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BIMODAL EFFECTS OF CELLULAR AMINO ACIDS ON Na^+-DEPENDENT AMINO ACID TRANSPORT IN EHRLICH CELLS

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Summary

Cells depleted of amino acids show lower rates of glycine or aminoisobutyric acid uptake than do freshly isolated cells. In the amino acid-depleted cells, addition of valinomycin stimulates amino acid influx at least to the level observed in freshly isolated cells. In cells containing high levels of cellular amino acids, valinomycin has little effect on influx of amino acids. It is concluded that the transport of amino acids in freshly isolated cells is elevated compared to depleted cells because the cells are hyperpolarized by the continuous loss of cellular amino acids during the transport assay. During this hyperpolarization by amino acid loss, transport of amino acids is not further stimulated by valinomycin at low external $[K^+]$ (10 mM ± 5 mM).

With the exception of preloading with glycine, cells preloaded with a single amino acid to a concentration greater than 20 mM show reduced rates of glycine and aminoisobutyric acid influx at early times (less than 15 min) compared to amino acid-depleted cells. The reduction of influx is transient and by 30 min, influx is greater in preloaded than in amino acid-depleted cells.

Knowing that increases and decreases in the membrane potential are achieved by using varying external $[K^+]$ in the presence of valinomycin and propranolol, and using amino acid-depleted cells, it can be shown that an increased membrane potential increases the V for glycine and aminoisobutyric acid influx. A decrease in the potential difference results in a decreased V. Changes in K_m also occur when the membrane potential is varied.

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Introduction

It has recently been shown that an increase in the membrane potential (inside negative) stimulates influx of amino acids by the Na^+-dependent route in a number of intact animal cells or plasma membrane vesicles [1—9]. Moreover, it has also been demonstrated that transport of amino acids coupled to Na^+ causes transient depolarization of tumour cells and intestinal cells [10—14].

This behaviour raises some interesting questions about the effect of changes in membrane potential per se on the kinetic characteristics of amino acid transport. Na^+ has been shown to alter K_m for amino acid transport under conditions where $\Delta\tilde{\mu}_{Na} = 0$ with relatively less effect on V [15]. Geck and Heinz [16] and Heinz and Geck [17] have put forth theoretical models which predict that in K_m systems (i.e., systems in which K_m changes as the Na^+ gradient changes) an increase in membrane potential will cause a decrease in K_m and an increase in V. V systems (i.e., systems in which V changes as the Na^+ gradient changes) will also show changes in both parameters, but with differences in which the relative values of the parameters change.

The addition of valinomycin and propranolol to Ehrlich cells causes a variable change in membrane potential dependent on external K^+ concentration [2—5]. By using valinomycin and propranolol at variable external K^+ concentrations (and in the presence of glucose to compensate for the effects of valinomycin on oxidative phosphorylation) we were in a position to monitor the effects of a variable membrane potential on the kinetic parameters for amino acid uptake in Ehrlich cells while maintaining Na^+ at constant levels internally and externally.

In addition to the fact that uptake of amino acids causes depolarization, we have recently shown that efflux of cellular amino acids may cause hyperpolarization of the Ehrlich cells [18]. This observation suggests that when a dilute cell suspension is incubated in vitro in an Na^+ medium, the cell may be exhibiting changes in the membrane potential which may, in turn, affect the parameters under investigation. Since we observed that on incubation in dilute suspension, Ehrlich cells gradually lost most of their cellular amino acid content and became depolarized [18], we turned our attention to the consequences of depleting the amino acid pool on influx of amino acids as well as the effect of elevating the cellular pool with a single amino acid on amino acid influx.

Materials and Methods

Ehrlich cells were maintained in Swiss white mice by weekly transfers and harvested as previously described [19]. The cells were washed at room temperature. After washing to remove serum and red cells, the cells were centrifuged for 2 min at $1400 \times g$ to obtain an approximate 'packed' cell volume. The cells were then diluted with a 300-fold excess (0.3% cytocrit) of Na^+/Ringer's medium containing 145 mM NaCl, 5 mM KCl, 1.5 mM $MgSO_4$ and 1.5 mM KH_2PO_4 buffered with 10 mM K^+/Hepes, pH 7.4. The cells were incubated in a warm room for 60—90 min using a magnetic stirrer to keep the cells agitated.

After preincubation to empty the cells of endogenous amino acids, the cells were recentrifuged, washed once with isotonic saline and resuspended in isotonic choline/Ringer's medium.

To obtain cells preloaded with various amino acids the above procedure was followed except that the preincubation medium contained 5—10 mM of the specified amino acid.

The incubation medium was of the same ionic composition as that used for preincubation with the following exceptions. When the Na^+ concentration was varied, choline chloride was used as replacement. The Na^+ concentration never exceeded 125 mM because the cells were introduced as a suspension in choline chloride. External $[K^+]$ was varied as given. Glucose (10 mM) was present in all incubations.

After resuspension in choline, the cells were brought to the temperature of the bath (5 min) and then introduced into the reaction flask containing the complete medium except the labeled amino acid. The final cell cytocrit in the incubation flask was 2.0—2.5%. The reaction was initiated by injection of amino acid. Samples (1-ml) were taken at specified intervals and injected into tared tubes containing 10 ml cold isotonic choline chloride. Wet and dry weights were determined as before [19]. In these experiments, 20 mg fresh wt. of tissue contained 11 μl cell water and 3 mg dry wt. Extracellular space was 30% of the fresh weight.

