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Excitatory Amino Acid-Stimulated Uptake of $^{22}Na^+$ in Primary Astrocyte Cultures

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In this study we have found that L-glutamic acid, as well as being taken up by a Na^+-dependent mechanism, will stimulate the uptake of $^{22}Na^+$ by primary astrocyte cultures from rat brain in the presence of ouabain. By simultaneously measuring the uptake of $^{22}Na^+$ and L-^{3}H-glutamate a stoichiometry of 2–3 Na^+ per glutamate was measured, implying electrogenic uptake. Increasing the medium K^+ concentration to depolarize the cells inhibited L-^{3}H-glutamate uptake, while calculations of the energetics of the observed L-^{3}H-glutamate accumulation also supported an electrogenic mechanism of at least 2 Na^+:1 glutamate. In contrast, kinetic analysis of the Na^+ dependence of L-^{3}H-glutamate uptake indicated a stoichiometry of Na^+ to glutamate of 1:1, but further analysis showed that the stoichiometry cannot be resolved by purely kinetic studies. Studies with glutamate analogs, however, showed that kainic acid was a very effective stimulant of $^{22}Na^+$ uptake, but ^{3}H-kainic acid showed no Na^+-dependent uptake. Furthermore, while L-^{3}H-glutamate uptake was very sensitive to lowered temperatures, glutamate-stimulated $^{22}Na^+$ uptake was relatively insensitive. These results indicate that glutamate-stimulated uptake of $^{22}Na^+$ in primary astrocytes cultures cannot be explained solely by cotransport of Na^+ with glutamate, and they suggest that direct kainic acid-type receptor induced stimulation of Na^+ uptake also occurs. Since both receptor and uptake effects involve transport of Na^+, accurate measurements of the Na^+:glutamate stoichiometry for uptake can only be done using completely specific inhibitors of these 2 systems.

It is well-established that astrocytes both *in situ* and *in vitro* have a very active Na^+-dependent uptake system for L-glutamate, presumed to be important for terminating the action of this major excitatory amino acid transmitter (Hertz, 1979; Shank and Campbell, 1983; Fonnum, 1984). Such uptake is thought to represent the small, rapidly turning over glutamate pool in brain tissue (Van den Berg and Garfinkel, 1971; Benjamin and Quastel, 1972; Berl, 1972; Balazs et al., 1973; Nicklas et al., 1987), which provides a substrate for glutamine synthetase, an astrocyte-specific enzyme (Norenberg, 1979). Although the dependence of L-^{3}H-glutamate uptake on the presence of Na^+ in the medium has been amply demonstrated in brain slices (Balcar and Johnston, 1972) and in astrocytes and other glial cells (Henn et al., 1974; Currie and Kelly, 1981; Wilkin et al., 1982; Gordon and Balazs, 1983), stimulation of Na^+ uptake by glutamate in glial cells has not been reported. Indeed, such a measurement has been reported only in a line of cultured cerebellar neurons, where a stoichiometry of 2 Na^+:1 glutamate was found (Stallcup et al., 1979). In contrast, it has been reported (Drejer et al., 1982) that Hill plots of the Na^+ dependence of L-^{3}H-glutamate uptake by rat primary astrocyte cultures gave a value for n of 1, which was interpreted as indicating a Na^+:glutamate stoichiometry of 1:1. However, in C_6 glioma cells a value for n of 2 was indicated by the kinetics of the dependence of D-^{3}H-aspartate accumulation on the medium Na^+ concentration (Erecinska et al., 1986). Because of these discrepancies and the importance of the Na^+:glutamate stoichiometry in considerations of the functional implications and energetics of L-glutamate uptake in astrocytes, we studied the simultaneous uptake of $^{22}Na^+$ and L-^{3}H-glutamate in primary astrocyte cultures in the same way as Stallcup et al. (1979) did for neuronal cultures, to obtain a direct, and hopefully more definitive, measure of this stoichiometry. We also studied the kinetics of the Na^+ dependence of L-^{3}H-glutamate uptake to compare our data with that of Drejer et al. (1982).

In addition, depolarization of astrocytes in primary culture by L-glutamic and kainic acid has been reported by Bowman and Kimelberg (1984), Kettenmann et al. (1984), and Kettenmann and Schachner (1985). These and other more recent studies have been interpreted either as indicating direct, receptor-mediated effects of excitatory amino acids on astrocytes and other glial cells or depolarization due to electrogenic Na^+-dependent uptake of L-glutamate (Tang and Orkand, 1986; Brew and Attwell, 1987; Sontheimer et al., 1988). Thus, we also examined the effects of kainic acid and other excitatory amino acid agonists and the effect of temperature on L-^{3}H-glutamate uptake and L-glutamate-stimulated $^{22}Na^+$ uptake to further test the hypothesis that all the glutamate-stimulated $^{22}Na^+$ uptake can be accounted for by Na^+ plus L-glutamate cotransport.

Materials and Methods

Cell culture. Primary astrocyte cultures were prepared from the cerebral cortices of 1-d-old rat pups as previously described (Frangakis and Kimelberg, 1984; Kimelberg and Katz, 1986). In brief the tissue was dissociated using the neutral bacterial protease Dispase and plated in 12-well trays at a density of 4×10^4 cells in 1 ml medium/well (ap-

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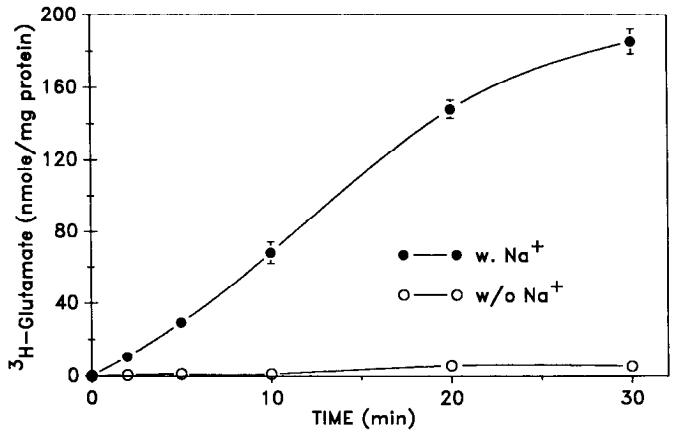

Figure 1. Time course of uptake of 10^{-4} M L-^{3}H-glutamate. Age of cultures was 29 d. Each data point represents the mean of the results from 4 wells, and error bars represent ±SEM. Where they are not shown, the SEM was smaller than the size of the symbols. NaCl was replaced with choline Cl and $NaHCO_3$ with triethyl ammonium bicarbonate (see Materials and Methods for further experimental details).

