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Na^+-dependent high-affinity uptake of L-glutamate in cultured fibroblasts

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Uptake of 1 μM [^{3}H]L-glutamate by cultured 3T3 fibroblasts was strongly dependent on extracellular Na^+; it was reduced by elevated concentrations of K^+ (60 mM) but it was not influenced by variations in the concentration of Ca^{2+} (0–9.6 mM). D- and L-Asparate, D- and L-*threo*-3-hydroxyaspartate DL-*threo*-3-methylaspartate and a few other glutamate derivatives and analogues inhibited the uptake but several close analogues of L-glutamate (including D-glutamate) had no effect, implying that the uptake system is highly structurally selective. The recently identified inhibitor of glutamate uptake in synaptosomal preparations, L-*trans*-pyrrolidine-2,4-dicarboxylate, was also among the inhibitors. Apparent K_m of the uptake was found to be <10 μM. The present observations indicate that Na^+-dependent 'high-affinity' uptake of L-glutamate may appear in structures which are apparently unrelated to glutamatergic synaptic transmission in the CNS.

Amino acid; L-Glutamate; Glutamate analogue; Agonist and antagonist; High-affinity uptake; Fibroblast 3T3

1. INTRODUCTION

L-Glutamate has been shown to be accumulated by 'high-affinity' (apparent K_m < 50 μM), Na^+-dependent uptake systems in rat brain synaptosomes [1], 'mini-slices' of rat cerebral cortex [2], glia-enriched fractions of rabbit cerebral homogenates [3], cat [4] and rat [5] spinal cord slices (but see also [6]), human brain tumours [7], dorsal spinal ganglia [8], preparations from human brain ([9] for a review see [10]), cell lines cultured from, respectively, glioma (C6) [3,11], mouse neuroblastoma (C1300) [12] and neonatal syrian hamster glial cells (NN) [13], as well as by primary cultures of both glia and neurons from newborn and embryonic mouse and rat brain [14–17]. The principal common aim of virtually all of those studies was to investigate the relationship of high affinity uptake of L-glutamate to the glutamatergic synaptic transmission in the nervous tissue. In contrast, the experiments discussed in the present article were conducted in order to examine whether a similar high-affinity uptake system of L-glutamate can be expressed in cells other than those originating from the central nervous tissue, for example in cultured fibroblasts of embryonic origin.

2. MATERIALS AND METHODS

Cell line, 3T3, was selected as a readily available model of embryonic fibroblasts in culture. The cells were grown in 35 mm culture dishes (Corning, Selby Anax and Disposable Products, Sydney) in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM, Sigma) containing D-glucose at 4.5 g/l (25 mM) and supplemented with 10% of heat-inactivated (55°C for 60 min) foetal bovine serum (FBS, Cytosystems). The dishes were kept at 37°C in a humidified atmosphere containing 5% CO_2. After 4–6 days, when the cells formed confluent monolayers, they were used for experiments.

A previously described technique was used to study uptake of [^{3}H]L-glutamate [13,17]. The culture medium was replaced with 2 ml of incubation medium (125 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$) buffered (pH 7.4) by 10 mM sodium phosphate or, when higher concentrations of $CaCl_2$ or choline chloride were present, by 25 mM Tricine-Na^+ (pH 7.4). Also, 1 g of D-glucose/l (5.5 mM) was added to the medium.

The cells were pre-incubated, in the presence of a potential inhibitor when necessary, at 25°C in a shaking water bath (about 60–70 strokes/min, dishes held in a custom-made rack). [^{3}H]L-Glutamate at a required concentration and specific activity was then added and the incubation continued for as long as necessary in each particular experiment (usually 5 min). At the end of the incubation, the medium was removed by suction and the cells were washed twice with 2 ml of fresh [^{3}H]L-glutamate-free medium (at room temperature 17–22°C). The cells were extracted with 1 ml of 0.25 mM NaOH overnight and 0.5 ml aliquots were taken for the determination of radioactivity by scintillation counting using 4.5 ml of Opti-Fluor (Canberra Packard) in plastic minivials and Packard 2000C scintillation counter. The remainder of the alkaline extract was used for protein estimation [18]. Kinetic constants were calculated usig Multifit II program (Cambridge, UK).