Despite the high accumulation of some amino acids, there was no apparent change in the cell water/dry weight ratio in these experiments which remained throughout at 3.6 ± 0.1 μl/mg dry wt. An increase in cell pellet fresh weight of 2.0 mg (av. wt. 22 mg) would not have been statistically significant in the spread of values obtained between replicates for fresh weight and hence such differences might have been overlooked. However, in a test run we examined the cell cytocrit of cells incubated without and with 10 mM α-aminoisobutyric acid. In these experiments containing $1.14 \cdot 10^8$ cells/ml suspension (depleted cells) and $1.27 \cdot 10^8$ cells (α-aminoisobutyric acid-incubated cells) the cell cytocrits were 22 and 20%, respectively. These values do not indicate major changes in cellular fluid volume on incubation with amino acids. Jacquez [20] reported a maximum increase of 13% in cell water with high levels of α-aminoisobutyric acid in the medium.

Although it is more appropriate to express fluxes in terms of a parameter such as cell protein which may more reliably relate to cell surface area, we have shown most of the data as cellular concentrations. Since the ratio between cellular water and dry weight remained apparently constant, conversion of the data to a dry weight basis may be attained using the factor of 3.6 μl cell water/mg dry wt.

Washed pellets were extracted with ethanol and a sample was counted with liquid scintillation using a modified Bray's solution.

Valinomycin was purchased from Calbiochem, U.S.A. It was used at a concentration of 5 μg/ml. (±)-Propranolol was obtained from Sigma, St. Louis, MO. All labelled amino acids were obtained from New England Nuclear Corp., Boston, MA.

Results

For some years we have observed that Ehrlich cells preincubated at 37°C for 30—60 min at a cytocrit of 3% show approx. 30—50% less [^{14}C]glycine uptake than fresh cells or cells kept on ice for the equivalent period (unpublished observation). Recently, the significance of this observation became apparent when it was observed that upon incubation there is a loss of cellular amino acids and that during the period of amino acid loss, there is concomitant hyperpolarization of the cell [18]. The extent of hyperpolarization is dependent on the concentration of cellular amino acids and on cell [Na^+] [18]. As the cellular amino acid pool is depleted, the cells depolarize and maintain a potential of approx. 30 ± 5 mV (inside negative) for 1—2 h [14]. Since the cellular amino acid pool influences the membrane potential and the membrane potential in turn influences amino acid uptake [1—5], it would be anticipated that amino acid uptake would be more rapid during the period of Na^+-dependent net amino acid efflux (i.e., freshly isolated cells) than in cells that have been preincubated in the absence of amino acids and hence have lost most of the amino acid pool.

The results in Fig. 1 show that this prediction is true and that the rate of

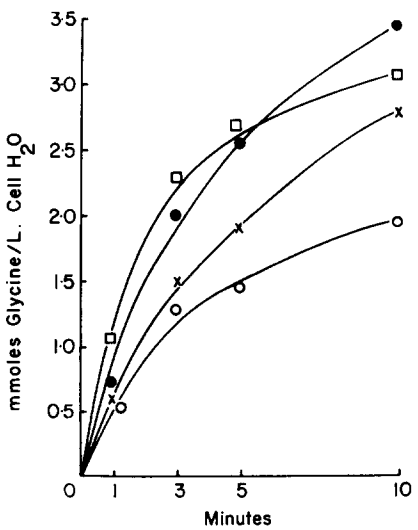

Fig. 1. Effect of preincubation on the response to valinomycin. Cells were preincubated in Krebs-Ringer medium at 0.3% cytocrit for 1 h at 37°C (○———○, □———□) or resuspended in the original ascitic fluid at 10% cytocrit (●———●, X———X). After this period, the cells were washed, resuspended in choline chloride and brought to the temperature of the bath (37°C) for 10 min. Then the cells were injected into the incubation media at a final cytocrit of 2%. The final $[Na^+]_o$ was 90 mM, $[K^+]$ was 10 mM and the buffer was 10 mM Hepes. Isotonicity was maintained with choline chloride. Uptake of [1-^{14}C]glycine from 0.5 mM was measured. Valinomycin, where present (□———□, X———X), was 5 μg/ml. To terminate the reaction, 1-ml samples were injected into cold isotonic choline chloride and centrifuged at 1400 X *g*. The pellets were wiped dry and weighed. Results are expressed as mmol glycine per l cell water (55% of the fresh weight as determined in separate experiments). A representative experiment from three separate experiments is shown. (●———●) stored at 10% cytocrit in ascitic fluid, (X———X) stored at 10% cytocrit in ascitic fluid + valinomycin (added during transport assay), (○———○) emptied at 0.3% cytocrit at 37°C, (□———□) emptied at 37°C at 0.3% cytocrit + valinomycin (added during transport assay).

glycine uptake is less in preincubated cells than in freshly isolated cells.

