UPTAKE OF α-KETOISOCAPROIC ACID IN LYMPHOBLAST LINE WI-L2

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SUMMARY

The uptake of α-ketoisocaproate by the cultured human lymphoblast line WI-L2 appears to be mediated by a transport system which has an apparent Km of 125 μM. The rate of uptake of α-ketoisocaproate decreases with increasing pH values, i.e., pH 6 > 7 > 8 and is stimulated by sodium at all pH values. Closely related branched chain α-ketoacids, α-keto-β-methylvaleric and α-ketoisovaleric exhibited the greatest inhibition of α-ketoisocaproate transport. Straight chain α-keto acids inhibited α-ketoisocaproic acid uptake to a lesser degree as did the α-hydroxy analogs of the branched chain α-keto acids. Inhibitors of the general anion transport system of erythrocytes, 1-anilino-8-napthalene sulfonic acid and 4-acetamido-4-isothiocyanostilbene-2-1'-disulfonic acid did not affect α-ketoisocaproate transport. A reduced sulfhydryl group is critical for α-ketoisocaproate acid uptake; transport is partially or completely inhibited by sulfhydryl reagents such as dithio-bis-nitrobenzoate, iodoacetamide, and p-chloromercuribenzoate. Inhibition by the sulfhydryl reagents is reversed with β-mercaptoethanol or partially with dithiothreitol.

The branched chain keto acids α-ketoisocaproic (KICA)[2], α-keto-β-methylvaleri and α-ketoisovaleric acids, are products of the first step in the catabolism of leucine, isoleucine, and valine (1,2), respectively; the reaction is mediated by the branched chain amino acid transaminase (E.C. 2.6.1.6) which carries out the following reaction: amino acid + α-ketoglutarate ⇆ ketoacid + glutamate. This enzymatic activity has been found in both mitochondrial and cytoplasmic fractions of the cell (3). Conceivably the concentration and compartmentalization of these ketoacids may influence the ability of the cell to metabolize their analogous amir acids. This study describes a transport system for the α-keto acid analog of L-leucine, α-ketoisocaproic acid, in the human lymphoblast cell line, WI-L2.

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[2] Abbreviations used: KICA, α-ketoisocaproic acid; ANS, 1-anilino-8-napthalene disulfonic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2-2'-disulfonic acid; DTNB, dithio-bis-nitrobenzoate; PCMB, p-chloromercuribenzoate; α-KG, α-ketoglutarate; DTT, dithiothreitol; BME, β-mercaptoethanol; NEM, N-ethylmaleimide; IAM, iodoacetamide; MH, minimal HEPES; BSA, bovine serum albumin.

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for the α-keto acid analogs of L-leucine, α-ketoisocaproic acid in human lymphoblast cell line, WI-L2.

MATERIALS AND METHODS

Chemicals. L-{U-^{14}C}leucine was obtained from New England Nuclear at a specific activity of 320 mCi/mmole. L-Amino acid oxidase of *Crotalus adamanteus* from Koch-Light Laboratories and thymol-free bovine liver catalase from Sigma were employed in the synthesis of KICA. All other chemicals from commercial sources were of the highest purity available. The general anion transport inhibitors, 1-anilino-8-napthalene sulfonic acid (ANS) and 4-acetamido-4-isothiocyanostilbene-2-2'-disulfonic acid (SITS) (6) were gifts from Dr. P.A. George Fortes, Department of Biology, UCSD.

Synthesis of {^{14}C}KICA. {^{14}C}KICA was synthesized from L-{U-^{14}C}leucine as follows: 50 μCi leucine, 1 mg L-amino acid oxidase, 2 mg catalase, and 5 μl octyl alcohol were brought to a final volume of 2 ml with double distilled H_2O and adjusted to pH 7.4 with 1 mM Tris-base. This mixture was incubated at 37° C for 5 hours with water saturated O_2 passed through the reaction flask. The mixture was transferred to an Amicon cone, pore size 224 CF-50 and centrifuged for 45 min at 3000 x g to remove protein. Unreacted leucine was removed from KICA by passing the clarified reaction mixtures through a 0.5 cm ID column containing 2 ml of Dowex 50 x 8 (200-400 mesh, chloride form) resin. Synthetic yield was generally 70-80%. The specific activity of the isotope was diluted to 2-5 μc/μmol and solutions were stored at -70° until used. Purity of the ketoacid was determined by thin-layer chromatography of hydrazone derivatives of the sample and KICA standard in a solvent system of ethyl formate: petroleum ether; glacial acetic acid (5:5:0.7); greater than 99% of the radioactivity co-chromatographed with the standard.