Possible metabolic conversion of [^{3}H]L-glutamate taken up by the cells was investigated as described in an earlier study [13], except that a high performance autoradiographic film (Hyperfilm-^{3}H, Amersham, UK), rather than a scanner, was used to visualise the distribution of radioactivity on the thin layer chromatogram. Also, following the autoradiography, samples of silicagel were collected from the plates (T-6145, Sigma, St. Louis, MO, USA) and assayed for radioactivity by scintillation counting. The experiments indicated that at least 80% of the recovered tritium was present in the form of [^{3}H]L-glutamate (R_f ~ 0.42 in *n*-butanol/acetic acid/water, 4:1:1 by vol.). There was no evidence, such as additional spots or peaks, of any ^{3}H-labelled metabolites.

[^{3}H]L-Glutamic acid (41.9 Ci/mmol, Lot 2646-293) was purchased from New England Nuclear (Boston, MA, USA). 3-((RS)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP), 3(4'-chlorophenyl)-DL-glutamic acid, L-cysteic acid (0.10), D- and L-*threo*-3-hydroxyaspartic

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acid (0.17, 0.17), *N*-methyl-D-aspartic acid, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX/FG 9065), L-cysteine sulphinic acid (0.26), dihydrokainic acid, L-homocysteic acid (0.22) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (0.38, bright yellow spot produced by ninhydrin reaction) were obtained from Tocris Neuramin, Bristol, UK. DL-2-Aminoadipic acid, 3-aminoadipic acid, 3-aminoglutaric acid (β-glutamic acid, no ninhydrin-positive or UV-sensitive spot), L-aspartate-4-hydroxamate (0.27), D-aspartate-4-hydroxamate, D-aspartic acid (0.36), L-aspartic acid (0.36), L-aspartate-4-benzylester (0.66), L-glutamate-5-benzylester (0.69), L-glutamic acid (0.44), D-glutamic acid (0.44), kainic acid, DL-methionine sulphoxide, L-methionine sulphone, DL-methionine-DL-sulphoximine, *N*-methyl-D-aspartate, L-aspartate-4-methylester (0.34), DL-2-methyl aspartic acid and DL-*threo*-3-methyl aspartic acid (0.47) came from Sigma, St. Louis, MO, USA. Chelidonic acid was purchased from Aldrich Chemical Company, Milwaukee, WI, USA. The figures in parentheses are the values of R_f obtained by thin layer chromatography (TLC) on polyester silicagel plates (Sigma, T-6145) in *n*-butanol/acetic acid/water (4:1:1, by vol.). No UV-sensitive or ninhdrine-positive impurities were detected in any of the compounds subjected to the TLC analysis. All other chemicals, not specifically listed, were bought from commercial suppliers and were of at least analytical grade.

3. RESULTS

The uptake was found to be linear with time for at least 7.5–10 min at 1 μM [^{3}H]L-glutamate and was within 5 and 10% of linearity after 5–7.5 min at 100 μM and 5 min at 1 mM concentrations of substrate, respectively. The uptake values obtained at 5 min (1–100 μM) or 2.5 min (0.1–1 mM) were considered as satisfactory approximations of initial rates of uptake within those concentration intervals [19]. Analysis of the initial rates using a model which included both saturable and non-saturable components [12–14] produces the values of K_m ~5–10 μM, V_{max} ~0.1–0.2 nmol/mg prot/min and k_{diff} ~3–4 μl/mg/min. Results of a representative kinetic study are shown in Table I.

Several compounds structurally similar to glutamate (Fig. 1) inhibited the uptake of 1 μM [^{3}H]L-glutamate

Table I

Kinetic characteristics of high-affinity uptake of L-glutamate in cultured 3T3 cells

K_m (μM)	V_{max} (pmol/mg protein/min)	k_{diff} (μl/mg protein/min)
7.91 ± 1.06	185 ± 6	3.27 ± 0.24

The constants, expressed as mean ± S.E.M. are based on the kinetic equation $v = V_{max} \times S/(K_m + S) + k_{diff} \times S$ where S is the concentration of substrate. Uptake was measured at the following substrate concentrations (μM): 1, 2, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750 and 1,000. Specific activity varied between 1 and 167 μCi/μmol for 1,000 and 1 μM concentrations, respectively. Incubations lasted 5 min (1–100 μM) or 2.5 min (250–1,000 μM). Blanks were zero-time (in reality, 5–10 s) incubations and accounted for about 12–20% of the total value of uptake. Four values were obtained at each concentration (two at 75 and 100 μM), i.e. the total of 60 experimental points were included in the calculation. Kinetic constants were computed from the values obtained at 1–100 μM concentrations (46 points), corrected for a non-saturable component determined by linear regression at 100–1,000 μM (18 points) concentrations [13,14]. The amount protein/dish varied from 274 to 496 μg, mean ± S.D. = 372 ± 54.