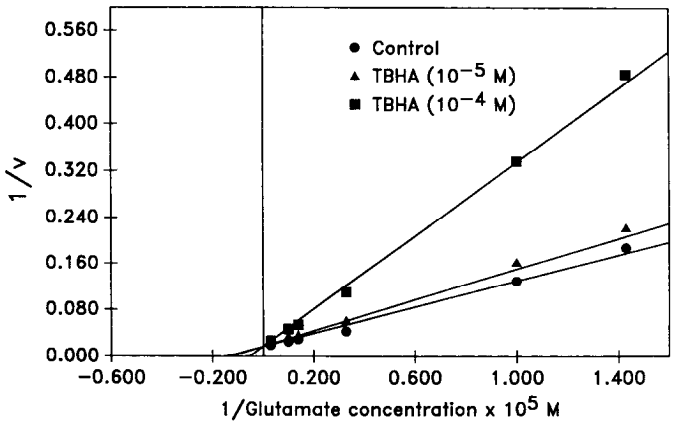

Figure 2. Kinetics of L-^{3}H-glutamate uptake and the effect of threo beta-hydroxy aspartate (TBHA). TBHA was added to the final concentrations shown at the same time as L-^{3}H-glutamate. The solid lines were calculated using a computer program fitting the data to the equation for competitive inhibition by a nonlinear regression method and plotted in the double-reciprocal form. v is in nmol L-^{3}H-glutamate/mg protein/5 min. Cells were 35 d old, and each data point represents the mean of 3 wells ± SEM. L-^{3}H-glutamate, 0.2 μCi, was added per 0.5 ml reaction medium per well. Time for uptake was 5 min with a 0 time uptake subtracted.

proximately 4 cm^2 growing area). The cells were grown in Eagle's minimal essential medium plus 10% fetal calf serum with added supplements and antibiotics (Frangakis and Kimelberg, 1984). They were cultured at 37°C in a 5% CO_2/95% atmosphere in a Forma Stericult incubator and were used when 3–5 weeks old. The cultures were ≥95% glial fibrillary acidic protein (GFAP) positive as indicated by immunocytochemistry.

Transport studies. These were performed essentially as previously described in detail for radionuclides (Kimelberg and Walz, 1988, and references therein). First, the growth medium was poured off and the cells washed 3 times with HCO_3^--buffered medium consisting of the following components (in mmol/liter): NaCl, 122; KCl, 3; $CaCl_2$, 1.3; $MgSO_4$, 0.4; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 10. The buffer was previously bubbled with a 5% CO_2/95% air mixture to obtain a pH of 7.4. The cells were then incubated in this medium at 37°C in a Wedco CO_2 incubator in a 5% CO_2/95% air atmosphere for 20–30 min. This incubator uses a fan to rapidly attain a uniform internal temperature and atmosphere. The medium was then rapidly removed and 0.5 ml of the same medium or medium with altered ionic composition, warmed to 37°C, bubbled with 5% CO_2/95% air, and containing the appropriate radioactivity was added to each well. The cells were then returned to the incubator. When $^{22}Na^+$ uptake was being studied, 1 mM ouabain was added at the same time as $^{22}Na^+$ to prevent it being pumped out of the cells. We determined that 1 mM ouabain was the minimal concentration needed to obtain maximal stimulation of $^{22}Na^+$ uptake by 10^{-4}M L-glutamate. Usually 0.2 μCi L-^{3}H-glutamate, 1 μCi ^{3}H-kainic acid, and 1–2 μCi $^{22}Na^+$ were added per well. Unlabeled L-glutamic or kainic acid was added to obtain the desired final concentration. Sodium chloride was replaced with choline chloride or *N*-methyl-D-glucamine to study the effects of the absence of sodium on L-^{3}H-glutamate uptake. In Na^+-free medium, $NaHCO_3$ was replaced with triethyl ammonium bicarbonate, which we determined by itself had no effect on Na^+-dependent uptake of L-^{3}H-glutamate. Na^+-free medium was added at 0 time with L-^{3}H-glutamate or ^{3}H-kainic acid. Cotransport of sodium and L-glutamate was studied by measuring the simultaneous uptake of $^{22}Na^+$ and L-^{3}H-glutamate in the presence of 1 mM ouabain. Ouabain was added at the same time as $^{22}Na^+$, but other inhibitors were usually preincubated for 30 min. At varying times, the medium was rapidly aspirated from each well and each well was washed 4 times within 15 sec with 1 ml per wash of ice-cold 0.29 M sucrose containing 10 mM Tris nitrate and 0.5 mM $Ca(NO_3)_2$, pH 7.4. The cell monolayer from each well was then solubilized in 1 ml of 1 N NaOH at room temperature for about 15–20 min. Aliquots were then taken for scintillation counting and protein assay. Protein content was determined by the Lowry-Folin method (Lowry et al., 1951).

Materials. L-3,4-^{3}H-glutamate was obtained from New England Nuclear with a specific activity of 52 Ci/mmol. G-^{3}H-kainic acid (8 Ci/mmol) and $^{22}NaCl$ were obtained from Amersham. L-Glutamic acid, kainic acid, kynurenic acid, quisqualate, DL-threo beta-hydroxy aspartic acid (TBHA), DL-aspartic acid, beta hydroxamate, and beta-methyl-DL-aspartate were all from Sigma. *N*-Methyl-D-aspartate (NMDA) was obtained from Cambridge Research Biochemicals Ltd. (Cambridge, England). 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) was from ICN Biochemicals, Inc.