Preincubation may lead to deterioration of transport activity. To demonstrate that the transport system had not deteriorated during preincubation and that the drop in transport could be restored by increasing the membrane potential, depleted and 'nondepleted' cells were challenged with valinomycin. The data (Fig. 1) show that in depleted cells challenged with valinomycin, uptake of glycine returned to approximately the same level as that seen in the nondepleted cells whereas valinomycin tended to inhibit glycine uptake in nondepleted cells. The effect of valinomycin on amino acid uptake is variable in nondepleted cells. At a K_o^+ level of 10—15 mM (the usual level in the basic medium in these experiments) valinomycin either had no effect (approx. 30% of experiments) or caused an inhibition of 15—25% in the rate of glycine uptake as seen in Fig. 1 ($n > 40$). At a K_o^+ level below 5 mM, a transient increase in uptake of 25—35% was observed at early times (1—5 min) in approx. 50% of the experiments and little or no effect in the remainder of the experiments ($n > 20$).

We have long been puzzled by the fact that in our hands valinomycin did not show a sustained stimulation of amino acid uptake in freshly isolated cells with 10 mM K^+ in the medium. In cells depleted of ATP, however, valinomycin did stimulate uptake of amino acids if the.cellular $[K^+]$ was maintained at 100 mM or more (unpublished data). Gibb and Eddy [2] reported a different result, i.e., stimulation of amino acid uptake by valinomycin in ATP-containing cells. It is possible that under their conditions of handling the cells, the cellular amino acid pool had been depleted.

Using propranolol instead of valinomycin, we have shown increases or decreases in membrane potential depending on the $[K^+]_o$ level [5]. Using propranolol (with $[K^+]_o = 10$ mM) instead of valinomycin to increase the membrane potential [5], stimulations of amino acid uptake were seen in nondepleted as well as amino acid-depleted cells. However, the stimulation of amino acid uptake by propranolol was approximately twice as high with amino acid-depleted cells as with nondepleted cells. We reported earlier [5] that propranolol did not stimulate amino acid uptake in approx. 25% of experiments with Ehrlich cells and we did not know why. Since introduction of the depletion procedure, propranolol stimulated uptake in all experiments ($n > 20$). Moreover, in nondepleted cells, $1 \cdot 10^{-3}$ M propranolol was necessary to increase the initial rate of glycine uptake 25—50%. With depleted cells, $5 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ M propranolol increased the initial rate by 40—80%. In depleted cells, concentrations of $1 \cdot 10^{-4}$ M propranolol gave optimal stimulations of amino acid transport.

Influence of the amino acid pool on the response to valinomycin

To demonstrate that valinomycin's efficacy in stimulating amino acid uptake was dependent on the amino acid pool, cells were preincubated without and with various amino acids to obtain cells containing variable cellular amino acid pools. The results show (Fig. 2) that glycine uptake is greatly stimulated by valinomycin only in cells depleted of amino acids. If the cellular amino acid level is maintained by preloading with glycine or aminoisobutyric acid, addition of valinomycin causes relatively little stimulation of glycine influx. The rate of

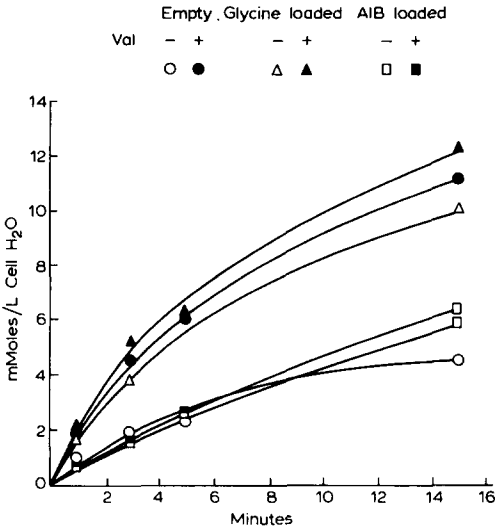

Fig. 2. Glycine uptake in glycine or aminoisobutyric acid-preloaded cells. Effects of valinomycin. Washed cells were incubated at 0.3% cytocrit for 60 min at 37°C without amino acid (○———○, ●———●), with 10 mM aminoisobutyric acid (AIB) (□———□, ■———■) and with 10 mM glycine (△———△, ▲———▲). After the preincubation, the cells were rewashed, suspended in isotonic choline chloride and brought to 37°C. The cells were injected into incubation flasks containing 105 mM Na^+ and 5 mM K^+ in Na^+/Hepes buffer, pH 7.4, at 37°C. The final cell cytocrit was 2%. The valinomycin concentration, where present (●———●, ▲———▲, ■———■), was 5 μg/ml medium. [1-^{14}C]Glycine (0.5 mM) was added to start the reaction. A typical experiment is shown from at least six similar experiments.

TABLE I

EFFECT OF PRELOADING WITH AMINO ACIDS ON THE RESPONSE OF GLYCINE AND AMINOISOBUTYRIC ACID TRANSPORT TO VALINOMYCIN

Cells at 0.3% cytocrit were preincubated for 1 h at 37°C with and without 10 mM of various amino acids. Then the cells were quickly spun down, washed and introduced into a normal Krebs-Ringer medium in Hepes buffer containing 7 mM K_o^+ and 10 mM glucose at a final cell cytocrit of 2%. Samples were incubated with and without 5 μg/nl valinomycin. [1-^{14}C]Glycine and [1-^{14}C]aminoisobutyric acid (AIB) were injected into the reaction flasks and samples were taken over 1—30 min interval. The results are expressed as the percent increase of uptake with valinomycin after 2—5 min incubation. Each experiment is representative of two similar experiments. The cellular level of the preloaded amino acid was in excess of 20 mM at the start of the experiment. ACPC, Aminocyclopentane carboxylic acid.

