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THE EFFECT OF MICROTUBULAR INHIBITORS ON TRANSPORT OF α-AMINOISOBUTYRIC ACID

INHIBITION OF UPHILL TRANSPORT WITHOUT CHANGES IN TRANSMEMBRANE GRADIENTS OF Na^+, K^+, or H^+

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Summary

A prior study indicated that vinca alkaloids partially inhibit the uphill transport of α-aminoisobutyric acid in Ehrlich ascites tumor cells. Maximum inhibition reduced the steady-state α-aminoisobutyric acid distribution ratio by only 25% leaving a large residual gradient. Studies were undertaken in two independent laboratories to correlate alterations in α-aminoisobutyric acid transport induced by vinca alkaloids with electrochemical potential differences for Na^+, K^+, or H^+ across the cell membrane. Vincristine reduced the transmembrane steady-state gradient for α-aminoisobutyric acid by 13% (Richmond) or 18% (Alabama), respectively. Vinblastine reduced this gradient by 14.5%. There was no concurrent change in the chemical gradients for Na^+, K^+, or H^+ across the cell membrane. A small, 4.08%, increase in the Cl^- distribution ratio would not account for the much larger change in α-aminoisobutyric acid gradients on the basis of a decrease in the electrochemical potential for intracellular Na^+. These data indicate that the decrease in the α-aminoisobutyric acid gradient across the Ehrlich ascites tumor cell membrane induced by vinca alkaloids cannot be attributed to a fall in the transmembrane electrochemical potential differences for Na^+, K^+, or H^+. The data suggest a role for cellular microtubules in the uphill transport of α-aminoisobutyric acid in this cell system.

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Introduction

Inhibitors of microtubular function, vinca alkaloids and colchicine, depress uphill transport of α-aminoisobutyric acid in Ehrlich ascites tumor cells, an effect which could not be attributed to an increase in the passive permeability of the cell membrane to α-aminoisobutyric acid [1]. Of particular interest was the observation that the inhibitory effect of vincristine (the vinca alkaloid studied most comprehensively), was only partial; maximum inhibition by this agent depressed the steady-state distribution ratio for α-aminoisobutyric acid by only 25%, leaving a large residual transmembrane α-aminoisobutyric acid gradient. This partial effect of vincristine suggested that there may be more than one source of energy which sustains uphill α-aminoisobutyric acid transport in these cells and that microtubular inhibitors may inhibit only one of these processes [1]. The sources of energy which sustain uphill transport of α-aminoisobutyric acid in Ehrlich ascites tumor cells have been attributed to (a) the electrochemical potential differences for Na^+ and possibly K^+ across the cell membrane, generated by the independent "Na^+ pump", and linked to the amino acid carrier by a cotransport with Na^+ or both of these ions, (b) a cotransport with H^+, and (c) the direct coupling of cellular energy metabolism to the amino acid carrier [2–5]. While a variety of approaches have been employed in an attempt to evaluate these possible mechanisms, an agent has not been identified which selectively depresses energy-dependent amino acid transport without concurrently decreasing at least one of these cation gradients. In this paper, data from two independent laboratories suggests that the inhibitory effect of vinca alkaloids on uphill transport of α-aminoisobutyric acid in the Ehrlich ascites tumor cannot be accounted for on the basis of changes in transmembrane electrochemical potential differences for Na^+, K^+, or H^+.

Methods

Cells and media. Ehrlich ascites tumor cells were maintained in both laboratories by weekly intraperitoneal inoculation and were used 6–8 days later. Contaminating erythrocytes were removed by low-speed centrifugation and the cells were washed and suspended into: (a) Alabama: Krebs-Ringer phosphate buffer containing: 130 mM NaCl, 8 mM KCl, 10 mM NaH_2PO_4/Na_2HPO_4, 2.0 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 1% bovine serum albumin; or (b) Richmond: 135 mM NaCl, 16 mM $NaHCO_3$, 1 mM Na_2HPO_4, 4.4 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$ with the pH maintained by passing warmed and humidified O_2/CO_2 (95 : 5, v/v) over the cell suspension. In both cases, the pH was 7.4, the buffer osmolality was 290 mosM and the final cytocrits were less than 3%.