Standard Composition of Minimal HEPES Buffer. Unless indicated otherwise, the composition of minimal HEPES (MH) buffer (8) was as follows: 135 mM sodium chloride; 40 mM sodium-HEPES, pH 7.4; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$; and 1% bovine serum albumin (BSA) adjusted to pH 7.4 with sodium hydroxide.

Culture and Preparation of Lymphoblasts. The conditions for suspension culture of WI-L2 have been described (7,8). For uptake assays, cells were harvested by centrifugation at 350 x g for 4 min, washed twice in MH buffer, and adjusted to a final concentration of 0.5 - 1.5 x 10^7 cells/ml of MH buffer. Cell suspensions were equilibrated at room temperature for 20 min before starting assays. Some experiments, as indicated, used lymphoblast line UM-30 (Obtained from A.D. Bloom, University of Michigan) which is deficient in the branched chain α-ketoacid decarboxylase (E.C.1.2.4.3.; 2-oxo-isocaproate dehydrogenase).

Transport Assays. 50 μl of cell suspension were incubated at 25° C with the concentrations of KICA indicated in a final volume of 100 μl with MH buffer at the designated pH and cation composition (i.e., incubation buffer). Assays were terminated by dilution with 1.0 ml MH buffer. A 1.0 ml sample was transferred to a buffer-moistened 2 cm x 2 cm Whatman GFC glass fiber filter. The sample was rapidly washed with 1 ml of incubation buffer by vacuum filtration. The filter was dried and placed in 2 ml of toluene-based scintillation fluid and counted; counting efficiency was 94%.

Alternatively, the conversion of KICA to leucine occurring during the uptake assay was demonstrated and quantitated. The assays were terminated by diluting the reaction mixture with 0.5 ml of MH buffer, immediate cooling of the samples in an ice slurry and then separating cells from media by centrifugation for 5 min at 300 x g. A 100 μl aliquot of supernatant was removed

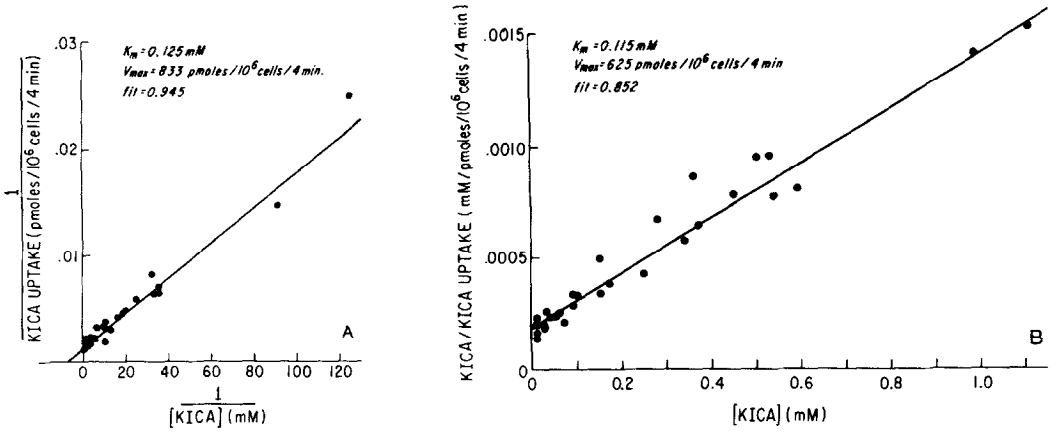

Figure 1. The determination of the apparent Km of KICA transport. All experiments were performed in MH buffer. The data presented are the mean values of quadruplicate determinations. Assay mixtures (100 µl) contained 3.5 x 10^5 cells. After four minutes of incubation at room temperature with {^{14}C}KICA (spec.act. 4 µc/µmol) the experiments were terminated by dilution of the mixture with 1 ml of MH buffer, collection of the cells by filtration of a 1 ml sample of the diluted mixture, and the filter was washed with 1 ml of MH buffer. Shown are the graphic representations of the data in the form of A) I/V vs. I/S and B) S/V vs. S

