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Na^+-dependent high affinity uptake of L-glutamate in primary cultures of human fibroblasts isolated from three different types of tissue

Vladimir J. Balcar[a,*], Jie Shen[b], Shisan Bao[b], Nicholas J.C. King[b]

[a]*Department of Anatomy and Histology, Anderson Stuart Building, F13, The University of Sydney, Sydney, NSW 2006, Australia*
[b]*Department of Pathology, The University of Sydney, Sydney, NSW 2006, Australia*

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Abstract

Cultured human fibroblasts isolated from embryonic muscle, skin and peripheral nerve tissues were found to accumulate [^{3}H]L-glutamate by a Na^+-dependent uptake process strongly inhibited by several glutamate/aspartate analogues including D- and L-aspartate, D- and L-*threo*-3-hydroxyaspartate and L-*trans*-pyrrolidine-2,4-dicarboxylate but not D-glutamate. It was also reduced by elevated concentrations of K^+, Rb^+ and Cs^+. The values of K_m's were 5–20 μM, well within the 'high affinity' region. Variations in the capacity (V_{max}) of [^{3}H]L-glutamate uptake did not correlate with the origin (muscle, skin or nerve tissue) of the fibroblasts. The uptake characteristics suggest that it is mediated by a transport system similar to that commonly observed only in brain tissue.

Key words: L-Glutamate; Transport; Uptake inhibitor; Fibroblast; Human

1. Introduction

High-affinity (apparent K_m < 50 μM), Na^+-dependent uptake of L-glutamate has been studied extensively in synaptosomes, brain slices, cultured glial and neuronal cells as well as in other preparations originating from the mammalian central nervous system (CNS) [1,2] (for review see [3]). Recently, L-glutamate uptake with properties similar to those of L-glutamate uptake in the CNS has been demonstrated in cultured 3T3 fibroblasts, i.e. in cells not directly related to any component of nervous tissue [4]. It has been previously reported, however, that, in cultured 3T3 fibroblasts, Na^+-dependent uptake of L-glutamate is either different from that in the CNS [5] or altogether absent [6,7]. Probable cause of the discrepancies is the variation in the potency of L-glutamate uptake among batches of 3T3 cells [4]; however, such observation must also raise more fundamental questions, e.g. is the Na^+-dependent, high affinity uptake of L-glutamate a general characteristic of the mammalian fibroblasts or is it just an incidental feature of some batches of 3T3 cells? What is the role of this particular type of L-glutamate uptake outside the CNS?

High affinity uptake of L-glutamate in brain tissue may be the principal means by which the extracellular concentrations of L-glutamate, released by excitatory nerve endings, are kept from reaching levels which could be toxic to neurons (for review see [8]). In the present series of experiments we studied L-glutamate uptake in fibroblasts cultured from three different human embryonic tissues muscle, skin and peripheral nerve. Thus any major differences (or absence of them) among the characteristics of glutamate uptake in the three types of fibroblasts would have potential significance for associating transport of L-glutamate with distinct structural and functional features of the tissue of origin. It could also help to test the hypothesis that the expression of L-glutamate uptake is influenced by the environment in which the cells originate [9–12].

2. Materials and methods

Fibroblasts were isolated from normal human embryos between 16–17 weeks post-fertilization. In all procedures relating to acquisition and use of these tissues, the guidelines issued by the National Health and Medical Research Council of Australia were strictly adhered to. The deeper layers of skin, the tendons of skeletal muscle and the brachial and lumbosacral nerve plexi, were isolated, minced and digested by 0.05% (w/v) trypsin in 1 mM EDTA (20 ml/g of tissue for 20 min at 24°C). The trypsinisation was stopped by addition of equal volumes of Dulbecco's modified Eagle's medium (Commonwealth Serum Laboratory) with 10% of foetal calf serum (DMEM/FCS) and the suspensions were centrifuged at 400×*g* for 10 min at 20°C. The resulting pellets were resuspended in DMEM/FCS, transferred into 175 cm^2 flasks and cultured under standard culture conditions (SCC), i.e. 37°C in a humidified atmosphere of 95% air/5% CO_2. When confluent, the cells were subcultured again using 175 cm^2 flasks and DMEM/FCS. The cycle of growth, confluency and subculturing was repeated at least twice. The cells were then frozen and stored in liquid N_2. When required, skin fibroblasts (SF), muscle fibroblasts (MF) and nerve fibroblasts (NF) from the same embryo were thawed, seeded at about 1.5×10^5/dish into 35 mm tissue culture dishes (Disposable Plastics, Cat. No. 23962) and grown under SCC. When confluent (in about 6 to 8 days), the fibroblast monolayers were used for glutamate uptake experiments.

