lag in the recovery supports the conclusion that chlorpromazine inhibits transport by interacting with a plasma membrane component. This may be a specific interaction as in the binding to calmodulin or a general hydrophobic interaction with the lipid bilayer as discussed by Landry and co-workers [7]. Upon removal of chlorpromazine from the transport medium, bound chlorpromazine would be released and normal membrane function would be rapidly restored. Intracellular binding of chlorpromazine to calmodulin is unlikely to be the cause of transport inhibition because removal of

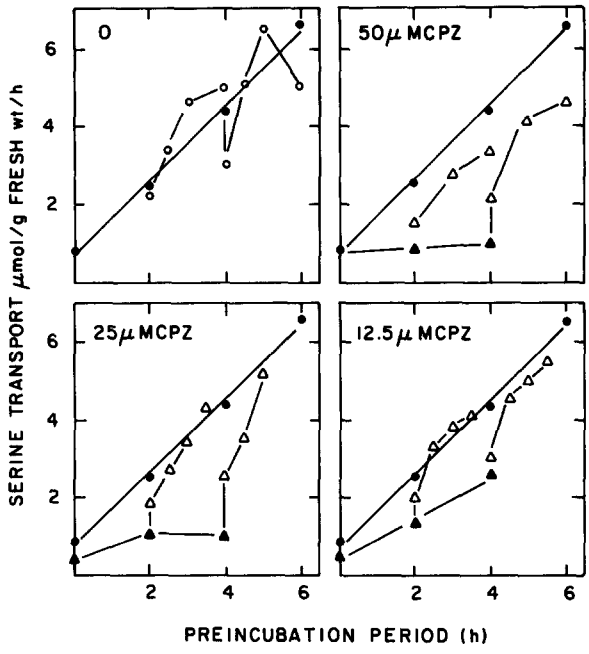

Fig. 2. Effect of chlorpromazine (CPZ) on serine transport into cultured tobacco cells. Cells were preincubated in 1% sucrose, 5 mM bisTris propane (pH 6) and 0.5 mM $CaCl_2$ for 2, 4, or 6 h before addition of [^{14}C]serine. At either 2 or 4 h some samples were harvested by vacuum filtration and placed in new medium and [^{14}C]serine added immediately or after 30, 60, 90 min. Transport was allowed to continue for 20 min. Symbols: ●———●, cells in preincubation medium; ○———○, cells harvested and transferred to fresh preincubation medium; ▲———▲, cells in preincubation medium containing chlorpromazine; △———△, cells preincubated in the presence of chlorpromazine for either 2 or 4 h before being transferred to preincubation medium.

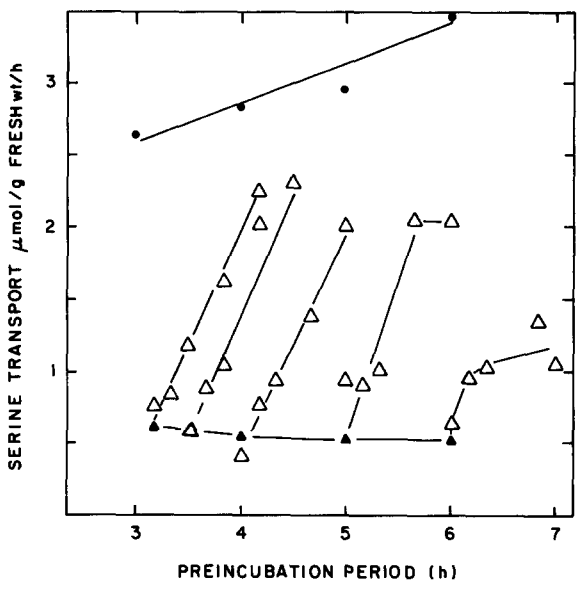

Fig. 3. Effect of chlorpromazine (CPZ) on serine transport into cultured tobacco cells. Cells were preincubated in 1% sucrose, 5 mM bisTris propane (pH 6) and 0.5 mM $CaCl_2$ for 3 h. In control cells (●———●) transport was initiated by the addition of [^{14}C]serine immediately (i.e. 3 h), 1 h, 2 h, or 3 h, later. Chlorpromazine (25 μM, final concentration) was added to the remaining samples. Either transport rates were determined in the presence of chlorpromazine after 10 min, 1 h, 2 h, or 3 h (▲———▲) or cells were harvested by vacuum filtration, at 10 min, 30 min, 1 h, 2 h, or 3 h, placed in new medium lacking chlorpromazine and transport measured immediately or after 10, 20, 40 and 60 min (△———△).

medium chlorpromazine would not immediately cause the release of intracellular calmodulin-bound chlorpromazine and therefore there would be a lag in the recovery.