and transferred to a 0.5 cm ID column containing 1 ml of Dowex 50 x 4 (200-400 mesh, chloride form) ion exchange resin. The remaining supernatant was aspirated. The cell pellet was resuspended in 0.5 ml double distilled H_2O and applied to a second column of Dowex 50 x 4 ion exchange resin. Columns were washed with 5 ml of H_2O and the eluent, containing anionic material such as KICA, collected. This was followed by an elution of the resin with 5 ml 6 N HCl, removing neutral substances, e.g., leucine. 1 ml aliquots of H_2O and acid washes were added to 10 ml of 33% Triton, toluene-based scintillation fluid and counted; counting efficiencies were 85% and 77%, respectively.

RESULTS AND DISCUSSION

K_m Studies. Concentrations of {^{14}C}KICA from 8 µM to 1.104 mM were employed All Km studies were carried out in MH buffer (8). The uptake of KICA was mediated by a saturable system which displays an apparent Km of 125 µM. Analysis of Lineweaver-Burke plots yielded a Km value of 0.125 mM (statistical correlation 0.945) (Fig.1A); analysis of Eadie-Hofstee plots indicated a Km of 0.115 mM (statistical correlation 0.852) (Fig.1B). Metabolism of KICA beyond transamination to leucine does not appear to occur rapidly enough to influence uptake since UM-30 lymphoblasts deficient in branched chain α-keto acid decarboxylase activity accumulated radioactivity from KICA at rates approximately those of the WI-L2 line (data not shown).

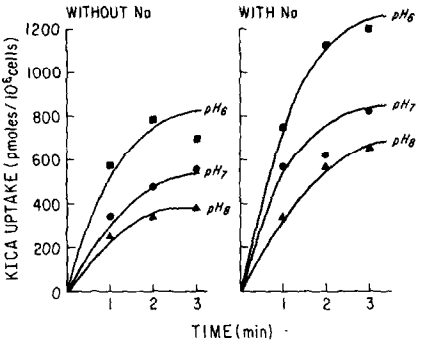

Figure 2. The pH and sodium dependence of KICA transport. Cells were harvested, washed once in MH buffer, resuspended in MH buffer, and divided into three equal portions. The cells were collected by centrifugation and resuspended in MH buffer prepared at pH 6, 7, and 8 with choline chloride replacing sodium chloride and Tris replacing the sodium in the sodium-HEPES buffer and sodium neutralized bovine serum albumin components of MH buffer. The suspensions (2 x 10^6 cells/ml) were incubated 20 minutes at 22°, collected by centrifugation, resuspended in the same buffers, and incubated 20 minutes further. The cells suspensions were divided, centrifuged and the divided portions were resuspended in the respective MH buffer prepared with or without sodium at pH 6, 7, and 8. The assay mixtures, 175 µl contained 5 x 10^5 cells and 0.375 mM {^{14}C}KICA (spec.act. 5 µc/µmol); 50 µl; samples were removed and diluted into 0.5 ml of the respective buffer and filtered to terminate uptake at the times indicated. The filtered cells were washed with 1 ml of the respective buffer. The results presented are the mean values of triplicate determinations.

pH and Na Dependence. To investigate the pH dependence of KICA uptake, 0.375 mM KICA (3 x Km of KICA) was employed in assay mixtures containing MH buffer prepared with choline chloride replacing sodium chloride, Tris-HEPES and Tris neutralized BSA replacing Na HEPES and NaOH neutralized BSA, respectively, at pH 6, 7, or 8. Sodium dependence was observed at all pH values studied and uptake appears to exhibit an acid optimum (Fig.2).