Test amino acid (0.5 mM)	Amino acid in preload	Percent increase in amino acid uptake with valinomycin
Glycine	none	120
	AIB	5
	glycine	20
	none	74
	methionine	7
	valine	55
	none	78
	methionine	25
	threonine	30
	none	147
	ACPC	3
AIB	none	105
	AIB	17
	methionine	19

amino acid uptake is also influenced by the nature of the amino acid used for preloading. Cells preloaded with glycine show higher rates of uptake than cells preloaded with aminoisobutyric acid. However, valinomycin has relatively little stimulatory effect on glycine uptake in cells preloaded with either glycine or aminoisobutyric acid. The general nature of this result is shown in Table I with a number of amino acids. With the exception of valine, cells preloaded with amino acids show less than half of the stimulation of uptake seen in 'empty cells' in response to valinomycin ($n > 50$).

In a previous communication, we reported that Ehrlich cells preloaded with amino acids like glycine and aminoisobutyric acid become significantly hyperpolarized compared to amino acid-depleted cells when the cells are introduced into an amino acid-free medium [18]. The present results show that the extent of stimulation of amino acid influx by valinomycin is influenced by the level of cellular amino acids, cells containing high levels of amino acids showing relatively little enhancement of amino acid influx when valinomycin is added. It is likely that cells already hyperpolarized due to the loss of cellular amino acids during the period when transport is assayed do not respond further to valinomycin.

Trans inhibition: effect of cellular amino acids on amino acid influx

While net efflux of amino acids may mask a stimulation of influx of amino acids by valinomycin, high cellular levels of several amino acids may also inhibit glycine or aminoisobutyric acid influx in a transient manner. Thus, we find that cells preloaded to high levels (greater than 20 mM) with amino acids such as aminoisobutyric acid, threonine, methionine and aminocyclopentane carboxylic acid, may show lower initial rates of uptake of aminoisobutyric acid or glycine than emptied cells. The exception is glycine since cells preloaded with glycine (up to levels greater than 50 mM) always show an increase in influx of aminoisobutyric acid or glycine compared to emptied cells.

The results in Table II show that cells preloaded to contain high concentrations (greater than 20 mM) of aminoisobutyric acid, methionine, threonine, or aminocyclopentane carboxylic acid show a decrease in the initial rate of glycine or aminoisobutyric acid uptake compared to amino acid-depleted cells. In 10 experiments, glycine uptake from a concentration of 0.5 mM dropped to 1.7 * ± 0.5 nmol/min per mg dry wt. in cells preloaded with 5 mM aminoisobutyric acid compared to 2.8 ± 0.8 nmol/min per mg dry wt. in amino acid-depleted cells. However, the inhibition of glycine (or aminoisobutyric acid) influx by cellular amino acids is transient. With time, the uptake of the labelled glycine in cells preloaded with amino acids is increased above the level seen in emptied cells. Fig. 3 shows a typical experiment of glycine influx in aminoisobutyric acid-preloaded cells. Table III shows several experiments illustrating the fact that, initially, glycine uptake is reduced in aminoisobutyric acid-containing cells and that during the course of the incubation, glycine uptake in aminoisobutyric acid-containing cells overtakes the uptake in control cells. The phenomenon is shown for glycine uptake with cells preloaded with different concentra-

* 0.48 nmol/min per ml cell water.

TABLE II

TRANS INHIBITION OF AMINO ACID UPTAKE

Washed cells (0.3% cytocrit) were incubated for 60 min at 37°C in the absence or presence of 10 mM amino acid (preloading). Then the cells were rewashed, brought to the temperature of the bath (37°C) and introduced into a normal Ringer's medium containing 10 mM K^+/Hepes buffer, pH 7.4, and 10 mM glucose. The final cell cytocrit was 2%. ^{14}C-labelled amino acid was injected and samples were taken over a 30 min period. The values after 1 min incubation are given. The average cellular amino acid level in the preloaded cells was in excess of 20 mM. Each set represents a different experiment executed on different days. Each is representative of at least two similar experiments. The variability in the response to preloading is evident (Expts. 1 and 2; 6 and 7).

Expt. no.	Test amino acid (0.5 mM)	Amino acid used in preloading	Uptake (mmol/min per l cell water)
1.	Glycine	none	0.98 *
		AIB	0.67
2.		none	0.87
		AIB	0.41
3.		none	1.20
		L-methionine	0.45
		L-valine	1.20
4.		none	1.16
		L-threonine	0.90
5.		none	0.77
		ACPC	0.53
6.	AIB	none	2.68
		AIB	1.60
7.		none	3.10
		AIB	1.40
		methionine	1.37

* An uptake of 0.98 mmol/min per l cell water is equivalent to 3.5 nmol/min per mg dry wt.