Transport did not completely recover when cells were incubated in chlorpromazine for long periods (from 2 to 6 h) at moderate concentration (25 to 50 μM) or short periods (1 h) at high concentrations (100 μM). This was due to the loss of cell viability (Table I).

One potential site of chlorpromazine action is the calmodulin-stimulated, Ca^{2+}-dependent, plasma membrane ATPase, which has been demonstrated in microsomal vesicles and whose physiological function is believed to be lowering of cytoplasmic Ca^{2+} by pumping Ca^{2+} out of the cells [12,13]. The Ca^{2+} content of tobacco cells was investigated by incubating cells in $^{45}Ca^{2+}$ for 3 h in the presence and absence of chlorpromazine. Because cell walls bind from 3 to 3.5 μmol Ca^{2+}/g fresh weight, an efflux analysis has to be done to measure intracellular Ca^{+} [1]. 90% of the ^{45}Ca associated with the cells is lost within 5 min after transfer to unlabeled medium and is interpreted as being externally bound Ca^{2+}. To confirm this conclusion, cells were killed by boiling and incubated in $^{45}Ca^{2+}$ for 3 h prior to efflux analysis. These killed cells retained less than 1% of the initially bound Ca^{2+} in contrast to living cells which retained 11%. The slower efflux from living cells, representing loss of intracellular Ca^{2+}, was composed of two exponential phases, the first of which had a $t_{1/2}$ of 15 ± 2 min and is referred to as fast and the second had a $t_{1/2}$ of 300 ± 50 min and is referred to as slow. Incubation of cells for 3 h in

TABLE I

EFFECT OF CHLORPROMAZINE (CPZ) ON CELL VIABILITY

Cells were suspended in 1% (w/v) sucrose, 5 mM bisTris propane, pH 6.0 and incubated for the indicated periods.

Additions (final concn., μM)	% Viability, incubation period		
	1 h	2 h	3 h
$CaCl_2$ (500)	98	98	97
$CaCl_2$ (500)+CPZ (100)	25	2	0
$CaCl_2$ (500)+CPZ (25)	93	90	88
$CaCl_2$ (50)	97	91	90
$CaCl_2$ (50)+CPZ (100)	40	15	4
$CaCl_2$ (50)+CPZ (25)	85	84	64
$La(NO_3)_3$ (500)	95	97	98
$La(NO_3)_3$ (500)+CPZ (100)	96	88	92

chlorpromazine caused a more than two-fold increase in total intracellular Ca^{2+} and increased the $t_{1/2}$ of the fast compartment to 24 ± 3 min (Table II). These results are consistent with chlorpromazine inhibition of a calmodulin-dependent ATPase which pumps Ca^{2+} out of the cells. However, the effect of chlorpromazine on intracellular Ca^{2+} does not correlate with its ability to inhibit transport. As reported above, transport is immediately inhibited by chlorpromazine and recovers within 1 h upon removal of chlorpromazine. In contrast, cells placed in 25 μM chlorpromazine for 1 h did not have significantly higher levels of intracellular Ca^{2+} and the Ca^{2+} content of chlorpromazine-treated cells transferred into fresh medium did not decline to control levels within 1 h. We suggest that the elevation of total intracellular Ca^{2+} by chlorpromazine is not the cause of transport inhibition, rather it is the inhibition of Ca^{2+} efflux by chlorpromazine which results in an elevation of the 'free' cytoplasmic Ca^{2+} (a small fraction of the total) which is the cause. If this is the case chlorpromazine would be less inhibitory under conditions where no Ca^{2+} could enter the cells. Amino acid transport into tobacco cells is usually low in the absence of Ca^{2+}, however, cells incubated in Ca^{2+} to obtain high transport rates retain high rates when transferred to La^{3+} [1]. Inhibition of amino acid transport by 25 μM chlorpromazine is significantly reduced by 0.5 or 5 mM La^{3+} (Table III). The possibility that these results are due to a chemical interaction between chlorpromazine and La^{3+} is unlikely because La^{3+} is less effective in the presence of Ca^{2+}. We conclude that La^{3+} prevents chlorpromazine inhibition of transport by blocking Ca^{2+} entry into the cell. La^{3+} also overcame chlorpromazine cytotoxicity (Table I).

