BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 63–67 (1997)
ARTICLE NO. RC977001

Transport of α-Ketoisocaproate in Neuroblastoma NB-2a Cells

Magdalena Bachowska-Mac,* Astrid Nehlig,† Maciej J. Nałęcz,* and Katarzyna A. Nałęcz*,[1]
**The Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland; and †INSERM U 398, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg, France*

Received May 21, 1997

Transport of α-ketoisocaproate (KIC), a ketoacid originating from leucine and proposed to be involved in the buffering of glutamate in neurones, was studied in neuroblastoma NB-2a cells. The accumulated KIC was mostly transaminated to leucine, while free ketoacid was detectable either only after prolonged times or after inhibiting transaminase with aminooxyacetate. Accumulation of KIC was found to be inhibited by other branched-chain ketoacids, while lactate and β-hydroxybutyrate were ineffective. The transport of KIC, resembling a facilitated diffusion, was decreased by phloretin, α-cyano-4-hydroxycinnamate, 4,4′-diisothiocyano-2,2′-stilbenedisulphonate, and p-chlorimercuribenzoate. The process of accumulation did not resemble a symport with protons; therefore an involvement of the known proton-coupled monocarboxylate transporters (MCT) was excluded. Distribution of KIC suggests a mechanism involving a cotransport with 2 [Na^+].

A high degree of integration of the central nervous system seems to take place due to communication and cooperation between very specialized cells. One of the important cases of such cooperation between different cell types is a "glutamate-glutamine cycle" (1, 2), a process crucial for the neuronal synthesis of glutamate from glutamine. All brain glutamine synthetase activity has been found in glial cells (3) and leucine was shown to be the main nitrogen donor (4, 5). Glutamine released to extracellular fluid can be taken up by neurones and inside these cells converted to glutamate *via* neuronal glutaminase. The carbon backbone of leucine is released from astrocytes as α-ketoisocaproate (6) and re-aminated in nerve endings.

Nothing is known to date how α-ketoisocaproate is accumulated in neural cells. A system capable of transporting this ketoacid was described to function in hepatocytes (7). Several transporters have been reported to transport another monocarboxylic acid - lactate, into different types of cells (for review, see (8)). The best characterized, isolated and cloned is the H^+-monocarboxylate transporter in the erythrocyte membrane -MCT1 (9). Other cells capable of monocarboxylate transport reveal significant differences in the properties of these transporters (8-12). Therefore the main aim of this study was to check if α-ketoisocaproate is transported to neurones and to characterize its transport mechanism.

MATERIALS AND METHODS

Materials. 2-Keto[1-^{14}C]isocaproic acid and inulin[^{14}C] carboxylic acid were from Amersham, ^3H_2O was supplied by Radioisotope Centre Polatom, Poland. Foetal bovine serum, culture media and antibiotics were from Gibco. Tissue culture plastics were purchased from Corning. All other reagents were delivered by Sigma.

Accumulation of α-ketoisocaproate. The neuroblastoma NB-2a cell line, derived from the C 1300 tumor (13) was grown to confluence, as reported elsewhere (14). For the accumulation experiments culture medium was removed, the cells were washed with PBS and subsequently incubated for the indicated times at 37°C in the presence of PBS, α-ketoisocaproate (0.2-0.5 Ci/mol) and the compounds indicated in the figure legends. The reaction was terminated and the total radioactivity accumulation estimated as described in (14). For separation of α-ketoisocaproate from leucine, the cellular pellet was treated with 10% TCA and the amount of radioactivity was quantified as given in (14). The material in supernatants, after extraction of TCA with diethylether, was subjected to TLC in n-butanol/acetic acid/water (8:2:2, v/v) (15). The position of α-ketoisocaproate and leucine was defined by comparison with the respective markers run in parallel and visualized with ninhydrin and 2,4-dinitrophenylhydrazine (16).

Miscellaneous techniques. For estimation of the cellular volume ^3H_2O (20 μCi/ml) and inulin [^{14}C]carboxylic acid (0.2 μCi/ml) were added to the cells for 30 min. The cellular space was determined as the inulin impermeable space, *versus* the space occupied by tritiated water, mea-

[1] To whom correspondence should be addressed. Fax: 48-22-22-53-42. E-mail: knal@nencki.gov.pl.