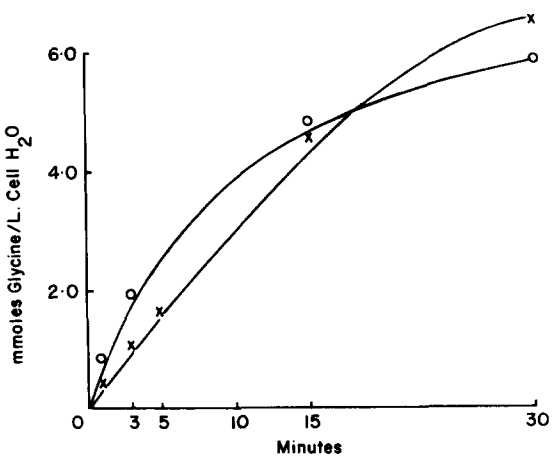

Fig. 3. Transient nature of inhibition by cellular aminoisobutyric acid on glycine uptake. For experimental conditions see Table III. (o———o) Control depleted cells, (X———X) cells preloaded with 10 mM aminoisobutyric acid.

TABLE III

PERCENT INHIBITION OF GLYCINE UPTAKE AT VARYING CELLULAR AMINOISOBUTYRIC ACID CONCENTRATIONS

Conditions as in Table II, except that during the preloading period, various concentrations of aminoisobutyric acid (AIB) were used to obtain the given cellular aminoisobutyric acid concentrations after 60 min incubation.

Expt. no.	Cellular amino acid after preloading (mM)		Percent control uptake at (min)			
			0.5	1	5	30
1.	$[AIB]_c$	0.8	—	100	100	100
		9.0	—	106	136	119
		32.0	—	74	125	149
		54.0	—	84	116	175
		78.0	—	78	115	160
2.	$[AIB]_c$	12.5	72	87	73	84
		26.0	55	64	60	112
		41.0	42	47	42	119
		69.0	24	25	38	110
		84.0	—	27	30	107
3.	$[AIB]_c$	22.0	80	73	75	140
		46.0	64	50	50	135
4.	$[Glycine]_c$	89.0	164	156	154	238

tions of aminoisobutyric acid. With different lots of cells, the time for switchover from inhibition to stimulation is very variable but usually occurs earlier than 15 min at 37°C.

Although attempts were made to determine the cellular aminoisobutyric acid concentration necessary to cause 50% inhibition of glycine uptake, the results are not conclusive. The data in Table III show qualitatively that a cellular level of over 25 mM aminoisobutyric acid is required to obtain 50% inhibition of glycine uptake from a 0.5 mM solution, but the problem is complicated by the transient nature of the inhibition and the changing cellular aminoisobutyric acid concentration. Clearly, however, the *trans* inhibition by aminoisobutyric acid of glycine uptake is less effective than inhibition from the *cis* side and is probably due to the fact that the K_m at the cytoplasmic side of the membrane is much greater than the K_m at the external side [15,21,23,24].

It is unlikely that inhibition of glycine uptake at early times in aminoisobutyric acid-containing cells is due to competition on the *cis* side between glycine and the aminoisobutyric acid lost from the cell during the experiment. The reasons for this conclusion are as follows.

(1) Inhibition due to preloading decreases with time (Fig. 3, Table III). If the inhibition were due to aminoisobutyric acid in the medium, the inhibition would increase with time as the concentration of aminoisobutyric acid in the medium rises.

(2) Assuming that all the cellular aminoisobutyric acid were lost to the medium, the effective aminoisobutyric acid concentration in the medium would be insufficient to bring about an inhibition equivalent to that observed. The data in Table IV show that to obtain equivalent inhibition from the *cis*

TABLE IV

COMPARISON BETWEEN INTRACELLULAR AND EXTRACELLULAR ACTION OF AMINOISOBUTYRIC ACID ON GLYCINE UPTAKE

Cells were incubated for 60 min without and with 10 mM aminoisobutyric acid (AIB) at 37°C at 0.3% cytocrit. In the latter cells the aminoisobutyric acid concentration was 73 mM at the end of 60 min. After the preincubation, cells were washed, brought to the temperature of the bath and introduced into a normal Ringer's medium with Hepes buffer and 10 mM glucose. The final cytocrit was 2%. The 'empty' cells were introduced into media containing aminoisobutyric acid at 0, 2.8 and 11.2 mM. No extracellular aminoisobutyric acid was added to the medium containing aminoisobutyric acid-preloaded cells. If all the cellular aminoisobutyric acid in the preloaded cells were released into the medium the concentration of aminoisobutyric acid would be less than 1 mM. [1-^{14}C]Glycine (final concentration 0.5 mM) was injected into the medium and samples taken at the intervals given. The experiment is representative of three similar experiments. Results are expressed as mmol/l cell water.

Time (min	Depleted cells			10 mM AIB in preload
$[AIB]_o$ (mM):	0	2.8	11.2	<1
1	0.70	0.32	0.19	0.19
3	1.50	0.64	0.35	0.46
5	2.10	0.89	0.48	0.73
15	2.50	1.57	0.85	1.79
30	5.30	2.50	1.21	3.37

side to that observed with preloaded cells would require 11 mM aminoisobutyric acid in the medium. Since a 2% cytocrit is used in these experiments and the cell water volume of the cells is 55—60% of the packed cell volume, the amino acid concentration of the medium will be approx. 1.2% of the original cellular amino acid concentration (less than 1 mM aminoisobutyric acid starting from an initial cellular aminoisobutyric acid concentration of 70 mM). Thus, under the present experimental conditions the amino acid lost to the medium is not sufficient to cause the inhibition of glycine uptake.