Results

Kinetics and Na^+ dependence of L-^{3}H-glutamate uptake

Figure 1 is a time course showing uptake of L-^{3}H-glutamate at a final glutamate concentration of 10^{-4} M with the normal concentration of medium Na^+ or with Na^+ completely replaced with choline, as indicated. As can be seen, the uptake was almost completely Na^+ dependent and the rate of uptake was linear for up to 20 min, giving an initial rate of 7.35 nmol/mg protein min^{-1}. The amount taken up at 30 min was 185 nmol/mg protein. Based on a cell volume of approximately 3.5 μl/mg protein (Kimelberg and Walz, 1988), this gives an average intracellular concentration of 53 × 10^{-3} M L-^{3}H-glutamate, or a 530-fold concentration gradient over the medium concentration of 10^{-4} M L-glutamate, assuming that the radioactivity remains as L-glutamate during this period. A similar dependence of L-^{3}H-glutamate uptake on medium Na^+ was seen when Na^+ was replaced with *N*-methyl-D-glucamine (not shown).

The initial rate of uptake of ^{3}H-glutamate, measured over 5 min, showed Michaelis-Menten kinetics with a K_m for ^{3}H-glutamate of 69.5 μM and a V_{max} of 12.3 nmol/mg protein/min (Fig. 2). This graph also shows that TBHA inhibited L-^{3}H-glutamate uptake in a competitive manner. The K_i was 55.3 μM. In Table 1 we show the effects of a number of other inhibitors of Na^+-dependent high-affinity glutamate uptake. These were all effective to some degree, confirming that we are indeed dealing with the Na^+-dependent L-glutamate uptake system described by others (Shank and Campbell, 1983; Fonnum, 1984; Waniewski and Martin, 1984).

In Figure 3 we show the dependence of the rate of uptake of 10^{-4} M L-^{3}H-glutamate on the medium Na^+ concentration. The drawn line was a calculated best-fit based on Michaelis-Menten

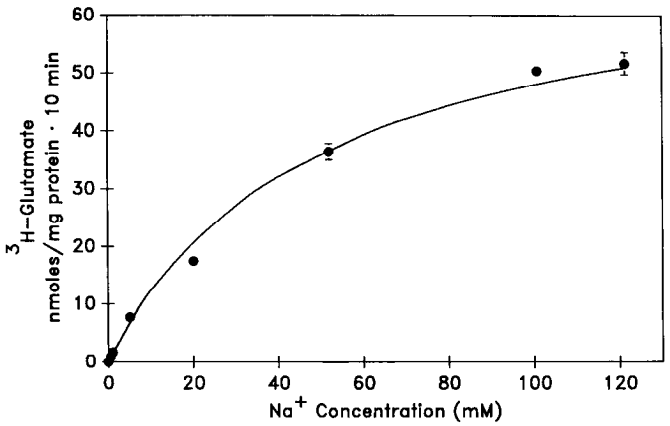

Figure 3. Effect of varying medium Na^+ concentration on L-^{3}H-glutamate uptake. The solid curve represents the fitted line for the equation $v = V \times S/K_m + S$, with values for V_{max} of 71.9 nmol L-^{3}H-glutamate/mg protein/10 min and a K_m of 49.7 mM Na^+. These values were best-fit to the data with a value for n in S^n of 1 using a nonlinear regression method. Fitting the equation for a value for n of 2 gave a much worse fit with the sum of the difference of the squares being about 10-fold more. L-^{3}H-glutamate, 0.3 μCi, was added per well per 0.5 ml reaction medium. Uptake time was 10 min. NaCl was replaced with choline Cl and $NaHCO_3$ triethyl ammonium bicarbonate. Age of culture was 29 d. Each point represents the mean of 3 wells ± SEM.

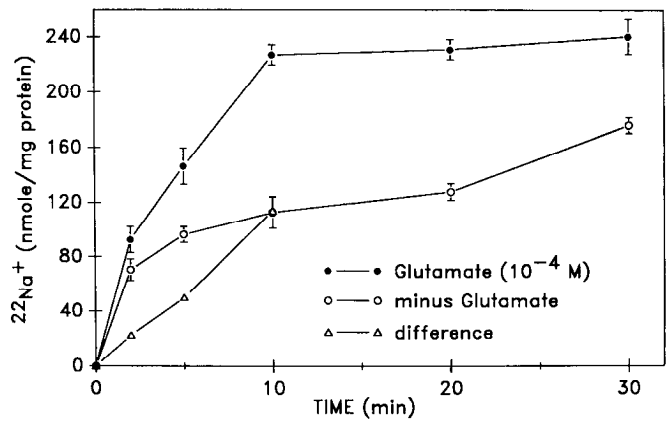

Figure 4. Effect of 10^{-4} M L-glutamate on $^{22}Na^+$ uptake. Ouabain, 1 mM, was added at same time as $^{22}Na^+$ and L-glutamate to prevent outward pumping of $^{22}Na^+$, but the cells were not pretreated with ouabain so as to minimize any changes in the transmembrane Na^+ and K^+ gradients due to inhibition of the (Na^+ + K^+) pump. The difference between the 2 curves representing the glutamate-stimulated uptake of $^{22}Na^+$ (△) is also shown. Cultures were 29 d old. Each data point represents mean of 4 wells ± SEM with $n = 4$. $^{22}Na^+$, 2 μCi, was added per well per 0.5 ml reaction medium.

kinetics with Na^+ as the varied substrate, giving a V_{max} of 7.2 nmol L-^{3}H-glutamate/mg protein/min and an apparent K_m for Na^+ of 49.7 mM at the concentration of glutamate used (10^{-4} M).