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonate; MCT, proton-coupled monocarboxylate transporter; pCMB, p-chloromercuribenzoate; PBS, phosphate buffered saline; TCA, trichloroacetic acid; TLC, thin layer chromatography.

0006-291X/97 $25.00

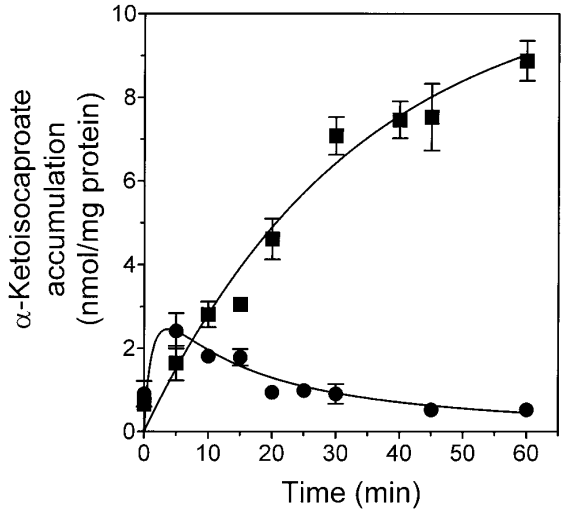

FIG. 1. Time dependence of α-ketoisocaproate accumulation in neuroblastoma NB-2a cells. Cells were preincubated for 30 min in PBS without any additions (squares) or with 10 mM aminooxyacetate (circles) which was removed prior to addition of 1 mM α-ketoisocaproate. The uptake was measured as described in the Materials and Methods section. The results represent means ± SEM from 4 independent experiments.

sured through the use of a double label counting programme. Protein concentration was determined by the Lowry procedure (17), modified by the addition of 1% sodium dodecyl phosphate (18).

RESULTS

Neuroblastoma NB-2a cells accumulate α-ketoisocaproate (Fig. 1). The amount of this compound taken up by these cells could be fitted to a first order rate equation, resulting in $k=0.030 \pm 0.005$ min $^{-1}$ as the velocity constant. The ketoacid could undergo transamination, therefore, accumulation of α-ketoisocaproate was analysed after treatment with aminooxyacetate, a known inhibitor of transminases (19). Unexpectedly, this compound strongly diminished the accumulation of radioactivity, although did not reveal any effect on the initial velocity of this process (Fig. 1). A more detailed analysis of α-ketoisocaproate metabolism was performed. As shown in Table 1, the ketoacid which enters NB-2a cells becomes a substrate of branched-chain amino acid transaminase, resulting in accumulation of leucine that can be detected in a free form, as well as incorporated into proteins (i.e. into a pellet after TCA precipitation). Free α-ketoisocaproate can only be detected after a prolonged time (1 h) and its relative content (0.4%) is very low. Treatment of NB-2a cells with aminooxyacetate results in a dramatic, although not complete inhibition of leucine formation. The amount of this amino acid is decreased by 90%. After preincubation with aminooxyacetate a low amount of α-ketoisocaproate could be detected even after a short incubation time and the relative content of this compound reached 10% of the overall accumulation of radioactivity. This analysis points to transaminase as the main driving force of α-ketoisocaproate uptake by NB-2a cells.

Accumulation of α-ketoisocaproate, measured as a function of its concentration, does not reach saturation even at such high concentrations as 10 mM (Fig. 2). Moreover, any attempt to fit the experimental data to equations of one or more Michaelis constants resulted in parameters beyond 95% confidence intervals. Interestingly, the accumulation of α-ketoisocaproate after pretreatment of the cells with aminooxyacetate also increased with the substrate concentration and could be fitted to a straight line function with a slope of 0.041 nmole/mg protein/min/mM. Taking into account this value, which could be treated as a diffusion constant, the diffusion component was subtracted from the total uptake. The differences obtained in such a way could be fitted to a Michaelis-Menten equation with a K_m of 70±15 μM. This value most probably represents the branched chain amino acid transferase affinity for α-ketoisocaproate and is found to be in the same range as the affinity for leucine of the transferase from astrocytes (20). These calculations, together with the obser-