*Corresponding author. Fax (61) (2) 552 2026.

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Samples of fibroblasts were taken to confirm the fibroblast nature and the homogeneity of cell populations. The cells were trypsinised, washed in phosphate buffered saline (PBS) and fixed in 1 ml of 100% ethanol at 24°C for 5 min. After the fixation they were twice washed in CMF-HBSS and once in DMEM/FCS and placed into two tubes for labelling with anti-human fibroblast monoclonal antibody (Dako, Cat. No. M877). Mouse IgG1 control antibody (Silenus, Cat. No. 12CONT01) was used as a non-specific isotype control antibody. The fixed cells were first resuspended in 100 μl of CMF-HBSS containing primary antibody (anti-fibroblast or isotype control antibody) and incubated at 4°C for 60 min, then centrifuged through an FCS bed to remove the residual primary antibody, resuspended and incubated in the presence of secondary sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (SAMIgG-FITC) for 60 min. All antibodies were used at concentrations previously determined by titration to give maximum specific labelling. After final centrifugation through an FCS bed to remove the residue of the secondary antibody, cells were resuspended in 300 μl of CMF-HBSS in preparation for flow cytometry.

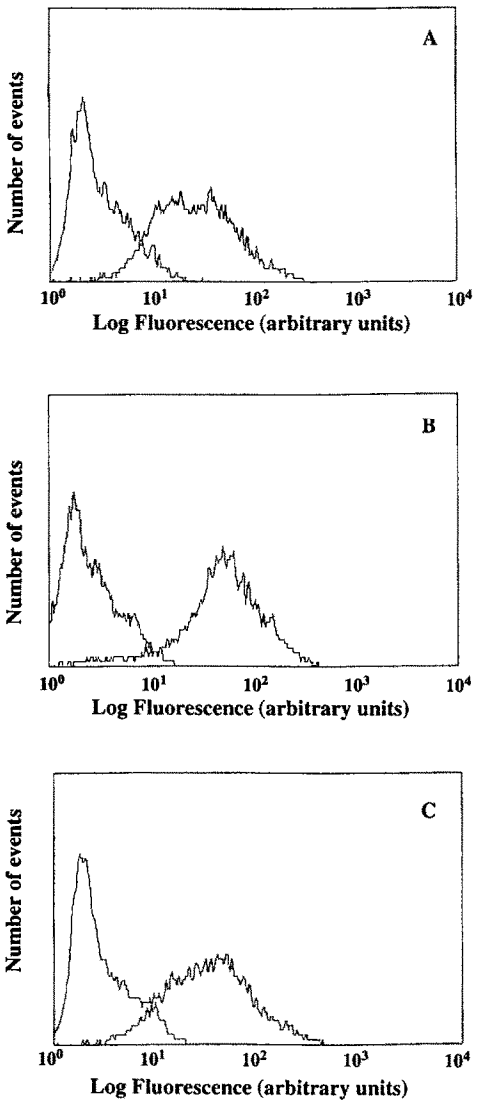

Fig. 1. Flow cytometric analysis of cultured human embryonic fibroblasts isolated from muscle (A), skin (B) and nerve (C). Cells were labelled with a primary anti-human fibroblast antibody and a secondary fluorescent antibody for flow cytometry (right trace). This is compared to samples labelled with a primary irrelevant isotypic control antibody and a secondary fluorescent antibody (left trace) to determine the purity of the preparation. The abscissa represents $\log_{10}$ fluorescence intensity; the ordinate represents number of events. (A) Fibroblasts of muscle origin. Comparison of the two traces shows at least 90% of cells staining positively for human fibroblast-specific antigen. (B) Fibroblasts of skin origin. Comparison of the two traces shows at least 95% of cells staining positively for human fibroblast-specific antigen. (C) Fibroblasts of nerve origin. Comparison of the two traces shows at least 90% of cells staining positively for human fibroblast-specific antigen.