Stimulation of $^{22}Na^+$ uptake by L-glutamate and $^{22}Na^+$:L-^{3}H-glutamate stoichiometry

Figure 4 shows the stimulation of $^{22}Na^+$ uptake, measured in the presence of 1 mM ouabain to inhibit active extrusion by the (Na^+ + K^+) pump, by 10^{-4} M L-glutamate. The difference line represents the glutamate-stimulated $^{22}Na^+$ uptake, which was linear over the first 10 min. In Table 2 we show results for the uptake of 10^{-5} and 10^{-4} M L-^{3}H-glutamate and glutamate-stimulated $^{22}Na^+$ uptake measured simultaneously for 10 min. Note that the presence of 1 mM ouabain reduced the uptake of L-^{3}H-glutamate by about 50% over the 10 min period, compared with the uptake of L-^{3}H-glutamate measured in the absence of ouabain (compare Table 2 with Figs. 1 and 3). This is possibly due to partial dissipation of the inwardly directed Na^+ gradient, with a consequent reduction of the driving force. This is supported by the results shown in Table 3, where it is shown that when uptake of 10^{-4} M L-^{3}H-glutamate was measured over a 2 min period after addition of 1 mM ouabain no inhibition of L-^{3}H-glutamate was seen, whereas uptake was inhibited by 58% after 10 min. In Table 2 the stoichiometry for $^{22}Na^+$:L-^{3}H-glutamate uptake is calculated from the difference between the mean of the $^{22}Na^+$ uptake in the presence and absence of 10^{-4} M L-^{3}H-glutamate measured on the same cultures in the same experiment, divided by the Na^+-dependent uptake of L-^{3}H-glutamate measured simultaneously with the glutamate-stimulated uptake of $^{22}Na^+$. The small amount of L-^{3}H-glutamate taken up in the absence of external Na^+ had, of course, to be measured separately and was also subtracted from the Na^+-dependent L-^{3}H-glutamate uptake. It can be seen that the ratio of $^{22}Na^+$:L-^{3}H-glutamate was significantly greater than 1; from these data, it was either 2 or 3 $^{22}Na^+$:1 ^{3}H-glutamate, since statistically we could not distinguish between these values.

In Table 4, we show the cotransport of $^{22}Na^+$ and L-^{3}H-glutamate (final concentration 10^{-4} M) when the cells were preequilibrated with $^{22}Na^+$ for 1 hr, thus measuring net glutamate-stimulated uptake of $^{22}Na^+$ and the concurrent influx of L-^{3}H-glutamate. As can be seen, for all time points up to 20 min a $^{22}Na^+$:L-^{3}H-glutamate stoichiometry close to 2 that was statistically different from 1 or 3 was obtained.

Effects of K^+-induced depolarization on L-^{3}H-glutamate uptake

A stoichiometry of 2 Na^+:1 glutamate implies that Na^+-dependent glutamate uptake will have a single net positive charge of 1 due to the extra Na^+ transported, and thus will be electrogenic.

Table 1. Effect of inhibitors on L-^{3}H-glutamate uptake

Inhibitors	Inhibitor concentration	L-^{3}H-glutamate uptake (nmol/mg protein/10 min)	% Control
Control (+Na^+)	None	31.05 ± 1.9	100
Control (−Na^+)	None	0.95 ± 0.03	
SITS	100 μM	29.64 ± 1.6 (NS)	95
	1 mM	16.90 ± 1.8[a]	54
β-methyl DL	100 μM	26.06 ± 2.9 (NS)	84
aspartic acid	1 mM	7.00 ± 0.4[a]	23
DL aspartic acid β	100 μM	35.70 ± 2.0 (NS)	115
hydroxamate	1 mM	11.63 ± 0.5[a]	37
DL-threo β-hydroxy	100 μM	22.26 ± 0.8 (NS)	72
aspartic acid	1 mM	4.27 ± 0.2[a]	14

L-^{3}H-glutamate concentration was 10^{-4} M in all cases. The cultures were preincubated with solutions containing inhibitors and without isotope for 20 min before adding L-^{3}H-glutamate in the same solutions. SITS, 4-acetamido-4′-isothiocyanostibene-2,2′-disulfonic acid. Dunnett's test was used for multiple comparisons with a single control. (NS), not significantly different from control (+Na^+).

[a] Different from control value (+Na^+) by $p < 0.01$.

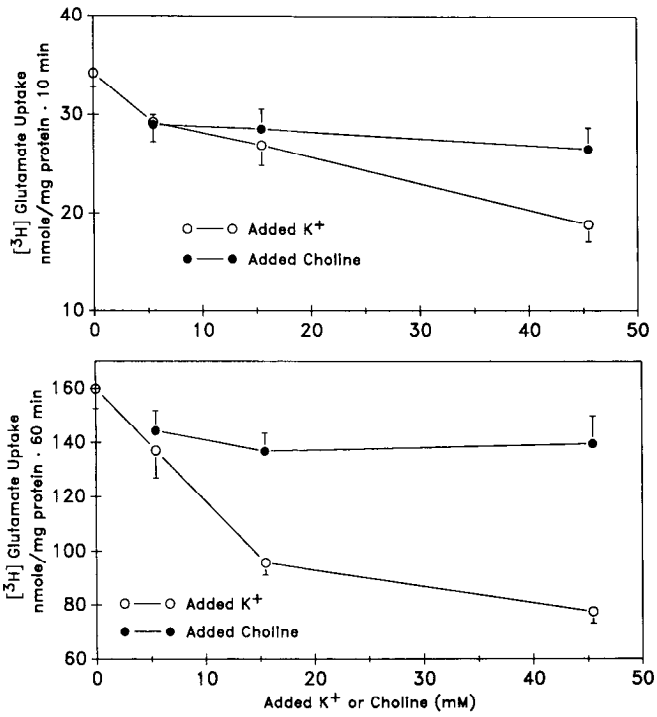

Figure 5. Effect of increasing medium $[K^+]$ on L-^{3}H-glutamate uptake. *Top,* Time for uptake was 10 min. Cultures were 24 d old. Each data point represents the mean of results from 4 wells ± SEM. L-^{3}H-glutamate, 0.4 μCi, was added per well per 0.5 ml reaction media. *Open circle* (○), NaCl replaced with KCl; *filled circle* (●), NaCl replaced with choline Cl. *Bottom,* Time for uptake was 60 min. Cultures were 28 d old. Other conditions same as in upper panel.