Incubation of cells in 0.5 mM Ca^{2+} for 6 h caused a 6-fold increase in the rate of amino acid

TABLE II

EFFECT OF CHLORPROMAZINE (CPZ) AND A23187 ON INTRACELLULAR $^{45}Ca^{2+}$

Two groups of cells were incubated in 1% (w/v) sucrose, 5 mM bisTris propane (pH 6), 0.5 mM $^{45}CaCl_2$, containing either 25 μM CPZ, 1 μM A23187 or no additions. After 3 h, one group of cells were harvested by vacuum filtration and the ^{45}Ca efflux measured as described in Materials and Methods. Efflux was composed of two exponential phases referred to as fast ($t_{1/2}$ = 15 ± 2 min) and slow ($t_{1/2}$ = 300 ± 50 min). The second group of cells were harvested by vacuum filtration, transferred to new medium and the intracellular ^{45}Ca measured after 1 h (4 h from the initiation of the experiment). Data is expressed as nmol $^{45}Ca^{2+}$/g fresh weight.

Initial medium	Ca distribution at 3 h		Transfer medium	Ca distribution at 4 h	
	Fast	Slow		Fast	Slow
0	30	250	0	40	240
0	30	250	25 μM CPZ	60	200
25 μM CPZ	70	660	0	60	500
25 μM CPZ	50	450	25 μM CPZ	60	600
0	40	260	0	40	270
0	50	320	1 μM A23187	80	700
1 μM A23187	65	750	0	55	900
1 μM A23187	55	750	1 μM A23187	55	800

TABLE III

EFFECT OF La^{3+} ON CHLORPROMAZINE (CPZ) INHIBITION OF SERINE TRANSPORT

Cells were incubated for 3 h in 1% (w/v) sucrose, 0.5 mM $CaCl_2$ and 5 mM bisTris propane (pH 6). These cells were transferred to 40 ml of 1% sucrose buffered with 5 mM bisTris propane (pH 6) and the indicated additions and incubated for 50 min before addition of [^{14}C]serine (0.5 mM, final concentration). The control transport rate was 4 μmol/g fresh weight per h.

Additions (final concn., μM)	% inhibition
$CaCl_2$ (500)+CPZ (25)	50
$La(NO_3)_3$ (5000)+CPZ (25)	8
$La(NO_3)_3$ (500)+CPZ (25)	12
$CaCl_2$ (500)+$La(NO_3)_3$ (5000)+CPZ (25)	37
$CaCl_2$ (500)+$La(NO_3)_3$ (500)+CPZ (25)	25

transport (Fig. 2). Calcium was more effective than Mg^{2+} as the stimulating cation and K^+ and La^{3+} were ineffective [1]. Chlorpromazine did not inhibit this process, because transport always recovered to the stimulated rate (Fig. 2). For instance, transport by cells preincubated in 25 μM chlorpromazine for 2 h recovered from 1 to 3.5 μmol/g fresh weight per h within 1 h whereas cells preincubated for 4 h recovered from 1 to 5.2 μmol/g fresh weight per h within 1 h. These results indicate that the stimulation of transport capacity by Ca^{2+} is a process independent of transport per se. In other systems, Ca^{2+} stimulates phospholipase activity resulting in an increase in membrane microviscosity [14,15]. We suggest that such a process would continue even when transport itself was inhibited and would be consistent with recovery to the stimulated rate upon removal of the inhibitor.