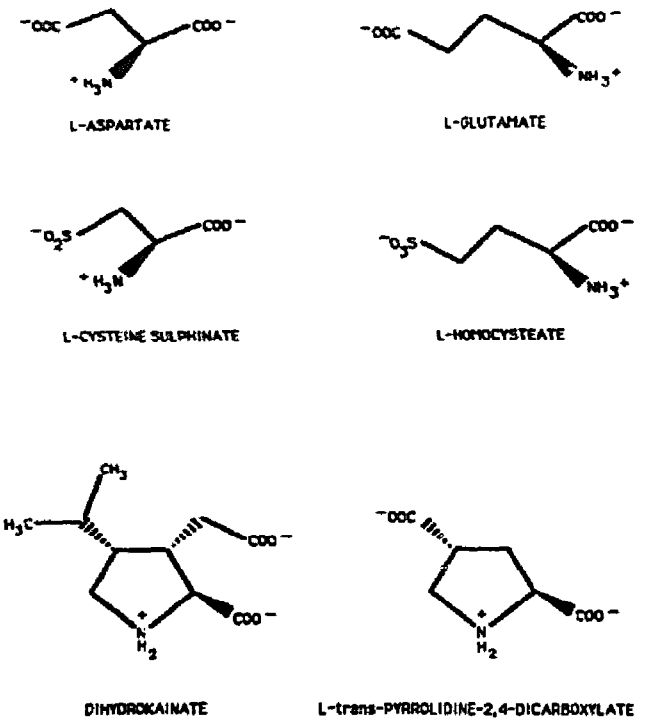

Fig. 1. Structural formulae of L-aspartate, L-glutamate, L-cysteine sulphinate, L-homocysteate, dihydrokainate and L-*trans*-pyrrolidine-2,4-dicarboxylate. The structures are represented in fully ionized forms. L-Aspartate, L-cysteine sulphinate and L-*trans*-pyrrolidine-2,4-dicarboxylate inhibited L-glutamate uptake. L-homocysteate was a weak inhibitor and dihydrokainate was inactive.

(Table II). Among them, L- and D-aspartate, together with their 3-substituted derivatives, *threo*-3-hydroxyaspartate and *threo*-3-methyl aspartate, as well as L-cysteinesulphinate and L-cysteate (i.e. analogues closer to aspartate than to glutamate in terms of the distance between the two negatively charged groups) inhibited strongly (> 50%) at 250 μM and in some cases at 25 μM concentration. In contrast, L-homocysteate, which is only one carbon atom longer than L-cysteate, was a relatively weak inhibitor even at 250 μM. Significantly, although D-aspartate was an inhibitor, albeit weaker than L-aspartate, D-glutamate had no effect, even at 250 μM concentration. Among the other types of structural alterations only the esterification or hydroxamation of the 4- or 5-carboxyl in aspartate or glutamate produced, in some cases, compounds capable of interacting with the uptake system. However, their inhibitory potency also depended on the configuration of the 2-carbon (L-enantiomer stronger than D-enantiomer) and on the length of the carbon-atom chain (5C a weaker inhibitor than 4C). Thus, L-aspartate-4-hydroxamate was a more potent inhibitor than the corresponding D-enantiomer and the L-glutamate derivatives (L-glutamate-5-benzylester and L-glutamate-5-hydroxamate) were weaker inhibitors than the corresponding L-aspartate derivatives (Table II). *N*-Substitution, 2-substitution, longer carbon atom chain (6C), as well as additional

conformational restrictions, such as those in 3-((*RS*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, antagonist, at *N*-methyl-D-aspartate receptors in the CNS [20]), kainate (active on another subclass of excitatory glutamate receptors [20]) and dihydrokainate, produced inactive compounds (Table II). However, the conformationally restricted glutamate analogue, L-*trans*-pyrrolidine-2,4-dicarboxylate, was an inhibitor of L-glutamate uptake (Table II). Several other compounds related to L-glutamate, such as 5-cyano-7-nitroquinoxaline-2,3-dione (CNQX, active at non-NMDA receptors in the CNS [20]) or chelidonic acid and DL-methionine-DL-sulphoximine (inhibitors of enzymes involved in the metabolism of L-glutamate [21,22]) were inactive.