TABLE 1

Distribution of Radioactivity after Incubation of NB-2a Cells in the Presence of α-Keto[1-^{14}C]isocaproate

Addition	Time (min)	Leucine (nmol/mg protein)		α-Ketoisocaproate	
		In proteins	Soluble	(nmol/mg protein)	(%)
None	30	4.14	1.19	0	0
	60	7.30	1.80	0.04	0.4
Aminooxyacetate	30	0.10	0.33	0.04	8.5
	60	0.19	0.62	0.09	10.0

Note. The NB-2a cells were incubated for the indicated time with 1 mM radioactive α-ketoisocaproate after 30 min preincubation without any additions (control) or with 10 mM aminooxyacetate. Separation of leucine incorporated into proteins by TCA precipitation and the further analysis of leucine and ketoisocaproate content was performed by TLC, as described in the Materials and Methods section. The results represent means from 2 separate experiments.

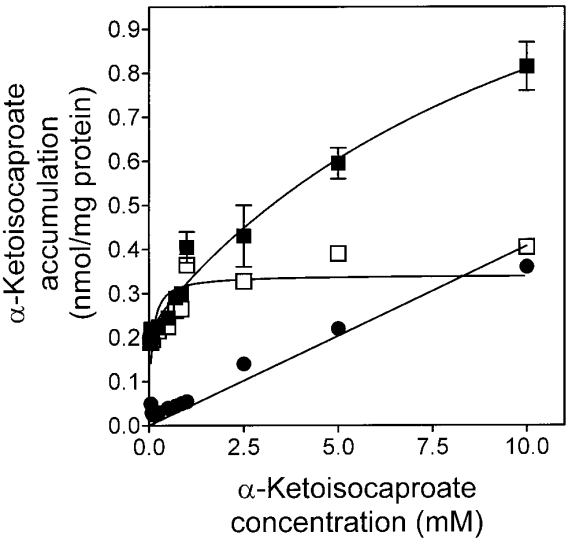

FIG. 2. Concentration dependence of α-ketoisocaproate accumulation in neuroblastoma NB-2a cells. Cells were preincubated for 30 min in PBS without any additions (filled squares) or with 10 mM aminooxyacetate (circles) which was removed prior to addition of α-ketoisocaproate at the indicated concentration. The velocities were estimated from the initial rates, measured after 5 min. The results represent means ± SEM from 3 independent measurements. The curve presented with open squares results from the calculated difference between the total uptake and the accumulation in aminooxyacetate-treated cells.

vation that α-ketoisocaproate is mainly metabolised to leucine (Table 1) would once more indicate that the process of transamination is responsible for this keto-acid uptake by NB-2a cells.

In order to elucidate the mechanism of α-ketoisocaproate transport through the plasma membrane, the effect of other short chain monocarboxylic acids was studied in more detail. As presented in Table 2, there was a strong inhibition of α-ketoisocaproate accumulation, when two other keto acids originating from the branched chain amino acids, valine and isoleucine, were added. Both, ketoisovalerate and keto-methylvalerate inhibited accumulation of radioactivity by ca. 80%. There was no effect observed after addition of β-hydroxybutyrate and lactate, a known substrate of the monocarboxylates' transporters in the plasma membrane. The detected inhibition by other branched-chain keto acids could reflect competition for the transaminase. Therefore, the other compounds reported to affect the transport of monocarboxylates through the plasma membrane were applied in order to verify an existence of a specific transporter for α-ketoisocaproate in NB-2a cells. As presented in Figure 3, the accumulation of α-ketoisocaproate was strongly inhibited by phloretin, reported to decrease the activity of MCT1 and MCT2 (9, 10). It was also inhibited, although to a lesser extent, by α-cyano-4-hydroxycinnamate and DIDS at concentrations reported to affect the specific monocarboxylate carrier in erythrocytes (8) and hepatocytes (7).