As a further test of this we studied the effect of K^+-induced membrane depolarization on L-^{3}H-glutamate uptake. These results are shown in Figure 5 as a function of increasing medium $[K^+]_o$ for initial uptake (10 min, top panel) and for 60 min uptake (bottom panel) when the L-^{3}H-glutamate level had approached a steady state. It can be seen that at higher $[K^+]_o$ there was a reduction of uptake relative to the case when Na^+ in the medium was reduced to the same extent with choline, and this effect was greater for the 60 min uptake values.

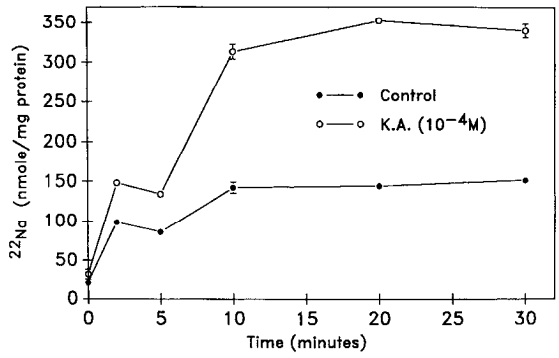

Figure 6. Effect of 10^{-4} M kainic acid on $^{22}Na^+$ uptake in the presence of 1 mM ouabain. Other conditions as in Figure 4.

Effects of excitatory amino acid agonists on $^{22}Na^+$ uptake

We also studied a number of excitatory amino acid agonists for their effects on $^{22}Na^+$ uptake to see if stimulation of Na^+ was unique to L-glutamate and also whether this effect correlated with the effectiveness of different excitatory amino acids in depolarizing astrocytes in primary culture (Bowman and Kimelberg, 1984; Kettenmann and Schachner, 1985). The results are shown in Table 5. The small stimulation by NMDA and quisqualate were not significant, while the effects of kainic acid and L-glutamate were statistically significant. A time course of stimulation of $^{22}Na^+$ uptake by kainic acid is also shown in Figure 6. As can be seen, maximum effects were first seen at 10 min.

In sharp contrast to L-glutamate, however, we show in Figure 7 that primary astrocyte cultures have no Na^+-dependent uptake of ^{3}H-kainic acid. Also, the uptake of ^{3}H-kainic acid at 10 min is 3 orders of magnitude lower than for L-^{3}H-glutamate (cf. Fig. 1).

Effects of temperature on L-glutamate-stimulated $^{22}Na^+$ uptake

In Figure 8*A* we show glutamate-stimulated and nonstimulated $^{22}Na^+$ uptake at different temperatures, both in the presence of ouabain. It can be seen from Figure 8*A* that the glutamate-stimulated component of $^{22}Na^+$ uptake was hardly affected until

Table 2. Stoichiometry of $^{22}Na^+$ to L-^{3}H-glutamate uptake in primary astrocyte cultures

Substrate concentration (M)			Uptake (nmol/mg protein)		
Sodium	Glutamate	*n*	Sodium	Glutamate	Ratio
0.147	0	7	138.7 ± 7.9	—	
0	10^{-5}	8	—	0.44 ± 0.039	
0.147	10^{-5}	7	164.2 ± 7.5	10.23 ± 0.93	
Cotransport			25.5 ± 10.9	9.79 ± 0.93	2.84 ± 0.66*
0.147	0	7	129.4 ± 8.7	—	
0	10^{-4}	7	—	1.68 ± 0.17	
0.147	10^{-4}	6	182.7 ± 6.2	26.08 ± 2.14	
Cotransport			53.2 ± 10.7	24.40 ± 2.15	2.29 ± 0.35†

Ouabain (1 mM) was added at the same time as $^{22}Na^+$ and L-^{3}H-glutamate to all wells, and uptake was measured for 10 min. All results shown are means ± SEM. The uptake of L-^{3}H-glutamate and $^{22}Na^+$ was measured simultaneously. The amount of substrate cotransported was then calculated by subtracting the amount of $^{22}Na^+$ or L-^{3}H-glutamate transported in the absence of the second substrate obtained separately in the same experiment. The ^{22}Na:L-^{3}H-glutamate cotransport ratio is significantly different from the ratio of 1 by Student's *t* test indicated by the following values: * $p < 0.01$, † $p < 0.0005$. The ratios are not significant different statistically from a ratio of 2 or 3 or from each other. *n* = number of separate wells.

Table 3. Effect on uptake of L-^{3}H-glutamate of different times of exposure to ouabain

Time for uptake (min)	L-^{3}H-glutamate uptake (nmol/mg protein)	
	No ouabain	Ouabain
0		0.27 ± 0.02
2	11.10 ± 0.50	10.27 ± 1.79
10	53.30 ± 0.62	22.48 ± 0.66

n = 4 wells, mean ± SEM. Ouabain, 1 mM, was added at same time as L-^{3}H-glutamate. Cells were 32 d old.

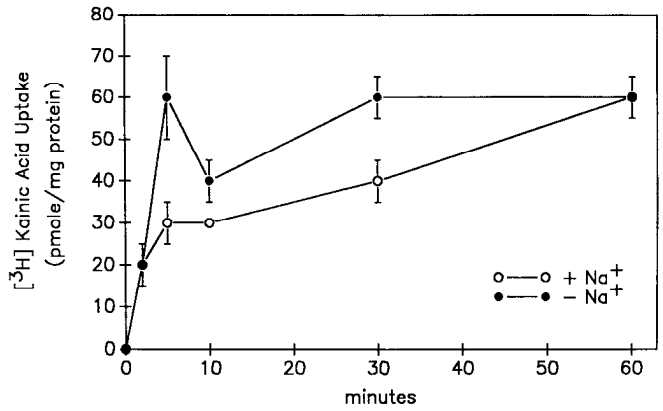

Figure 7. Time course of uptake of 10^{-4} M kainic acid. Cultures were 31 d old. ^{3}H-kainic acid, 2 μCi, was added per 0.5 ml reaction medium per well plus unlabeled kainic acid to obtain a final concentration of 10^{-4} M. Filled circle (●), NaCl replaced with choline Cl. Other conditions as in Figure 1.

a temperature of 4°C was reached. In Figure 8*B* it is shown that no stimulation of $^{22}Na^+$ uptake by glutamate was seen when ouabain was not added, which is why we always added ouabain for $^{22}Na^+$ uptake experiments.