Table II

Inhibition of the uptake of 1 μM [^{3}H]L-glutamate in 3T3 cells

Compound	Inhibitor concentration (μM)	% inhibition
L-Glutamate	5	36 ± 2
	25	50 ± 3
	250	84 ± 1
D-Glutamate	250	n.s.
L-Aspartate	25	54 ± 2
	250	66 ± 3
D-Aspartate	25	27 ± 7
	250	53 ± 6
DL-*threo*-3-Methylaspartate	25	40 ± 2
	250	55 ± 7
L-Cysteinesulphinate	250	66 ± 5
L-*threo*-3-Hydroxyaspartate	25	65 ± 5
	250	76 ± 2
D-*threo*-3-Hydroxyaspartate	25	27 ± 3
	250	58 ± 4
L-*trans*-Pyrrolidine-2,4-dicarboxylate	25	42 ± 4
	250	71 ± 3
L-Cysteate	[illegible]	79 [illegible] 1
3-Aminoglutarate	250	52 ± 9
L-Homocysteate	250	25 ± 4
L-Aspartate-4-hydroxamate	250	60 ± 5
D-Aspartate-4-hydroxamate	250	n.s.
L-Aspartate-4-benzylester	250	59 ± 6
L-Aspartate-4-methylester	250	62 ± 5
L-Glutamate-5-hydroxamate	500	n.s.
L-Glutamate-5-benzylester	250	18 ± 4
	500	29 ± 8
DL-3-(4'-chlorophenyl)-glutamate**	*	37 ± 7

Tested compounds were present in the medium during the 5 min pre-incubation, 1 μM [^{3}H]L-glutamate (usually 250 μCi/μmol) was then added and the incubation continued for another 5 min. Zero-time (~5–10 s) incubations were used as blanks accounting for 10–15% of the total. The results are expressed as % inhibition (decrease in uptake as compared to an inhibitor-free control) ± S.D. ($n = 4$). If the presence of a compound in the incubation medium resulted in less than 17% difference from controls (and $P > 0.02$% by Student's t-test) the inhibition was marked n.s. (not significant) or the compound was listed as inactive. Typical control values were about 20 pmol/mg protein/min (not corrected for diffusion which would account for about 14% of uptake at 1 μM substrate concentration, cf. Table I) and although constant within a batch of cells, could vary considerably from batch to batch from as low as 9 to as high as 40 pmol/mg protein/min.

Inactive as 250 μM: DL-2-Aminoadipate, 3-aminoadipate, 3-((*RS*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP)**, chelidonic acid**, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, FG9065))**, dihydrokainate, kainate, L-methionine sulphoxide, DL-methionine-DL-sulphoximine, 2-methyl-DL-aspartate, 2-methyl-DL-glutamate and *N*-methyl-D-aspartate (NMDA).

*Saturated solution (< 250 μM).

**Stock solution prepared in 50% DMSO (final concentration 2.5%) and compared to controls containing 2.5% DMSO which were about 33–43% lower than DMSO-free controls.

Uptake of 1 μM [^{3}H]L-glutamate was virtually abolished when the concentration of Na^+ was reduced to 5 mM (Fig. 2a). However, neither the absence (in the phosphate-buffered medium, not shown) nor an 8-fold increase in the concentration of Ca^{2+} had any effect, although an increase in the K^+ concentration to 60 mM reduced the uptake significantly (Fig. 2a). This reduction was even higher when the increase in K^+ concentration was offset by the corresponding decrease in the concentration of Na^+ to maintain constant osmolarity (Fig. 2c). Replacement of Na^+ by choline or Li^+ rather than by K^+ resulted in a smaller decrease in the uptake (Fig. 2c, replacement by Li^+ not shown). When the concentration of 100 μM [^{3}H]L-glutamate was used, only the reduction of Na^+ concentration to 5 mM resulted in a decrease of uptake, which was, moreover, rather modest when compared with that observed at 1 μM [^{3}H]L-glutamate (Fig. 2b).