In contrast to preloading with most amino acids, preloading with glycine always results in a stimulation of [^{14}C]glycine influx. The results (Fig. 2, Table III) show that even when the cellular level of glycine is greater than 50 mM there is no *trans* inhibition of glycine influx. Indeed, glycine uptake is stimulated in glycine-preloaded cells as was originally reported by Heinz and Walsh [25]. In six experiments using glycine at 0.5 mM, the control influx was 2.2 ± 0.7 nmol/mg dry wt. in depleted cells and 4.1 ± 1 nmol/mg dry wt. in glycine-preloaded cells. If *trans* inhibition by cellular glycine on glycine uptake can occur, it is too small to overcome the stimulation of uptake by cellular glycine. The lack of inhibition even at high cellular glycine levels is in line with the high K_m for glycine efflux at the cytoplasmic surface [15,21,23,24]. It appears that cellular amino acids may stimulate or inhibit influx of amino acids depending on the experimental situation. The stimulation by cellular glycine (or aminoisobutyric acid) on glycine influx described here is not due to exchange diffusion (defined as an obligatory, stoichiometric exchange between cellular and medium amino acids). The phenomenon described here is Na^+-dependent, is abolished by agents which depolarize the cell and has a variable stoichiometry. This contrasts with exchange diffusion of the L type [31].

Trans effects at steady state

Jacquez [20] observed that in cells brought to steady state with non-labelled aminoisobutyric acid, tracer aminoisobutyric acid influx was less than net aminoisobutyric acid influx in the pre-steady state. From these data, Jacquez concluded that cellular aminoisobutyric acid inhibits aminoisobutyric acid influx at cellular aminoisobutyric acid concentrations as low as 10 mM. In the studies quoted, Jacquez [20] used freshly isolated cells which were not depleted of their amino acid pools. Using cells depleted of amino acids, the observations in the present experiments are at variance with those reported by Jacquez [20].

The data in Fig. 4 show that when aminoisobutyric acid is at steady state, isotope influx is not less than net uptake. In fact, isotope influx may appear marginally greater especially at later times when net uptake is no longer linear. No difference between tracer one-way influx at steady state and net influx at pre-steady state was obtained with cellular aminisobutyric acid levels up to 40 mM.

A major difference between the present experiments and those reported by Jacquez is that the present experiments were carried out with cells which were preincubated to deplete the endogenous amino acid pools.

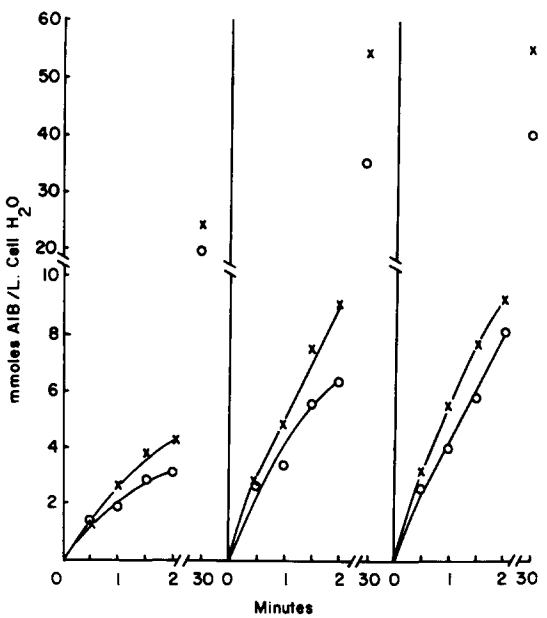

Fig. 4. Aminoisobutyric acid (AIB) uptake before and at steady state. Washed cells were incubated at 37°C for 1 h at 0.3% cytocrit in normal Krebs-Ringer medium. The cells were re-isolated, washed once and divided equally into six tubes. One set of three was preincubated for 45 min at 37°C at 2.5% cytocrit in Krebs-Ringer medium with 2, 10 and 20 mM unlabelled aminoisobutyric acid, respectively. The other set of three was incubated in an identical fashion except that no aminoisobutyric acid was present. At the end of the 45 min, tracer amino[1-^{14}C]isobutyric acid was injected into the former series while tracer plus carrier aminoisobutyric acid was injected into the latter series. In this way, in both series, the external aminoisobutyric acid concentration was identical. The panel on the left was incubated with 2 mM, the central one with 10 mM and the one on the right with 20 mM aminoisobutyric acid. o———o, represents net uptake; X———X, represents uptake of isotope at steady state. The internal aminoisobutyric acid concentration was 19 mM (2 mM external), 41.4 mM (10 mM external), 46 mM (20 mM external) at the time of adding tracer.

Since non-preincubated cells tend to become relatively hyperpolarized compared to preincubated cells [18] and non-preincubated cells show relatively higher rates of amino acid uptake (e.g., Fig. 1), the data reported by Jacquez may have been due to an increased uptake caused by hyperpolarization in the control cells rather than *trans* inhibition caused by cellular aminoisobutyric acid at steady state.

It is unlikely that exchange diffusion contributes significantly to aminoisobutyric acid uptake since control experiments have shown that in the absence of Na^+, aminoisobutyric acid uptake is not increased by the presence of cellular aminoisobutyric acid. It is known that aminoisobutyric acid does not exchange readily [21,22].

Changes in V with membrane potential

It was reported earlier that propranolol stimulates or inhibits amino acid influx depending on the external K^+ concentration [5]. Using amino acid-depleted cells, similar results are also obtained with valinomycin in line with an earlier report by Gibb and Eddy [2]. Since these agents appear to influence amino acid uptake by changing the membrane potential [2,5,14], we were prompted to determine whether such changes in membrane potential altered K_m and/or V for amino acid uptake.