In Figure 9*A* we have plotted L-^{3}H-glutamate uptake, and the L-glutamate-stimulated $^{22}Na^+$ uptake shown in Figure 8*A*, as a function of temperature. It can be seen that while L-^{3}H-glutamate uptake was almost completely abolished at 10°C, essentially no effect was seen at this temperature on L-glutamate-stimulated $^{22}Na^+$ uptake. At 4°C, glutamate-stimulated $^{22}Na^+$ uptake was sharply inhibited. In Figure 9*B* we show the $^{22}Na^+$/L-^{3}H-glutamate ratios calculated from the data in Figure 9*A*. It can be seen that very high apparent ratios are obtained at 10°C, while these progressively decline to lower values at higher temperatures because of the increase in ^{3}H-glutamate uptake.

Discussion

Na^+ dependence of L-glutamate uptake by astrocytes

An important unresolved question about the astrocytic uptake system is the stoichiometry of the Na^+ to glutamate taken up. If the Na^+:glutamate uptake ratio is >1, then this should greatly increase the driving force for glutamate uptake since it will now be driven by both the chemical and electrical inward gradients for the presumed positive complex (Martin, 1976; Erecinska, 1987). Such an electrogenic uptake might also be the mechanism for the Na^+-dependent, glutamate-induced depolarization seen in astrocytes in primary culture and other glial cells (see introductory remarks), which otherwise might be presumed to be due to a receptor-mediated increase in Na^+ conductance. We followed the method of Stallcup et al. (1979), who found a Na:glutamate ratio of 2:1 by measuring both $^{22}Na^+$ and L-^{3}H-glutamate uptake simultaneously in a cerebellar neuronal cell line in the presence of ouabain. Using the same methodology, we found a ratio of 2–3:1 for primary astrocyte cultures (Table 2) and 2:1 when the net glutamate-stimulated increase in $^{22}Na^+$ content was measured (Table 4). Stallcup et al. (1979) also showed a graph of the dependence of ^{3}H-glutamate uptake on $[Na^+]_o$, and we found that the Michaelis-Menten equation fitted their curve quite well and gave K_m = 184 ± 42 mM Na^+ (not shown). These results are very similar to ours, except our K_m was 50 mM (Fig. 3). Drejer et al. (1982) also found Michaelis-Menten kinetics for the Na^+ dependence of L-^{3}H-glutamate uptake in rat primary astrocyte cultures but concluded that this indicated that the stoichiometry for Na^+:glutamate uptake was 1:1. In contrast they found clear evidence of sigmoid, cooperative kinetics in neuronal cultures with a Hill coefficient of 2, indicating a requirement for 2 Na^+ for each transport cycle. One possible

Table 4. Stoichiometry of net glutamate-stimulated $^{22}Na^+$ uptake to L-^{3}H-glutamate uptake

Uptake time (min)	$^{22}Na^+$ content (nmol/mg protein)		L-^{3}H-glutamate uptake (nmol/mg protein)	Ratio of stimulated $^{22}Na^+$ uptake to L-^{3}H-glutamate uptake
	L-glutamate	No L-glutamate		
2	60.39 ± 0.19	54.60 ± 4.47	3.67 ± 0.32	1.47 ± 0.03
5	93.39 ± 2.74	71.83 ± 2.60	11.90 ± 0.79	1.81 ± 0.19
10	102.97 ± 3.55	76.10 ± 7.51	13.79 ± 1.01	1.88 ± 0.17
20	127.53 ± 10.54	80.68 ± 6.81	25.47 ± 1.95	1.80 ± 0.31
				Combined mean 1.74 ± 0.05 (n = 14)

Cells were preincubated with $^{22}Na^+$ (1 μCi/well containing 0.5 ml) for 1 hr without glutamate or ouabain and then 0.5 ml medium was added containing $^{22}Na^+$ (1 μCi/well) and 1 mM ouabain with or without 10^{-4} M L-^{3}H-glutamate (0.2 μCi/well). The amount of $^{22}Na^+$ and ^{3}H-glutamate taken up under these conditions for the times indicated was then determined as described in Materials and Methods. Cells were 28 d old. Values given as means ± SEM, n = 3–4 wells. The ratio in the final column was calculated separately for each well by subtracting the mean of the "No L-glutamate" column from each "L-glutamate" value and dividing this glutamate-stimulated $^{22}Na^+$ uptake by the L-^{3}H-glutamate taken up in the same well (third column). Two extreme values for $^{22}Na^+$ uptake with L-glutamate were rejected as outliers. The values for each time point and the combined mean are significantly greater than 1 and less than 3 by Student's t test ($p < 0.001$).