The flow cytometry was carried out using a FACScan (Becton Dickinson) equipped with an Argon ion laser set at 488 nm for excitation. Emitted fluorescence between 515 and 545 nm was measured. Forward and side scatter measurements were within the same range for all populations.

Glutamate uptake was studied by a previously described technique [4,13,14] The culture medium was replaced, following two brief washes, with 2 ml of incubation medium (120 mM NaCl, 4.5 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.5 mM D-glucose, buffered at pH 7.4 by 10 mM sodium phosphate, or, when higher concentrations of $CaCl_2$ or choline chloride were present, by 25 mM Tricine). The Petri dishes with cell monolayers were preincubated for 5 min at 25°C in a shaking water bath, [^{3}H]L-glutamate at required concentration and specific activity was then added and the incubation continued, usually for a further 5 min. At the end of the incubation the medium was removed and the cell monolayers were washed (2×15 s) with non-radioactive glutamate-free medium at room temperature (usually 21–24°C). The cells were extracted with 0.25 M NaOH overnight and both protein [15] and radioactivity (liquid scintillation counting) were determined in each Petri dish. The results were expressed as CPM's (or pmol of L-glutamate) per mg protein. Kinetic constants were computed as described earlier [4,14]. Diffusion coefficients (k_{diff}) were computed from the portion of glutamate uptake which increased linearly with substrate concentration (i.e. at 100, 250, 500 and 1,000 μM L-glutamate) as described in previous publications [4,13,16] (k_{diff} corresponds to the slope of the line).

[3,4-^{3}H]L-Glutamic acid (57.4 Ci/mmol, 2123.8 GBq/mmol, Lot No. 2950–049) was purchased from New England Nuclear, MA, USA. D- and L-*threo*-3-hydroxyaspartic acid, *N*-methyl-D-aspartic acid L-*trans*-pyrrolidine-2,4-dicarboxylic acid, L-cysteic acid and L-cysteinesulphinic acid were purchased from Tocris Neuramin, Bristol, UK and DL-2-aminoadipic acid, L-glutamic acid (monosodium salt), D-glutamic acid, L- and D-aspartic acid and DL-*threo*-3-methylaspartic acid were obtained from Sigma, St. Louis, MO, USA. All other chemicals were purchased from commercial suppliers and were of at least analytical grade.

3. Results

Fig. 1A,B and C are the histograms representing MF, SF and NF labelled with either anti-human fibroblast or isotype control antibodies, respectively. Fig. 1A and C show that more than 90% of cells were positively stained with anti-human fibroblast antibody, while figure 1B shows that more than 95% are positively stained. These variations can be considered negligible and may be explained by differences in the concentrations of the antigen molecules among individual cells.

Kinetic constants of the saturable and unsaturable components of the uptake of L-glutamate are listed in Table 1. Although the values of constants appear to vary significantly among the three different types of fibroblasts, there were also substantial inter-experimental variations (up to 3-fold in some cases).

Uptake of 1 μM [^{3}H]L-glutamate was strongly Na^+-dependent (Fig. 2). Table 2 summarizes the effects of glutamate analogues on the uptake of 1 μM [^{3}H]L-glutamate. While D-glutamate causes no inhibition even at 250 μM, both enantiomers of aspartate and *threo*-3-hydroxy-

Table 1
Kinetic characteristics of high affinity uptake of L-glutamate in primary cultures of human fibroblasts

	MF	SF	NF
K_m (μM)	16.9 ± 5.6	8.7 ± 1.0	19.8 ± 3.4
V_{max} (pmol/mg prot/min)	44.9 ± 6.4	125.7 ± 4.7	165.0 ± 12.7
	(n = 13)	(n = 16)	(n = 16)
K_{diff} (μl/mg prot/min)	0.79 ± 0.09	1.00 ± 0.06	1.74 ± 0.08
	(n = 15)	(n = 16)	(n = 16)