The MCT transporters belong to the group of H^+-monocarboxylate cotransporters. If α-ketoisocaproate crosses the plasma membrane of NB-2a cells by a symport with protons, one would expect a higher accumulation of such a compound at lower external pH. As shown in Table 3, this was not the case, as a lowering of the pH by 0.6 units even inhibited the process of accumulation by 11%. A strong decrease of α-ketoisocaproate accumulation at pH 8 would rather indicate an involvement of a basic amino acid residue with a pKa below this value. Histidine would therefore be a good candidate in the binding site. Many compounds which are able to cross the plasma membrane are transported by a process coupled to the transport of Na^+ ions down their free energy gradient. In such a case, an inhibition of Na,K-ATPase, responsible for maintaining the $[Na^+]$ gradient, would inhibit accumulation of the compound under study. Ouabain, a well established inhibitor of this ATPase (21) reduced the accumulation of α-ketoisocaproate by almost 70%. Moreover, uptake of this ketoacid was even more strongly inhibited by p-CMB (Table 3), a compound known to react with SH groups (22).

TABLE 2

Effect of Various Monocarboxylates on Accumulation of α-Ketoisocaproate in NB-2a Cells

	α-Ketoisocaproate accumulation			
	10 min		30 min	
Monocarboxylate added	(nmol/mg protein)	(%)	(nmol/mg protein)	(%)
None	2.8 ± 0.3 (13)	100	7.0 ± 0.4 (13)	100
α-Ketoisovalerate	0.5 ± 0.1 (3)	18	1.5 ± 0.2 (3)	22
α-Keto-β-methylvalerate	0.4 ± 0.1 (3)	14	0.6 ± 0.1 (3)	8
β-Hydroxybutyrate	3.4 ± 0.5 (3)	123	9.2 ± 1.1 (3)	132
Lactate	2.0 ± 0.3 (5)	73	9.2 ± 1.0 (6)	131

Note. The uptake measurements were performed for indicated time, as described in the Materials and Methods section. All monocarboxylates, at 10 mM concentration were added simultaneously with 1 mM α-ketoisocaproate. The results are means ± SEM; the number of experiments is given in parentheses.

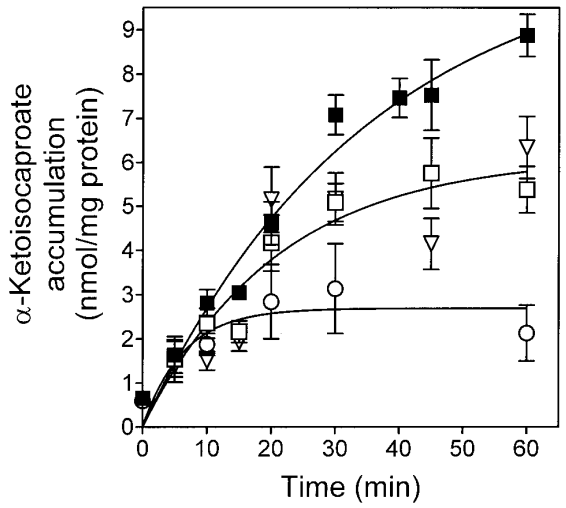

FIG. 3. Effect of inhibitors of monocarboxylate transporters on the accumulation of α-ketoisocaproate in neuroblastoma NB-2a cells. Cells were preincubated in PBS either without any additions (filled squares) or the following compounds were added: 5 mM α-cyano-4-hydroxycinnamate (open squares), 100 μM phloretin (open circles), 20 μM DIDS (open triangles) before addition of 1 mM α-ketoisicaproate. Preincubation with α-cyano-4-hydroxycinnamate and phloretin lasted 5 min, that with DIDS lasted 20 min, and the concentration of this compound was increased during incubation to 50 μM (7). The results represent means ± SEM from 3 independent experiments.