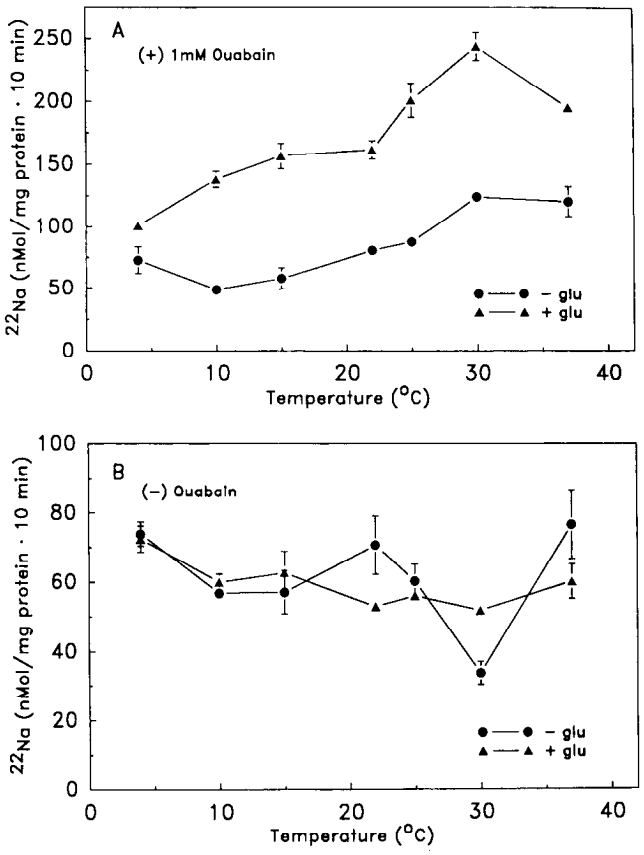

Figure 8. Effect of temperature on 10^{-4} M L-glutamate-stimulated uptake of $^{22}Na^+$. Uptake was measured for 10 min in the presence (*A*) or absence (*B*) of ouabain. HEPES, 25 mM, was used to buffer the medium instead of HCO_3^- since the different temperatures could not be achieved easily and still maintain a 5% CO_2/95% air atmosphere. pH was 7.4 and was adjusted for each temperature. Cells were 27 d old.

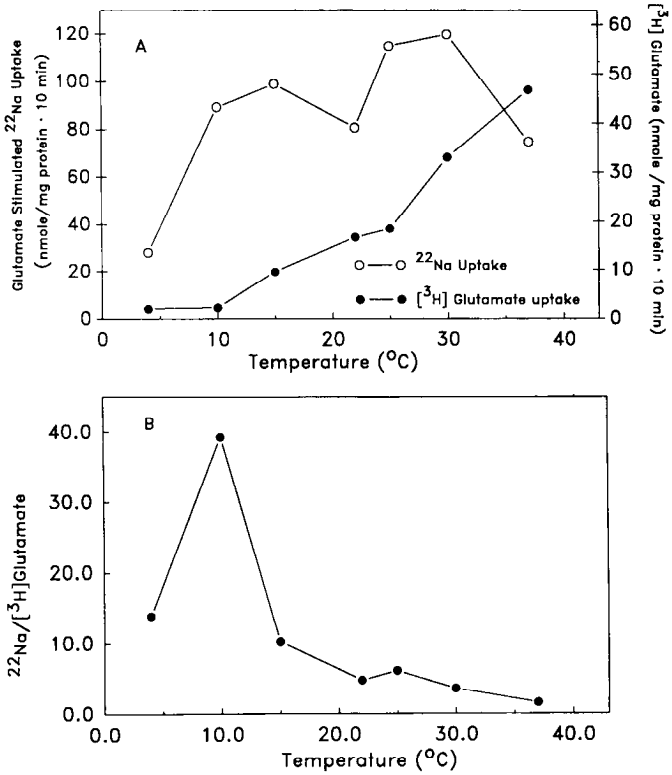

Figure 9. *A*, Uptake of 10^{-4} M L-^{3}H-glutamate and 10^{-4} M glutamate-stimulated Na^+ uptake as a function of temperature. $^{22}Na^+$ data from Figure 8*A*. L-^{3}H-glutamate uptake was measured in a separate experiment. *B*, Calculated Na^+:glutamate ratios at the different temperatures from the data shown in *A*.

reason for this discrepancy is that the rate equation for a ter (3-substrate) mechanism can give Michaelis-Menten kinetics even if 2 Na^+ are required. The equation for a rapid equilibrium random ter mechanism is

$$\frac{v}{V_{max}} = \frac{1}{\left[\left(1 + \frac{K_{glu}}{[Glut.]}\right)\left(1 + \frac{K_H}{[Na^+]}\right)\left(1 + \frac{K_L}{[Na^+]}\right)\right]}, \quad (1)$$

where K_L and K_H are the equilibrium dissociation constants for the low- and high-affinity sites for sodium, respectively. If these are equal, then sigmoid, cooperative, kinetics are obtained (see Fig. 10, furthest right-hand curve). It they are not equal, the high-affinity site will become saturated at lower concentrations of Na^+ and $K_H/[Na^+]$ will approach 0 and this part of the expression will reduce to 1. At a fixed concentration of glutamate, the entire expression then reduces to a Michaelis-Menten dependence of velocity on Na^+ concentration for K_L (Fig. 10, left-hand curve) with the observed K_m corresponding to the K_D of the low-affinity site. Thus, the direct determination of a Na: stoichiometry of 2:1 for Na^+-dependent glutamate uptake is not contradicted by kinetic behavior, indicating a stoichiometry of 1:1 because alternative models can be postulated to give Michaelis-Menten kinetics under the same conditions. A greater than 1:1 Na^+:glutamate ratio also seems indicated by considerations of the energetics of L-^{3}H-glutamate accumulation, as discussed in the next section.

Table 5. Effects of excitatory amino acid agonists on $^{22}Na^+$ uptake

Conditions	$^{22}Na^+$ uptake (nmol/mg protein/10 min)	*n*	% Control
Control	104.8 ± 6.67	20	100
10^{-4} M L-Glutamate	145.61 ± 14.90[a]	12	138
10^{-4} M Quisqualate	115.88 ± 8.02 (NS)	8	110
10^{-4} M NMDA	123.17 ± 10.87 (NS)	16	117
10^{-4} M Kainic acid	227.31 ± 15.50[a]	8	216

All agonists were added with 1 mM ouabain, and $^{22}Na^+$ uptake was measured for 10 min. Cells were 27 d old. *n* = number of wells, and uptake values represent means ± SEM. (NS), not significantly different from control.