4. DISCUSSION

The high-affinity uptake of L-glutamate has not yet been extensively studied in cultured fibroblasts although there is at least one report in the literature of L-glutamate uptake in 3T3 cells which is of 'high affinity' ($K_m = 16$ μM). This uptake system was described as moderately Na^+-dependent, not affected by elevated concentration of K^+ but sensitive to the absence of Ca^{2+} [11], thus perhaps indicating that it might have been similar to the Ca^{2+}/Cl^--dependent glutamate uptake observed in glial cells and brain homogenates [23–27]. However, a more recent attempt to study the high-affinity component of L-glutamate uptake in 3T3 fibroblasts was unsuccessful [28]. These contradictory observations could be explained by the variations in the potency of L-glutamate uptake (sometimes encountered in the present experiments, too, cf. Table II, legend) among batches of 3T3 cells.

The strong Na^+-dependence, insensitivity to the variations in Ca^{2+}-concentration and inhibition by 3-substituted glutamate analogues demonstrate that the high-affinity uptake for glutamate in 3T3 cells observed under the present experimental conditions is clearly different from the Ca^{2+}- and Cl^--dependent glutamate uptake [23–27]. Rather, the kinetic properties and ionic requirements would seem to relate it to the class of high affinity Na^+-dependent uptake systems usually dis-

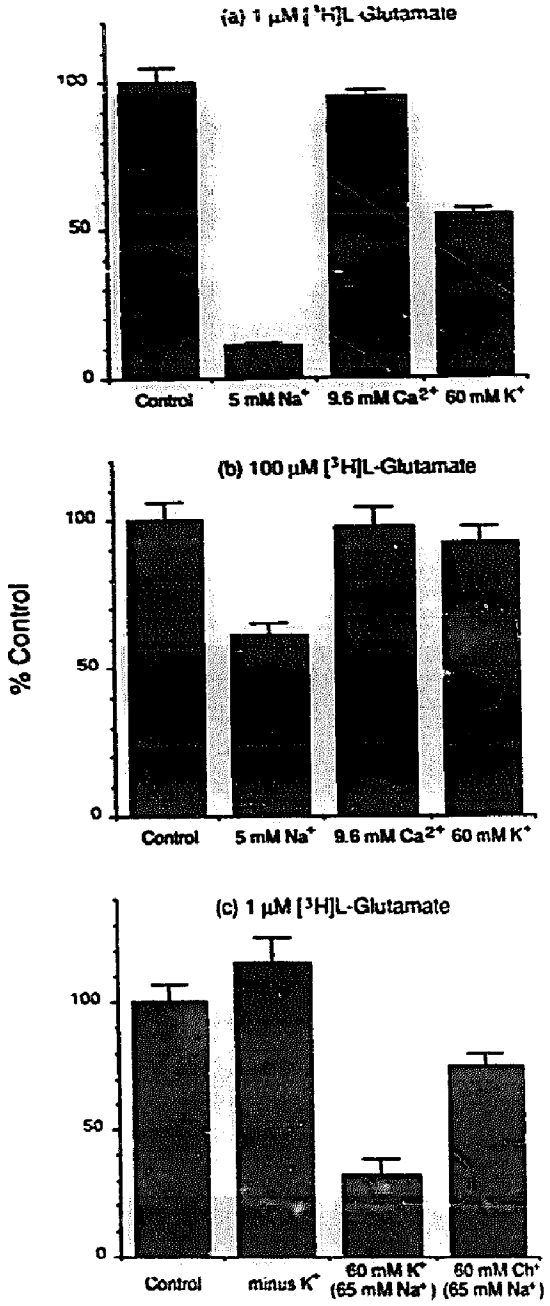

Fig. 2. Effects of variations in ionic concentrations on the uptake of 1 μM [³H]L-glutamate by 3T3 cells. The bars are means ± S.D. (n = 4), normalized, so that the controls = 100%. Control concentrations of Na^+ were 125 mM in tricine-buffered medium. In (a) and (b) the increases in Ca^{2+} and K^+ concentrations were not compensated for by decreases in Na^+ concentrations. At 5 mM Na^+, 120 mM choline (Ch^+) chloride was used to replace NaCl. In (c) the increases in $[K^+]$ and decreases in $[Na^+]$ were counterbalanced by corresponding changes in $[Na^+]$ or by addition of Ch^+. The effects of reduced $[Na^+]$ (5 mM in (a) and (b) and 65 mM in (c)) were statistically significant at $P < 0.02$ by Student's t-test. Also, uptake at 60 mM $[K^+]$ is significantly ($P < 0.02$) lower than that at 60 mM $[Ch^+]$ (both at 65 mM $[Na^+]$). Absolute values of controls were not always determined for the experiments in (a) and (c) but the values of CPM/mg protein indicated that they were similar to those in Table II. Controls in (b) were about 800 600 pmol/mg min which is close to 498 pmol/mg min (about 65% by diffusion) predicted from the kinetic equation and constants in Table I.

cussed within the context of synaptic events in the central nervous tissue [29,30].