Experimental and analytical procedures. Intracellular Na^+, K^+, Cl^- and physical properties of cells were determined in Alabama as described in detail previously [7]. Experimental and analytical technology employed in Richmond were as follows:

Cells were incubated with [^{14}C]inulin and α-amino[^{3}H]isobutyric acid or [^{3}H]inulin and 5,5-di[^{14}C]methyloxazolidine-2,4-dione for 3 min then divided into equal portions, one a control, the other containing vincristine. 30 min

later, 3—4 samples were obtained for analysis of $[H^+]_i$ in those suspensions incubated with [^{3}H]inulin and 5,5-di[^{14}C]methyloxazolidine-2,4-dione. 30—70 min later, six samples were taken for analysis of [α-aminoisobutyric acid]$_i$, $[Na^+]_i$, $[K^+]_i$, and $[Cl^-]_i$ in those suspensions incubated with [^{14}C]inulin and α-amino[^{3}H]isobutyric acid.

For analysis of $[Na^+]_i$, $[K^+]_i$, and $[Cl^-]_i$ on the same cell sample, cells were separated from the suspension by centrifugation. A cell pellet was drawn up into the tip of a Pasteur pipet, extruded in approximately two equal portions onto two separate glassine tares and wet weights taken on a Beckman LM-800 automatic balance. The two specimens were then processed further as follows: One specimen was dried overnight at 70°C, a dry weight obtained and the dried cell pellet digested in 0.25 ml of 1 M KOH (30 min at 70°C in a small glass vial). Chloride was determined on a 0.1 ml portion of the digest with the Buchler-Cotlove chloridimeter (Buchler Instruments, Inc., Fort Lee, N.J.). [^{14}C]Inulin was determined on another 0.1 ml portion as previously described [8,9]. From these measurements, the intracellular and extracellular water of a cell pellet could be determined as well as the ratio of the dry to wet weight (dry weight/wet weight), extracellular water to wet weight ($[H_2O]_e$/wet weight), and intracellular water to dry weight ($[H_2O]_i$/dry weight).

The other pellet on its glassine tare was transferred to a small glass vial, 0.3 ml of concentrated HNO_3 was added, and the pellet along with the tare was completely digested at room temperature. A 0.1 ml portion was analyzed for Na^+ and K^+ using a Model 143-03 IL flame photometer (Instrument Laboratory, Watertown, Mass.). Appropriate controls were obtained to assess Na^+ or K^+ associated with the tare, glassware or HNO_3. The [^{14}C]inulin content was determined in another 0.1 ml portion to correct for extracellular Na^+ and K^+. Intracellular water was calculated from the measured wet weight of this cell pellet and the parameters of the other half of the pellet which had been subjected to drying (see above), $[H_2O]_i$ = (dry weight/wet weight) ($[H_2O]_i$/dry weight) (wet weight). Analysis of Na^+ and K^+ in a "wet pellet" was required because of the difficulty in achieving acid digestion of dried cells.

Intracellular α-amino[^{3}H]isobutyric acid was determined in all cases on a cell pellet washed three times with 0°C buffered (pH 7.4) 0.85% saline. Intracellular 5,5-di[^{14}C]methyloxazolidine-2,4-dione and the accompanying [^{3}H]inulin extracellular marker were determined on the hydrolysate of a single unwashed cell pellet in the same manner as described above for chloride except for simultaneous determination of ^{14}C and ^{3}H on the liquid scintillation spectrometer.