DISCUSSION

Measurements of α-ketoisocaproate accumulation in neuroblastoma NB-2a cells reflect the ability of this ketoacid to cross the plasma membrane. α-Ketoisocaproate does not undergo further decarboxylation, as this process demands a presence of CO_2 and ATP, both absent in our experimental system. Moreover, supplementation of incubation medium with glucose, a condition which should increase the formation of ATP, does not influence the overall accumulation of α-ketoisocaproate (not shown). This demonstrates that the process of decarboxylation could be neglected, especially that the activity of branched-chain ketoacid dehydrogenase was observed to be low in neurones (23). After entering the neural cells, α-ketoisocaproate is observed to be quickly transaminated. Moreover, the branched chain amino acid transaminase, an enzyme that also catalyzes the reverse reaction (i.e. amino acid formation) seems to be the main driving force for α-ketoisocaproate, confirming an important role of this ketoacid in "glutamate-glutamine" cycle. The observed immediate conversion of α-ketoisocaproate into leucine would explain the reported extremely low α-ketoisocaproate concentration in the brain (24, 25), apart from the fact that the branched chain ketoacids easily cross the blood-brain barrier (26, 27).

The mechanism of α-ketoisocaproate transport across the plasma membrane of NB-2a cells resembles "facilitated diffusion", although the concentration of free ketoacid inside these cells does not reach the external concentration. Taking into account the amount of 90 pmoles of α-ketoisocaproate per mg protein observed to accumulate in NB-2a cells after inhibition of transaminases (Table 1) and the cell volume of $3.5\times10^{-5} \pm 0.4\times10^{-5}$ l/mg protein, one can estimate the intracellular α-ketoisocaproate concentration being 2.6 μM *versus* 1 mM outside. Such a low uptake excluded a ΔpH dependent transport mechanism. This conclusion was confirmed by the absence of an effect by lactate, a monocarboxylate known to be transported by symport with H^+ (8). Inhibition by several compounds (DIDS, α-cyano-4-hydroxycinnamate, phloretin, pCMB), known to interact with functional groups of several proteins, would point, anyhow, to an involvement of a protein in transport processes. Inhibition by ouabain can indicate that the process of transport occurs at the expense of the $[Na^+]$ gradient. A ratio of internal to outside α-ketoisocaproate concentration could be explained as a result of a process involving 2 $[Na^+]$ per one ketoacid anion. A symport of monocarboxylate with Na^+ was reported to function in renal microvilli (28), moreover, a stoichiometry of 2 Na^+ per 1 lactate was estimated (29), leading to a hypothesis for the electrogenic nature of the monovalent organic anion transporter. Most probably a similar transporter functions in neurones. It is worth mentioning that a similar mechanism of transport (electrogenic symport with 2-3 Na^+ ions) was described for glutamate (30). Such a mechanism would suggest that α-ketoisocaproate accumulates in nonstimulated cells at resting potential. It has to be emphasized, that the main features of α-ketoisocaproate accumulation in neurones can confirm the physiological role of this ketoacid as an acceptor of amino groups.

TABLE 3

Effect of Inhibitors and Different External pH on Accumulation of α-Ketoisocaproate

Addition to preincubation	pH	α-Ketoisocaproate accumulation (nmol/mg protein)	(%)
None	7.4	2.8 ± 0.3 (13)	100
None	6.8	2.5 ± 0.4 (3)	89
None	8.0	1.3 ± 0.2 (3)	45
Ouabain (0.5 mM)	7.4	0.9 ± 0.1 (3)	33
pCMB (0.3 mM)	7.4	0.34 ± 0.05 (3)	12

Note. Neuroblastoma NB-2a cells were incubated in PBS at the pH indicated in the table. The accumulation of 1 mM α-ketoisocaproate was performed as given in the Materials and Methods section. Inhibitors were added either 5 min (pCMB) or 20 min (oaubain) before the substrate. The results represents the means ± SEM, the number of experiments performed is given in parentheses.

ACKNOWLEDGMENT

This work was supported by a grant of the Institut National de la Sante et de la Recherche Medicale Réseau de collaboration Est–Ouest, No 4E008B.