Values of K_m and V_{max} are means ± S.E. obtained by fitting the initial rates of uptake, corrected for the non-saturable component, as described previously [4,13,16], to the equation $v = V_{max} \times S/(K_m + S)$ where S is the substrate concentration. For further details see [4,14]. The substrate concentrations were (μM) 5, 10, 20, 50, 100, 250, 500 and 1,000. K_m and V_{max} were determined in the 10–50 μM range, the values of k_{diff} were computed from the rates obtained at 100–1,000 μM. Either 3 or 4 dishes were used per each concentration, n is the the total number of tissue culture dishes in each experiment. The three types of cell cultures were prepared and cultured at the same time and processed in parallel experiments.

aspartate (*t*-3OHA) cause strong inhibitions. Inhibitions by 5 μM L-aspartate are about as great as those caused by 25 μM D-aspartate, while 5 μM L-*t*-3OHA appears to be a stronger inhibitor than the corresponding enantiomer at 25 μM, at least in case of skin- and muscle-derived fibroblasts ($P<0.02$ by Student's *t*-test). Also, L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*t*-PDC) [17] appears to be a weaker inhibitor than L-*t*-3OHA [2,18] (Table 2). However, the observed inhibitions may have been influenced by the presence of the 'non-specific diffusion' (Na^+-independent, inhibition-insensitive component of L-glutamate uptake, cf. [13,16]). While the constants in Table 1 would predict about 9–24% diffusion at 1 μM concentration of substrate for the three types of fibroblasts, respectively, combination of the lowest V_{max} (44.9 pmol/mg prot/min, muscle-derived fibroblasts), the highest K_m (19.8 μM, nerve-derived cells) and the highest k_{diff} (2.2 μl/mg prot/min, nerve- and skin-derived fibroblasts) gives 50% non-specific diffusion even at 1 μM substrate concentration. The inhibitions caused by 250 μM non-labelled L-glutamate, however, were rather constant, at 70–80%, suggesting that the extreme variations in the diffusion component either among the cells, or from one experiment to another were infrequent or absent.

Dependence of L-glutamate uptake on the presence of Na^+ in the external media was demonstrated by experiments shown in Fig. 2. Reduction of Na^+ concentration from 125 mM to 5 mM by replacing Na^+ with choline ion caused about 75% decrease in the uptake (the residual 5 mM Na^+ corresponds to the approximate concentration of Na^+ in Tricine buffer). Also, when the extracellular concentration of K^+ was increased from the usual 4.5 mM to 60 mM (osmolarity maintained constant by corresponding reduction in the concentration of Na^+) uptake was reduced by 50–70%, i.e. significantly more