It has been proposed that the high-affinity uptake is the chief means of limiting the excitatory action of L-glutamate released from glutamatergic synaptic terminals [31]. High-affinity uptake of L-glutamate has even been used as a marker for glutamatergic synapses, presumably on the assumption that it is localized within the glutamatergic terminals or adjacent glial cells [32–38], although this approach has recently been criticized [24]. In fact, several studies using synaptosomes and brain slices have demonstrated regional variations in the brain [39–41] and thus provided an indication that glutamate uptake may not be a homogeneous process linked to a single aspect of the neuronal function, but rather a family of distinct, though mutually similar, uptake systems. This view has been supported by data obtained in cultured cells which have, furthermore, suggested that, according to their structural requirements, glutamate-specific, Na^+-dependent high-affinity uptake systems could be broadly classified into two groups. Those found in cultured neuroblastoma [12,42] do not take up aspartate very efficiently but they interact with the longer carbon atom-chain analogue of L- glutamate, 2-aminoadipate. In contrast, L-glutamate uptake in cultured glial cells readily interacts with aspartate and is only very weakly, or not at all, affected by 2-aminoadipate [13,42]. Other differences, along the same lines, have also been reported [43].

The high affinity uptake of L-glutamate discussed in the present communication is, using the above criteria, similar to the 'glial' Na^+-dependent high-affinity glutamate uptake system. It is strongly inhibited by aspartate, especially by the L-enantiomer, and it is insensitive to 2-aminoadipate. This is paralleled by the action of sulphur-containing aspartate/glutamate analogues, L-cysteate (shorter analogue, stronger inhibitor) and L-homocysteate (longer analogue, weaker inhibitor). The same principle holds for hydroxamates and benzyl esters of L-aspartate and L-glutamate. It would seem, therefore, that the binding site on the putative carrier interacts preferentially with a folded (shorter), rather than extended (longer) conformation of L-glutamate, but this simple interpretation is not entirely consistent with the strong inhibition shown by the recently identified specific inhibitor of synaptosomal uptake of L-glutamate, L-*trans*-pyrrolidine-2,4-dicarboxylate [44] which maintains, through a conformationally restricting presence of a rigid pyrrolidine ring within its structure, a distance between the two negatively charged (carboxyl) groups closer to that in the extended, rather than folded, conformation of glutamate (Fig. 1). In addition, dihydrokainate [45], in which a non-bonded interaction between 4-isopropyl and 3-carboxymethyl would seem to force the glutamate-like part of the molecule away from the extended conformation, was inactive. The latter observation could be explained by an

analogy between the nitrogen atom within the pyrrolidine ring of dihydrokainate and the *N*-substitution which makes *N*-methyl- D-asparate inactive as a glutamate uptake inhibitor. However, this explanation disregards the fact that the same structural feature in L-*trans*-pyrrolidine-2,4-dicarboxylate does not have a similar, inactivating, effect.

High-affinity uptake of L-glutamate has been studied in blood platelets [46,47] which are known to have several distinct neuronal characteristics [48] and a similar system has been detected in brain capillaries [49]. However, the presence of a Na^+-dependent high affinity uptake of L-glutamate in fibroblasts and the potential ubiquity of such cells in vivo would seem to indicate that a type of glutamate uptake usually found only in nervous tissue may sometimes appear in structures which are in no way related either to the central or peripheral nervous system. The present findings may thus help to explain the recent failure of an antibody raised against the glycoprotein thought to form the glutamate transporter [50] to specifically label the neuronal structures known to be glutamatergic in the rat brain [51]. Also, the existence of fibroblast-located glutamate uptake may have important implications for the experiments in which *Xenopus laevis* oocyte system is utilized to study the characteristics of glutamate transporter expressed from fragments of mRNA obtained in early postnatal tissues [52].

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