REFERENCES

1. Shank, R. P., and Aprison, M. H. (1977) *Life Sci.* **28,** 837–842.
2. Hertz, L. (1979) *Prog. Neurobiol.* **13,** 277–323.
3. Martinez-Hernandez, A., Bell, K. P., and Norenberg, M. D. (1977) *Science* **195,** 1356–1358.
4. Yudkoff, M., Daikhin, Y., Lin, Z., Nissim, I., Stern, J., Pleasure, D., and Nissim, I. (1994) *J. Neurochem.* **62,** 1192–1202.
5. Hutson, S. M., Wallin, R., and Hall, T. R. (1992) *J. Biol. Chem.* **267,** 15681–15686.
6. Bixel, M. G., and Hamprecht, B. (1995) *J. Neurochem.* **65**(Suppl), S115B.
7. Nałęcz, K. A., Wojtczak, A. B., and Wojtczak, L. (1984) *Biochim. Biophys. Acta* **805,** 1–11.
8. Poole, R. C., and Halestrap, A. P. (1993) *Am. J. Physiol.* **264,** C761–C782.
9. Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. W., and Brown, M. S. (1994) *Cell* **76,** 865–873.
10. Garcia, C. K., Brown, M. S., Pathak, R. K., and Goldstein, J. L. (1995) *J. Biol. Chem.* **270,** 1843–1849.
11. Mengual, R., Schlageter, M. H., and Sudaka, P. (1990) *J. Biol. Chem.* **265,** 292–299.
12. Nord, E. P., Wright, S. H., Kippen, I., and Wright, E. M. (1983) *J. Membr. Biol.* **72,** 213–221.
13. Augusti-Tocco, G., and Sato, G. (1969) *Biochemistry* **64,** 311–315.
14. Nałęcz, K. A., Korzon, D., Wawrzeńczyk, A., and Nałęcz, M. J. (1995) *Archiv. Biochem. Biophys.* **322,** 214–220.
15. Brenner, M., Niederwasser, A., and Pataki, G. (1969) *in* Thin Layer Chromatography (Stahl, E., Ed.), pp. 730–786, Springer-Verlag, Berlin/Heidelberg/New York.
16. Krebs, K. G., Hensser, D., and Himmer, H. (1969) *in* Thin Layer Chromatography (Stahl, E., Ed.), pp. 854–908, Springer-Verlag, Berlin/Heidelberg/New York.
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Nałęcz, M. J., Nałęcz, K. A., and Azzi, A. (1986) *in* Membrane Proteins. Isolation and Characterization (Azzi, A., Masotti, L., and Vecli, A., Eds.) pp. 67–75, Springer-Verlag, Berlin/Heidelberg/New York.
19. Hotta, S. S. (1968) *Archiv. Biochem. Biophys.* **127,** 132–139.
20. Yudkoff, M., Daikhin, Y., Grunstein, L., Nissim, I., Stern, J., Pleasure, D., and Nissim, I. (1996) *J. Neurochem.* **66,** 378–385.
21. Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., and Hokin, L. E. (1971) *J. Biol. Chem.* **246,** 531–543.
22. Benesh, R., and Benesh, R. E. (1962) *in* Methods of Biochemical Analysis (Glick, D., Ed.), Vol. 10, pp. 43–70, Wiley, New York/London.
23. Smith, Q. R., Momma, S., Aoyagi, M., and Rapoport, S. I. (1987) *J. Neurochem.* **49,** 1651–1658.
24. Keen, R. E., Nissenson, C. H., and Bario, J. R. (1993) *Anal. Biochem.* **213,** 23–28.
25. Matsuo, Y., Yagi, M., and Walser, M. (1993) *J. Lab. Clin. Med.* **121,** 779–784.
26. Oldendorf, W. H. (1971) *Am. J. Physiol.* **224,** 1450–1453.
27. Conn, A. R., and Steele, R. D. (1982) *Am. J. Physiol.* **243,** E272–E277.
28. Barac-Nieto, M. (1984) *Am. J. Physiol.* **239,** F496–F506.
29. Mengual, R., Leblanc, G., and Sudaka, P. (1983) *J. Biol. Chem.* **258,** 15071–15078.
30. Clark, J. E., and Amara, S. G. (1993) *BioEssays* **15,** 323–332.