Chemicals. [^{14}C]-, [^{3}H]Inulin, 5,5-di[^{14}C]methyloxazolidine-2,4-dione, and [^{14}C]sorbitol (the extracellular marker used in Alabama) were obtained from New England Nuclear (Boston, Mass.). α-Amino[^{3}H]isobutyric acid was obtained from ICN (Cleveland, Ohio). Vincristine and vinblastine sulfate were generously provided by Dr. Robert Hosley of Eli Lilly and Co., Indianapolis, Ind.

Results

The effects of vinca alkaloids on [α-aminoisobutyric acid]$_i$, $[Na^+]_i$, $[K^+]_i$, and the chloride distribution ratio

Fig. 1 is a representative experiment (Richmond) which illustrates the inhibi-

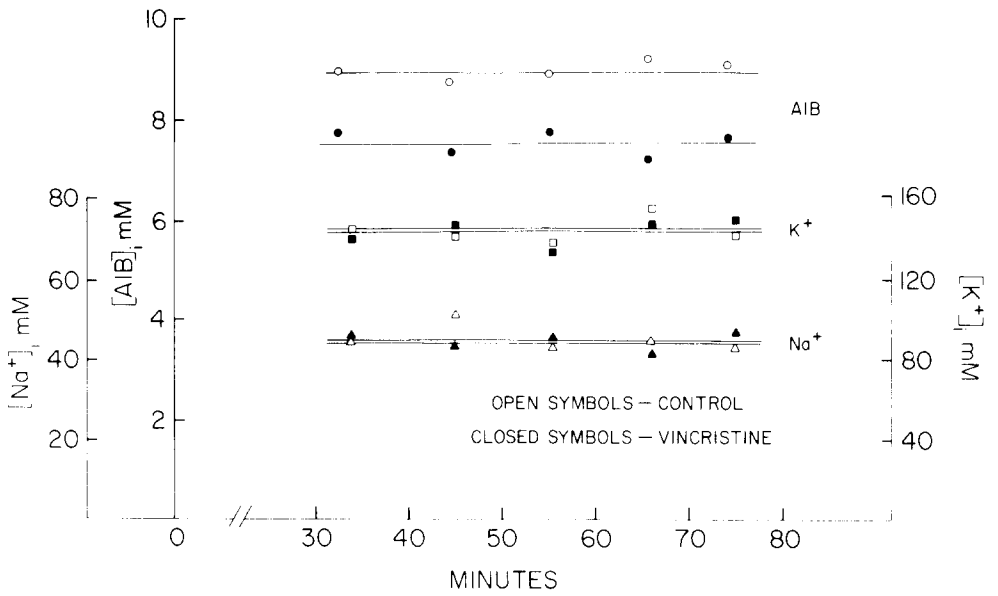

Fig. 1. The effects of 30 μM vincristine on steady-state intracellular concentrations of α-aminoisobutyric acid (AIB), Na^+ and K^+. The elapsed time after beginning the incubation at 37°C is shown on the abscissa. The extracellular α-aminoisobutyric acid concentration was 1 mM.

TABLE I

THE EFFECTS OF VINCA ALKALOIDS ON NET TRANSPORT OF α-AMINOISOBUTYRIC ACID, Na^+, K^+, Cl^- AND PHYSICAL PROPERTIES OF EHRLICH ASCITES TUMOR CELLS

(A) Richmond[1]	Control	30 μM vincristine	%Δ	P
$[Na^+]_i$ (mM)	49.56 ± 2.32[3]	49.57 ± 2.26	—	>0.5
$[K^+]_i$ (mM)	154.1 ± 2.3	153.4 ± 2.8	—	>0.5
$[Cl^-]_i/[Cl^-]_e$	0.505 ± 0.023	0.538 ± 0.018	+6.6	>0.1
$[AIB]_i$ (mM)[4]	9.16 ± 0.33	7.98 ± 0.33	−12.9	>0.001
$[H_2O]_e$/wet weight (μl/mg)[5]	0.2067 ± 0.0088	0.2039 ± 0.0071	—	>0.5
Dry weight/wet weight	0.1650 ± 0.0015	0.1706 ± 0.0014	+3.4	<0.001
$[H_2O]_i$/dry weight (μl/mg)[5]	3.808 ± 0.059	3.665 ± 0.0445	−3.8	<0.01