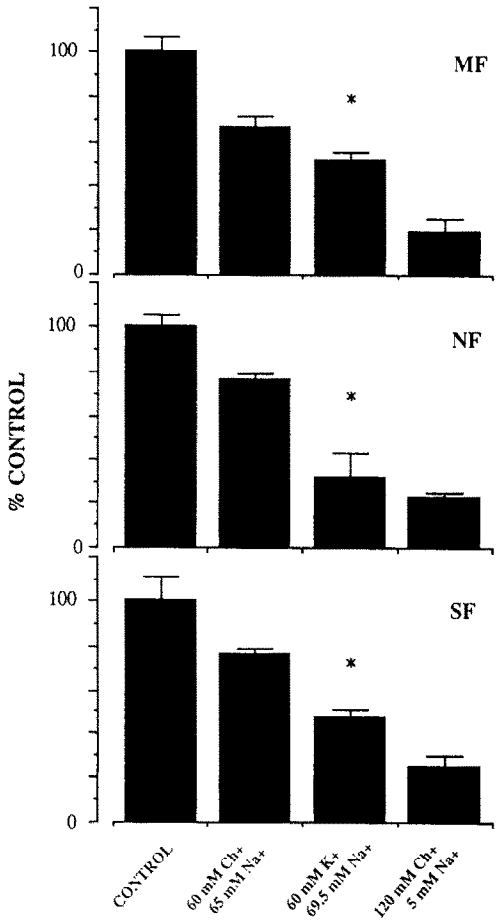

Fig. 2. The bars represent uptake of 1 μM [^{3}H]L-glutamate normalized so as to controls = 100%. They are means ± S.D. of 4 values. All preparations with reduced Na^+ or increased K^+ are significantly different from controls ($P<0.01$ at least by two-tailed Student's *t*-test). Asterisks mark statistically significant difference of 60 mM K^+ preparations from those containing 60 mM Ch^+/65 mM Na^+ at $P<0.01$, by two-tailed Student's *t*-test. The abbreviation Ch^+ is used for choline ion.

than when Na^+ was partially replaced by equimolar concentration (60 mM) of Ch^+. Similar large reductions (up to 70%) in L-glutamate uptake were obtained when 60 mM Rb^+ or 60 mM Cs^+ were used instead of 60 mM K^+ (not shown).

4. Discussion

The present results are similar to those obtained using cultured 3T3 fibroblasts [4] and thus further support the notion that Na^+-dependent, high affinity uptake of L-glutamate, similar to that found in brain, may exist in mammalian cells unrelated to central nervous tissue (cf. also [19,20]).

The values of apparent K_m (5–20 μM, well within the high affinity range), agree with those found earlier in cultured glial cells (primary cultures of actrocytes [4,14,16,21,22]; cultured glioma C6 cells [5,21,23]; non-

Table 2
Inhibition of 1 μM [^{3}H]L-glutamate uptake in primary cultures of human fibroblasts by glutamate and aspartate analogues

	Inhibitor concentration (μM)	% Inhibition		
		NF	SF	MF
L-Glutamate	250	71 ± 3	75 ± 2	78 ± 2
D-Glutamate	250	n.s.	n.s.	n.s.
DL-2-Aminoadipate	250	33 ± 6	17 ± 6	n.s.
L-Aspartate	5	31 ± 2	48 ± 2	52 ± 2
	25	63 ± 2	63 ± 4	53 ± 1
	250	66 ± 1	80 ± 1	84 ± 0
D-Aspartate	5	17 ± 3	28 ± 2	15 ± 3
	25	62 ± 3	51 ± 3	28 ± 1
	250	66 ± 3	76 ± 2	77 ± 1
L-Cysteate	250	68 ± 2	77 ± 7	71 ± 4
L-Cysteinesulphinate	5	31 ± 3	50 ± 3	45 ± 6
	25	74 ± 4	62 ± 2	79 ± 1
L-Homocysteate	250	n.s.	17 ± 3	n.s.
L-*trans*-Pyrrolidine-2,4-dicarboxylate	5	14 ± 4	15 ± 2	n.s.
	25	39 ± 1	45 ± 6	27 ± 7
	250	61 ± 3	69 ± 3	62 ± 4
L-*threo*-3-Hydroxy-aspartate	5	42 ± 5	53 ± 3	51 ± 3
	25	72 ± 3	54 ± 9	57 ± 4
	250	76 ± 4	80 ± 1	83 ± 1
D-*threo*-3-Hydroxy-aspartate	25	39 ± 6	29 ± 8	21 ± 3
	250	72 ± 2	60 ± 4	74 ± 1
DL-*treo*-3-Methyl-aspartate	25	44 ± 4	45 ± 4	39 ± 4
	250	68 ± 4	62 ± 2	83 ± 3

The values are means ± standard deviations (S.D.) of four determinations. The cells were pre-incubated in the presence of inhibitor for 5 min, [^{3}H]L-glutamate (1 μM) was then added and the incubation was terminated 5 min later. Zero-time (<15 s) incubations were used as blanks. The values of controls were, typically, 8–14 pmol/mg prot/min.

tumoral glial cell line [13]), cultured neurons (neuroblastoma [21,24,25]; primary cultures of cerebellar granule cells [26]) preparations enriched in various components of the cerebellar cortex [27], slices of rat cerebral cortex and cat spinal cord [2,28] and synaptosomes [1]; K_m's obtained in the present series of experiments are actually lower than those sometimes reported for glutamate uptake in primary cultures of astrocytes [9] or in slices of rat spinal cord [29].