Competition Studies. The specificity of uptake of KICA was examined using a series of compounds showing structural similarities to KICA. These substances were added at a final concentration 20 x that of KICA. Assays were initiated by simultaneous addition of the test compound and KICA and terminated after 4 minutes of incubation. The results are shown in Table 1, Experiment A. KICA, α-keto-β-methylvaleric, and α-ketoisovaleric provided the greatest inhibition of KICA uptake. The straight chain α-keto acids, α-keto-

TABLE I

The Effects of Structurally Related Compounds and Analogs on KICA Uptake.[1]

Additions	pmoles/10^6cells	% inhibition of uptake
Experiment A		
None	464	0
α-Ketoisocaproic	63	86
α-keto-β-methylvaleric	135	71
α-ketoisovaleric	91	80
α-ketocaproic	180	61
α-ketobutyric	232	50
α-hydroxyisocaproic	287	38
α-hydroxyisovaleric	392	16
α-hydroxy-β-methylvaleric	188	59
α-ketoglutarate	1605	246 stimulation
succinate	1246	169 stimulation
glutarate	1216	162 stimulation
malate	2068	346 stimulation
oxalate	1969	285 stimulation
Experiment B		
None	511	0
Leucine	197	61
Valine	30	94
Cycloleucine	14	97
Norleucine	103	80
Norvaline	55	89
Experiment C	pmoles KICA/10^6cells	
None	523	
ANS	596	
SITS	574	
ANS + SITS	537	

[1] Experiments A, B, and C were performed on different days. The assays were initiat by addition of 50 µl of cell suspension (approx. 1 x 10^7 - 1.5 x 10^7 cells/ml) in buffer to tubes containing 50 µl of 0.75 mM {^{14}C}-KICA and 15 mM of the indicated compound in MH buffer. Experiments A and B; the solutions of test compounds were adjusted to pH 7.4 with NaOH or HCl solutions when required. The assays were term ted as described in Figure 1 after four minutes of incubation. Experiment C was p formed as described for Experiments A and B, except ANS and SITS were present at final concentrations of 250 µM and 0.5 mg/ml, respectively. The data indicated re present the mean values of triplicate determinations. No additions represent samp receiving ^{14}C-KICA only.

caproic and α-ketobutyric, and the α-hydroxy analog of the branched chain keto-acid, α-hydroxy-β-methylvaleric acid inhibited KICA to a lesser degree.

Leucine and other amino acids substantially inhibited the uptake of KICA

TABLE II

Conversion of {^{14}C}KICA to Leucine in the Presence of KICA Analogs.[1]

	Intracellular Accumulation		Accumulation in Media	
litions	KICA	LEUCINE	LEUCINE	TOTAL
	pmoles/10^6cells	pmoles/10^6cells	pmoles/10^6cells	pmoles/10^6cells
ıe	50	249	335	634
:loleucine	23	N.D.*	540	563
line	N.D.	23	448	471
late	261	392	357	1010
alate	1832	97	197	2126

he experiments were performed with cell suspensions (9.6 x 10^6 cells/ml) in MH uffer as described in Table I, Experiments A and B; except termination of the ssay by dilution with MH buffer, the cell and medium samples were collected and ınalyzed separately after centrifugation of the diluted mixture as described un- ler Methods. *Total* represents accumulated KICA and leucine within the cells as ell as leucine in the medium.

N.D. represents values below limits of detection. The data indicated represents the mean value of triplicate determinations.

as shown in Table 1, Experiment B. Assays using filtration which measures only radioactivity within the cell, showed 61%, 94%, and 97% inhibition of KICA uptake by leucine, valine, and cycloleucine, respectively. These results are in contrast to results obtained by identifying the radioactive components accumulated by the cell and appearing in the medium. The presence of cycloleucine or valine in the mixture does not inhibit KICA uptake but does stimulate the exit from the cell into the medium of the transamination product leucine thereby reducing the level of radioactivity inside the cell by amounts equivalent to the leucine excreted. It appears from this data that although the branched chain amino acids may lower the total amount of radioactivity within the cell, the effect is dependent on leucine counter-transport (9), rather than KICA transport (Table II).