(B) Alabama[2]	Control	30 μM vinblastine	%Δ	P
$[AIB]_i/[AIB]_e$[4]	17.58 ± 0.70[3]	15.02 ± 0.34	−14.5	<0.01
$[Na^+]_i$ (mM)	17.00 ± 3.05	16.85 ± 2.85	—	>0.5
$[K^+]_i$ (mM)	137.7 ± 5.6	141.5 ± 4.3	—	>0.1
$[H_2O]_i$/dry weight[5]	3.421 ± 0.083	3.324 ± 0.089	−2.8	<0.01

[1] Cells were incubated with 1 mM α-amino[^{3}H]isobutyric acid and [^{14}C]inulin for 30 min then divided into two portions, one a control, the other exposed to 30 μM vincristine. 30—75 min later, portions of the cell suspensions were analyzed for the parameters noted above.

[2] Cells were brought to the steady-state in the presence or absence of vinblastine over 90 min following which the parameters noted above were measured. The extracellular α-aminoisobutyric acid concentration at the steady state was 3.4 mM.

[3] The data represents the mean ± S.E. of the average values from ten (Richmond) or four experments (Alabama) performed on different days. In each experiment average values were determined from 5—6 or three replicate measurements, respectively.

[4] AIB, α-aminoisobutyric acid.

[5] $[H_2O]_e$ and $[H_2O]_i$ are the extracellular and intracellular water of a cell pellet, respectively. Dry weight and wet weight are the dry and wet weights of a cell pellet, respectively.

TABLE II

THE EFFECTS OF VINCRISTINE ON THE TRANSMEMBRANE H^+ GRADIENT

A cell suspension was incubated with 12 μM 5,5-di[^{14}C]methyloxazolidine-2,4-dione and [^{3}H]inulin for 30 min in the presence or absence of the indicated agents. Following this, 3—4 determinations of intracellular and extracellular 5,5-dimethyloxazolidine-2,4-dione were obtained. The table indicates the mean ± S.E. of the average values from seven experiments done on different days. The *P* value was determined from an analysis of variance. $[H^+]_i$ was calculated according to the method of Poole [20] (Richmond).

Condition	$[H^+]_i/[H^+]_e$	*P*
Control	1.69 ± 0.17	—
Vincristine (1 μM)	1.90 ± 0.21	>0.2
Vincristine (30 μM)	1.81 ± 0.14	>0.1

tory effect of 30 μM vincristine on net α-aminoisobutyric acid transport under steady state conditions for this amino acid, $[Na^+]_i$, and $[K^+]_i$. Table I (section A) is a composite of 10 such experiments. There was no change in $[Na^+]_i$ or $[K^+]_i$ under conditions in which [α-aminoisobutyric acid]$_i$ fell by 13%. In this series of experiments, the chloride distribution ratio rose by 6.6%, a value which reached a level of significance of only $P > 0.1$. However, when this data was combined with 12 other experiments, a small increase in the chloride distribution ratio (4.08%) reached the 5% level of significance. Vincristine produced a small (3.8%) but significant ($P < 0.01$) decrease in $[H_2O]_i$/dry weight along with a 3.4% ($P < 0.001$) increase in dry weight/wet weight of a cell pellet, compatible with a small decrease in cell volume. The inulin space of the cell pellet, $[H_2O]_e$/wet weight, was unaffected by vincristine.

Similarly studies from Alabama indicate that 30 μM vincristine (not shown) or 30 μM vinblastine, another vinca alkaloid (section B, Table I) reduce the net α-aminoisobutyric acid level by 18.3 and 14.5%, respectively, without an alteration in $[Na^+]_i$ or $[K^+]_i$. Vinblastine produced a small, but significant, decrease in $[H_2O]_i$/dry weight.