Maximum rates of uptake (V_{max}, 45–280 pmol/mg prot/min) appear relatively low when compared with V_{max} usually reported for glutamate uptake by cultured glial cells (primary cultures [21]; cultured glioma C6 [21]). However, studies of glutamate uptake in other systems such as cultured neuroblastoma [24] or a non-tumoral glial cell line [13] produced values of V_{max} almost as low as those observed in the present experiments.

A Na^+-dependent L-glutamate uptake with K_m in the low μM region has been reported in human skin fibroblasts [20]. However, present results suggest that the high affinity uptake of L-glutamate in fibroblasts is not only Na^+- but also K^+-dependent. A simple, but plausible, interpretation of the results in Fig. 2 is that the uptake is driven by a Na^+ concentration gradient ($[Na^+]_o/[Na^+]_i >> 1$) but the regeneration of the transporter depends on, or is facilitated by, a K^+ concentration gradient in the opposite direction ($[K^+]_o/[K^+]_i < 1$). This is, in fact, in accordance with the mechanism proposed by Kanner [30] for the high affinity uptake of L-glutamate in the central nervous tissue. Thus not only the high affinity and structural specificity but also the ionic requirements of glutamate uptake in cultured fibroblasts are similar to those of glutamate uptake in the brain.

An intriguing aspect of glutamate uptake in fibroblasts is its variation, not only in 3T3 cells [4–7] but also in the cultures of human embryonic fibroblasts. (Although we have never observed, in any of the three types of cultured human fibroblasts, the complete absence of Na^+-dependent glutamate uptake as it has been sometimes seen in 3T3 cells [6,7].) A possible explanation of the variations is that the expression of glutamate uptake in fibroblasts is particularly susceptible to a variety of regulatory factors. Preliminary experiments, using conditions similar to those described by Casado et al. [31], indicated that 1 μM L-glutamate uptake in fibroblasts was not influenced by a phorbol ester (phorbol 12-myristate 13-acetate, 3.2 nM for 24 h). It was, however, significantly reduced (by about 25–30%, $P<0.01$ by two-tailed Student's t-test, in all three types of primary cultures) by interferon-γ (100 IU/ml, 24 h).

A part of the 'non-specific diffusion' could actually be mediated by a Na^+-independent low affinity transport shown to be expressed by cultured human fibroblasts [32]. We have, however, observed no inhibition by D-glutamate and, since the Na^+-independent uptake handles both D- and L-glutamate [32], this result would seem to indicate that any contribution from the Na^+-independent, low-affinity uptake system is, under presently used experimental conditions, negligible.

There are no indications that fibroblasts possess any of the receptor-linked ionic channels commonly found on neurons and glial cells (for review see [8]). There have been some controversies about the exact stoichiometry of the Na^+-dependence of L-glutamate uptake, the effects of fluctuations in membrane potentials associated with the activation of the glutamate receptor-operated ionic channels and the electrogenicity of glutamate uptake [22,33,34]; the primary cultures of fibroblasts, assuming they are indeed free of 'excitatory' glutamate receptors, could provide a suitable experimental model to study these and related problems.

In conclusion, the present studies demonstrate that primary cultures of human fibroblasts take up L-glutamate by a system which is, like that in cultured 3T3

fibroblasts, similar to the Na^+-dependent, high-affinity, acidic amino acid-specific uptake system usually observed in preparations derived from the central nervous tissue. The present experiments detected variability in the values of kinetic constants but there were no indications that either the affinity (K_m) or the capacity (V_{max}) correlated with the tissue origin of the cultured fibroblasts. Apparent absence of receptor-linked ionic channels in the fibroblast membrane should make the primary cultures of fibroblasts an ideal experimental model to study the energetics and the mechanisms of ionic dependence of the Na^+-dependent, high affinity L-glutamate uptake.

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