The data indicate that both K_m and V are changed when the membrane potential is varied by changing external $[K^+]$ in the presence of valinomycin or propranolol (Fig. 5, Table V). At low $[K^+]_o$ (5 mM), addition of valinomycin or propranolol increases the membrane potential (inside negative) [2,5,14]. Under these conditions V is increased 2—3-fold, ($n > 10$). At elevated $[K^+]_o$

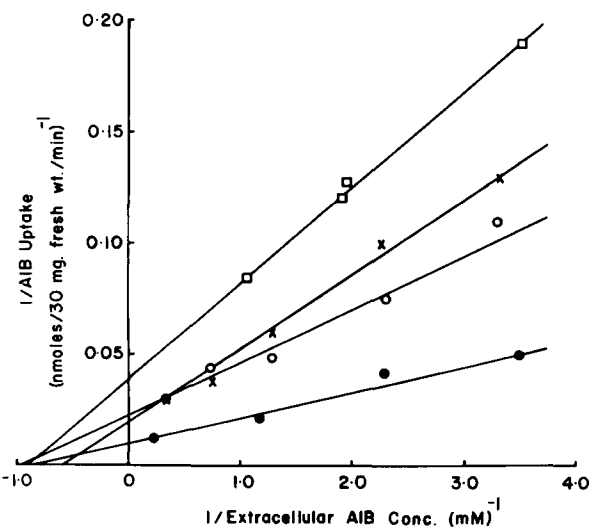

Fig. 5. Kinetic effect of a change in membrane potential. Conditions as described in Table V. The final Na^+ concentration was 80 mM and (±)-propranolol, where present, was at $1 \cdot 10^{-3}$ M. 2 mM $CaCl_2$ was present throughout. ●———●, $[K^+]_o$ = 5 mM, propranolol was present; ○———○, $[K^+]_o$ = 5 mM, no propranolol; X———X, $[K^+]_o$ = 75 mM, no propranolol; □———□, $[K^+]_o$ = 75 mM, propranolol was present.

TABLE V

CHANGES IN K_m AND V IN RESPONSE TO PROPRANOLOL AND VALINOMYCIN AT VARIABLE $[K^+]_o$

Cells were preincubated at 0.3% cytocrit for 1.5 h at 37°C. The preincubated cells were washed and brought to the temperature of the incubation bath. Propranolol was used at a concentration of $1 \cdot 10^{-3}$ M and valinomycin at 5 μg/ml. Glucose was present throughout at 10 mM. The Na^+ concentration was 75 mM in the glycine series and 60 mM with aminoisobutyric acid (AIB). Isotonicity was maintained with choline chloride. The reaction was initiated by adding [1-^{14}C]glycine or amino[1-^{14}C]isobutyric acid. 1 min later, the reaction was stopped by injecting 10 ml ice-cold choline chloride and rapid centrifugation. $CaCl_2$ (2 mM) was present in experiments with (±)-propranolol.

Test amino acid	$[K^+]_o$	Polarizing agent	K_m ± S.D. (mM)	V ± S.D. (nmol/min per mg protein)
Glycine	5	—	3.7 ± 1.3 (n = 5)	20 ± 7 (n = 5)
	5	valinomycin	5.4 ± 1.0 (n = 5)	39 ± 12 (n = 5)
	75	—	3.4 ± 0.6 (n = 5)	17 ± 6 (n = 3)
	75	valinomycin	5.4 ± 1.3 (n = 4)	15 ± 6 (n = 4)
AIB	5	—	1.3 ± 0.3 (n = 4)	17 ± 5 (n = 4)
	5	(±)-propranolol	2.3 ± 0.5 (n = 5)	33 ± 9 (n = 5)
	90	—	1.5 ± 0.2 (n = 3)	13 ± 3 (n = 3)
	90	(±)-propranolol	2.1 ± 0.7 (n = 3)	10 ± 3 (n = 3)

(75 mM) where the membrane potential is decreased in the presence of propranolol or valinomycin, there is a concomitant decrease in V. The K_m values also change with changes in membrane potential difference. But, there is an inherent problem in these measurements which introduce difficulties in interpretation as already pointed out by Heinz and Geck [17]. As amino acid uptake increases with increased amino acid concentration there will be an increased tendency to depolarize the cell [3,4,10,11,14,17,18]. Hence, the membrane potential does not remain constant at variable external amino acid levels and experimentally this results in an apparent increase in K_m and an increase less than expected in V. In this respect, the values have an unavoidable built-in error. However, in the presence of valinomycin or propranolol, the potential is more likely to remain 'clamped' even in the presence of amino acids. It is noteworthy (Table V) that if values for K_m and V are compared at variable $[K^+]_o$ in the presence of either of these agents, there is less change in K_m and a substantial change in V on altering the membrane potential with external $[K^+]_o$. Despite the unavoidable experimental problems, the changes in the kinetic parameters obtained by changing the membrane potential alone should be contrasted with those caused by Na^+ concentration alone. A change in external $[Na^+]$ effected primarily a change in the K_m value both when $\Delta\tilde{\mu}_{Na} = 0$ [15] and in normally metabolizing cells [19,21,26].