The effect of vincristine on the transmembrane H^+ gradient

Table II indicates that these cells maintain a transmembrane chemical gradient for H^+ (intracellular > extracellular). Although vincristine (1 or 30 μM) tended to produce a small increase rather than a decrease in this gradient, in no case was this change statistically significant.

Discussion

Previous studies indicated that a small component of the uphill transport of α-aminoisobutyric acid in Ehrlich ascites tumor cells can be inhibited by vinca alkaloids and colchicine, compounds which interact with and inhibit cellular microtubules [1]. In this paper, studies from two independent laboratories indicate that this vinca alkaloid-induced decrease in the uphill transport of α-aminoisobutyric acid is not associated with a decrease in the chemical gradients of Na^+, K^+ or H^+ across the cell membrane. Although a small decline in the chloride distribution ratio was induced by vincristine, suggesting a decrease in the membrane potential [10,11], a small decrease in the electrochemical differ-

ence for Na^+ across the cell membrane would not account for the much larger fall in the α-aminoisobutyric acid gradient. As considered previously by Jacquez and Schafer [12], if each mole of α-aminoisobutyric acid which enters the cell is coupled to the influx of Na^+ and the countertransport of K^+, so that the system is not electrogenic, there would be no decrease in the α-aminoisobutyric acid gradient with a change in membrane potential unless the chemical Na^+ gradient was reduced. On the other hand, assuming the opposite extreme, if the Na^+ flux by the α-aminoisobutyric acid carrier was not coupled to the K^+ flux at all, then a decline in the α-aminoisobutyric acid distribution ratio would be expected with a decline in the membrane potential. However, if the transmembrane voltage is −17.8 mV in control cells and −16.1 mV in cells exposed to 30 μM vincristine (based upon the chloride distribution ratios from Table I) the change of 1.7 mV in the transmembrane voltage would predict a maximum decrease of 6% in the α-aminoisobutyric acid distribution ratio or less than one-half the decrease observed in Richmond and one-third the decrease observed in Alabama. Further, we have conducted other studies in which we find that to produce comparable depression of net α-aminoisobutyric transport on the basis of a change in Na^+ gradient alone, as with ouabain, requires a 36% decrease in the transmembrane Na^+ gradient [13].

It is, of course, possible that vinca alkaloids might produce shifts in the distribution of Na^+ between cytoplasmic and nuclear pools or alter the ratio of bound to free cell Na^+, thereby increasing the true cytoplasmic Na^+ concentration without changing the total cell Na^+. Such a mechanism was suggested by Heinz [3] as an explanation for metabolic inhibition without apparent changes in intracellular cation concentrations. Alternatively, vinca alkaloids might decrease the coupling efficiencies of the Na^+ and/or K^+ electrochemical potential gradients to the α-aminoisobutyric acid carrier, or in other ways alter the properties of the translocation of the cation-amino acid-carrier complex within the cell membrane or inhibit the coupling to an energy source other than these gradients. While this energy source could involve coupling to other transmembrane solute gradients, a role for the H^+ gradient seems to be excluded by the observation that vincristine did not decrease the H^+ gradient across the cell membrane.

While vinca alkaloids at high concentrations non-specifically interact with cellular proteins (10^{-3} M; ref. 14) and inhibit Na^+ transport as well as other functional properties of cell membranes ($>10^{-4}$ M; refs. 15—17), the concentration of vinca alkaloids which maximally inhibits net α-aminoisobutyric acid transport ($<5 \cdot 10^{-7}$ M; ref. 1) is orders of magnitude lower and similar to the binding constants of these agents for isolated tubulin [18,19]. Hence, the data suggest that the interaction between vinca alkaloids and the α-aminoisobutyric acid transport system represents a specific effect of these agents on cellular microtubules which play a role in the uphill transport of α-aminoisobutyric acid in the Ehrlich ascites tumor.

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