Inhibition of serine transport by A23187

The inhibition of serine transport by chlorpromazine was interpreted as being due to an elevation of 'free' cytoplasmic Ca^{2+} resulting from the inhibition of a Ca^{2+} efflux pump. The effect of A23187, a calcium ionophore, on serine transport was examined since this compound elevates intracellular Ca^{2+} by a completely different mechanism [9]. Serine transport was inhibited 50% by incubating cells in 2 μM A23187 for either 2 or 4 h

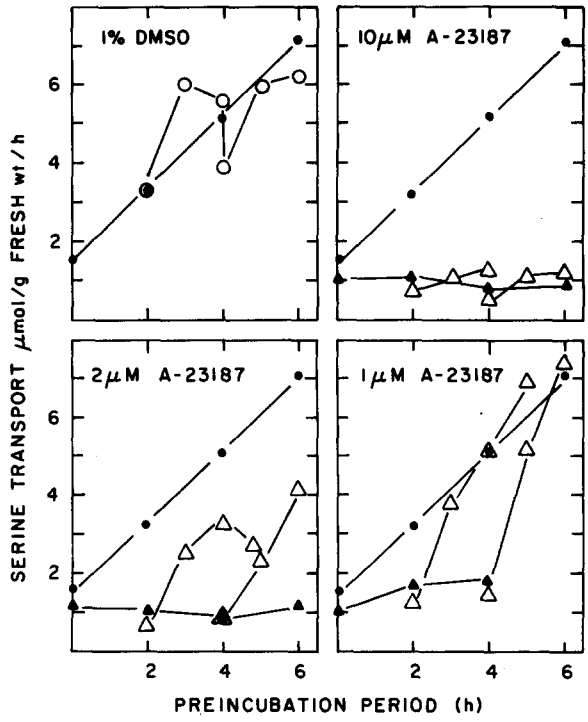

Fig. 4. Effect of A23187 on serine transport into cultured tobacco cells. Cells were preincubated in 1% sucrose, 5 mM bisTris propane (pH 6) and 0.5 mM $CaCl_2$ for 2, 4, or 6 h before addition of [^{14}C]serine. At either 2 or 4 h some samples were harvested by vacuum filtration and placed in new medium and [^{14}C]serine added immediately or after 1, 2, or 3 h. Symbols: ●——●, cells in preincubation medium; ○——○, cells harvested and transferred to fresh preincubation medium; ▲——▲, cells in preincubation medium containing A23187; △——△, cells preincubated in the presence of A23187 for either 2 or 4 h before being transferred to preincubation medium.

(Fig. 4). At low concentrations of A23187, transport was partially or totally restored when the cells were transferred to medium lacking the inhibitor. At high concentrations (10 μM), transport did not recover but this was not due to the death of the cells because cell viability was only reduced 20% by 50 μM A23187 (data not shown). A23187 increased the total Ca^{2+} content of the cells (Table II), but as in the case of chlorpromazine, recovery of transport occurred before reestablishment of control Ca^{2+} levels.

Inhibition of sulfate transport by chlorpromazine and A23187

The specificity of chlorpromazine and A23187

TABLE IV

EFFECT OF CHLORPROMAZINE (CPZ) AND A23187 ON SERINE AND SULFATE TRANSPORT

Transport methods are in Material and Methods. Sulfate transport medium was adjusted to pH 8 and the sulfate concentration was 50 μM. Control rates of transport were 85 nmol/g fresh weight per h for sulfate and 3.5 μmol/g fresh weight per h for serine.

Additions (final concn., μM)	% Inhibition of transport	
	Sulfate	Serine
CPZ (50)	82	87
CPZ (25)	75	28
CPZ (12.5)	43	27
A23187 (4)	72	72
A23187 (2)	56	43
A23187 (1)	33	10

as inhibitors of transport was examined. These compounds also inhibited sulfate transport (Table IV). The similarity of response of these different transport systems suggests that the inhibitors interfere with a process common to both, perhaps the supply of energy. Diverse transport systems in plants are believed to be energized in the same way [16].

Acknowledgements

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