Several substances including succinate, α-KG, malate and oxalate, produced a significant stimulation of KICA uptake; Table I, Experiment A. Experiments with oxalate and malate in which radioactivity was resolved between α-keto and amino acids indicate oxalate and malate may affect uptake results by different

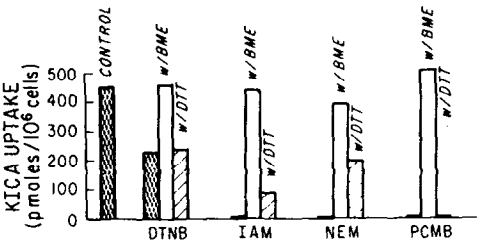

Figure 3. The effect of sulfhydryl reagents on KICA transport. A 50 µl sample of washed cell suspension (5.5×10^6 cells/ml) in MH buffer was incubated for two minutes with 8.5 µl of 7 mM DTNB, IAM, NEM or PCMB prepared in 7 mM NaOH; controls contained 8.5 µl of 7 mM NaOH. The assays were initiated by addition of KICA to a final concentration of 0.375 mM and a final volume of the assay mixture of 100 µl. In experiments examining reversibility of the inhibition; 4 µl of 125 mM BME or DTT were added two minutes after the addition of the DTNB, IAM, NEM, or PCMB and incubation was continued for two minutes before initiating the assay. Assays were terminated 105 seconds after addition of KICA by dilution of the mixture with 1 ml of MH buffer and collection by filtration of 1 ml of the diluted mixture. The results are the mean values of the triplicate determinations.

methods: Table II. While oxalate increased the total amounts of KICA within the cell, over 30-fold, it decreased the leucine content to 25% of control values. By contrast, malate served to elevate the intracellular concentration of both KIC (over 5-fold) and leucine (over 1.5-fold).

Further experiments are required to determine the nature of stimulation of KICA uptake by these compounds. The decrease in leucine formation in the presence of oxalate suggests an inhibition of the transamination of α-ketoisocaproate.

Effects of General Anion Transport Inhibitors. A general anion transport system operating via facilitated exchange diffusion in human red cells can be inhibited by the fluorescent probe ANS and by the disulfonic stilbene derivative SITS (6). Pyruvate transport in erythrocytes appears to be mediated by this system and pyruvate uptake is inhibited in the presence of KICA (10). 250 µM ANS and 0.5 mg/ml SITS were added to assay mixtures, alone or in combination, to assess the possibility that KICA might enter on such a system. However, these compounds did not significantly affect KICA uptake at the concentrations employed (Table 1, Experiment C).

Effects of Sulfhydryl Inhibitors. Sulfhydryl group inhibitors dithio-bis-nitrobenzoate (DTNB), iodoacetamide (IAM), N-ethyl maleimide (NEM), and p-chloro-

mercuribenzoate (PCMB) inhibited KICA uptake substantially. Incubation of 50 µl cells for two minutes with the sulfhydryl binding reagents before the addition of KICA to reaction mix greatly inhibited KICA uptake, (Fig.3). The addition of the reducing agents β-mercaptoethanol (BME) or dithiothreitol (DTT) to reaction mixtures two minutes after the addition of the sulfhydryl group inhibitors reversed the inhibition to varying degrees, (Fig.3). IAM, NEM, and PCMB eliminated uptake; the inhibition was reversed by BME but only partially reversed by DTT. DTNB partially inhibited uptake; BME provided complete restoration but DTT produced no significant effect.

The diminution of KICA uptake by all sulfhydryl group inhibitors suggests the presence of a reduced sulfhydryl group which may be essential for uptake. However, it is possible these critical sulfhydryl groups are not directly linked to KICA transport, but rather serve an important role in maintaining cell function or cellular integrity on which the transport system is dependent.

KICA apparently enters the lymphocyte on a transport system which has some degree of specificity for the branched chain α-keto acids. However, it has been difficult to separate the transport of KICA from accumulation of radioactivity through conversion to KICA to leucine by the branched chain amino acid transaminase. Attempts to block the transaminase with the vitamin B_6 analog, isonicotinic hydrazide, and amino oxyacetic acid were unsuccessful under the conditions employed. The possibility that oxalate may inhibit transamination without affecting transport is presently being explored.

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