Discussion

The present experiments bring to light a hitherto neglected problem in studies on amino acid transport. The problem centres on the role of the cellular amino acid pool on influx of amino acids. Although questions such as the effect of the cellular amino acid pool on amino acid influx have been examined

earlier, it was not recognized that efflux of amino acids may hyperpolarize the cell and alter the kinetic characteristics of amino acid uptake. Thus, cellular amino acids may have three distinct types of effect on uptake of amino acids.

(1) With some amino acids, particularly those transported by the L system, the amino acids may undergo Na^+-independent exchange diffusion. Experimentally this is seen as an enhanced uptake of amino acids which does not depend on Na^+ [27]. None of the data in this communication deal with the Na^+-independent phenomenon.

(2) With many amino acids transported by the Na^+-dependent route, efflux via the Na^+-dependent transport system will hyperpolarize the cell. Under conditions where efflux exceeds uptake (e.g., such as when a cell containing a high concentration of amino acids is placed in a medium containing little amino acid) the net loss of amino acids may hyperpolarize the cell and stimulate the Na^+-coupled uptake of a labelled amino acid from the medium. In this situation, the extent of stimulation of influx will depend not only on the size of the amino acid pool but also on the cellular Na^+ concentration. It may be predicted that cells preincubated in the cold will show a greater enhancement of influx of labelled amino acids than cells containing an identical amino acid pool but maintained at room temperature because the cellular Na^+ levels will be higher in cold stored cells. Hyperpolarization due to Na^+-coupled amino acid efflux as well as enhanced $(Na^+ + K^+)$-ATPase activity would increase the degree of hyperpolarization in cold-stored cells and increase influx of labelled amino acids.

(3) The third effect of cellular amino acids is the inhibition of influx by high cellular levels of amino acids (*trans* inhibition). *Trans* inhibition may be offset by hyperpolarization. The expression of *trans* inhibition will depend upon a number of experimental variables, such as concentration of cellular amino acids, type of amino acid, as well as cellular Na^+ concentration. From the present work, it may be seen that even at cellular levels of 80—90 mM, cellular glycine stimulates uptake of labelled glycine from the medium. This may be due to the fact that the K_m for glycine efflux even at elevated cell $[Na^+]$ (greater than 50 mM Na_i) is more than an order of magnitude greater than the K_m for influx at an equivalent Na^+ concentration in the medium. In contrast to cellular glycine, cellular aminoisobutyric acid and other amino acids at concentrations above 20—25 mM may inhibit glycine influx. However, the degree of inhibition by cellular amino acids is not constant over the experimental period, since the inhibition is gradually reversed during the experimental period as a result of loss of cellular amino acid as well as the concomitant tendency for the amino acid loss to cause membrane hyperpolarization and thereby stimulate influx of amino acids. There is, therefore, a bimodal effect of cellular amino acids on Na^+-dependent influx of amino acids from the medium. The time at which the '*trans* inhibition' disappears and '*trans* stimulation' appears is highly variable and may depend on cell $[Na^+]$, although the latter has not been verified.

It might be construed that some of the *trans* stimulations reported here are due to an obligatory exchange diffusion of the L type. This is not so. Control experiments have shown, in fact, that these *trans* stimulations of glycine and aminoisobutyric acid uptake do not occur in Na^+-free media [31] unlike

exchange diffusion of methionine [27]. Moreover, if exchange diffusion contributed to the *trans* stimulations reported here, it would have been evident in the experiments shown in Fig. 4 with aminoisobutyric and the similar experiments performed with glycine (not shown).

This report also shows that changes in the membrane potential at constant cellular and extracellular [Na^+] and [K^+] alter the V. Changes in K_m are also seen with changes in membrane potential. The K_m shifts seen are frequently opposite to those predicted on the basis of theoretical considerations [16,17]. Moreover, the changes in K_m tend to counteract the effects of changes in V. Since it is not possible to maintain a constant potential with increasing amino acid levels, the concomitant depolarization with increased amino acid concentration would tend to lower the observed velocity from the 'true' velocity to an increasing extent with increasing amino acid levels, resulting experimentally in a lower V and an apparent increase in K_m. It is clear that these problems need to be resolved before making firm conclusions on the kinetic parameters changed by altering the membrane potential.

Despite some of the remaining uncertainties, the present study has shown that the intracellular amino acid pool has a marked influence on amino acid uptake. The efflux of neutral amino acids may be an important determinant (and a hitherto unsuspected one) of the membrane potential and of the influx of solutes coupled to Na^+. For example, Villereal and Cook [28] have recently concluded that contact inhibited cells have lower membrane potentials than cells during rapid growth based on the relative levels of amino acid uptake and the relative responses to valinomycin in the two states. Similar results might be obtained if the cells had different cellular amino acid pools in the two states. Similar arguments may apply to recent observations that the V (construed to be equivalent to the number of transport sites) increases at different stages of cell development [29,30]. The V changes may be due to differences in membrane potential at different stages of development.

It is premature to conclude that the present explanation is more likely than those proposed by Villereal and Cook [28], and others [29,30]. However, the fact that influx and efflux of solutes coupled to Na^+ have substantial effects on the magnitude of membrane potential and, conversely, that changes in membrane potential are reflected in different rates of Na^+-dependent solute uptake and efflux, introduces new complexities in the interpretation of data on solute transport.

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