[a] Significantly different from control by Dunnett's test (see Table 1 and Dunnett, 1964) with $p < 0.01$.

Energetics of L-glutamate uptake

The form of glutamate transport as an electrogenic process is clearly advantageous in that the driving force has both a Na^+ concentration gradient and a net inward electrical component. Based on the following reaction formulation for Na^+-dependent transport of glutamate from outside to inside,

$$\text{glutamate}_o + n\ Na^+_o \leftrightarrow \text{glutamate}_i + n\ Na^+_i, \quad (2)$$

we obtain the following equation to calculate the maximum ratio possible for L-glutamate [equation modified from that used by Martin (1976) for GABA]:

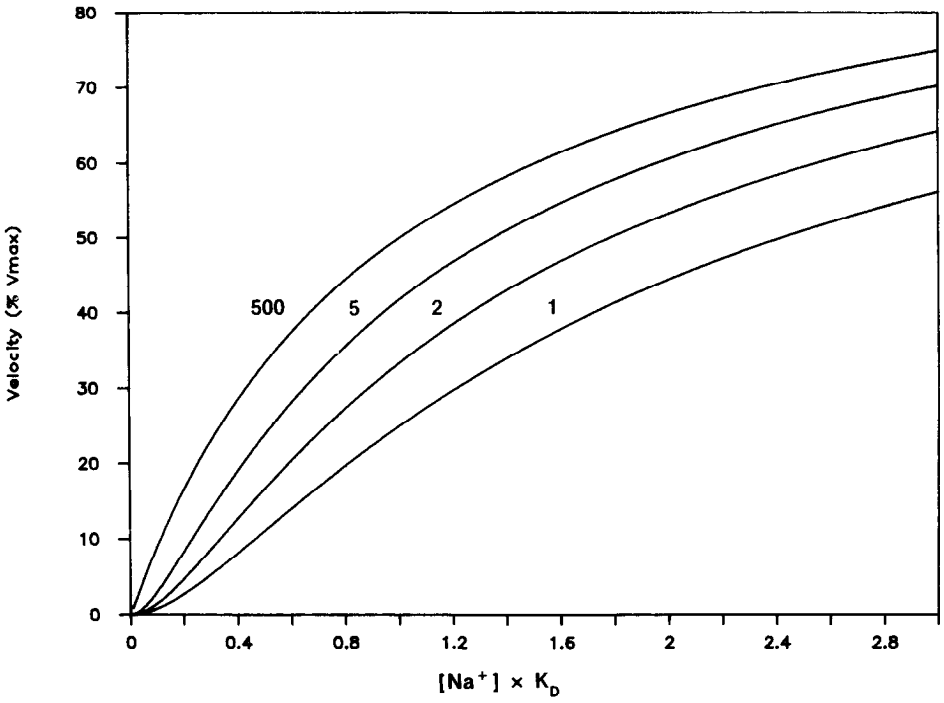

Figure 10. Theoretical dependence of glutamate transport upon [Na^+] for systems transporting 2 Na^+ per glutamate. The curves are, reading left to right, for the high-affinity Na^+ site having affinities 500, 5, 2, or 1 times greater than the affinity of the low-affinity site. The x axis represents the Na^+ concentration as a multiple of the K_D of the low-affinity site K_L (see text for further details).

$$[G_i]/[G_o] = ([Na^+{}_o]/[Na^+{}_i])^n \exp[-(n-1)(F\Delta E/RT)] \quad (3)$$

$[G_i]$ and $[G_o]$ represent the glutamate concentrations inside and outside the cell, respectively; n is the number of Na^+ ions transported per glutamate; E is the membrane potential; and F, R, and T have their usual meanings. In Table 6 we show the values of $[G_i]/[G_o]$ calculated for different values of n. It can be seen that the experimental concentration gradient we found from Figure 1 at 30 min, even when the system had not yet reached a steady state, is best fitted by a value for n of 2. We have also measured the intracellular glutamate level by HPLC (Waniewski and Martin, 1986) under the same conditions and found a ratio of 250 and 366 glutamate inside to outside measured after 30 min in 2 experiments (unpublished observations). Comparing this with the value obtained using tritium label (Table 6), we can see that 53–36% metabolism of L-^{3}H-glutamate is indicated. Such metabolism is not likely to be a problem when initial rates of L-^{3}H-glutamate are measured as in the experiments described in Tables 2 and 4 since the stoichiometry of the unidirectional inward fluxes is being measured. Also, to maintain even the minimum ratio of 250 found for actual glutamate inside over outside measured by HPLC would also clearly require a stoichiometry of 2 Na^+:1 glutamate (see Table 6).

Table 6. Different calculated ratios for intracellular to extracellular glutamate ($[G_i]/[G_o]$) for different values for the number (n) of Na^+ cotransported with glutamate

	$[G_i]/[G_o]$	
n	Calculated	Experimental
1	7.25	
2	729	530
3	73,361	

Based on Equation 3 with $[Na^+]_o$ = 145 and $[Na^+]_i$ = 20 mM. E = −0.07 V. Experimental value based on data in Figure 1 (see text).

Glutamate uptake and glutamate-induced depolarization of astrocytes

There are some similarities between the glutamate-induced depolarization (see introductory remarks) of astrocytes and glutamate uptake. In astrocyte cultures the EC_{50} value for the depolarization was around 50 μM, close to the K_m for L-^{3}H-glutamate uptake of 70 μM (Fig. 2). The cells were depolarized by L- and D-aspartate but not D-glutamate (Bowman and Kimelberg, 1984), and this corresponds to the specificity of the L-glutamate uptake system in brain slices (Balcar and Johnston, 1972) and glial cells (Henn et al., 1974). One major difference, however, is that kainic acid was as effective (Bowman and Kimelberg, 1984), or about half as effective (Kettenmann and Schachner, 1985), as L-glutamate in depolarizing the cells, and we have found that rat primary astrocyte cultures, like rat brain slices (Johnston et al., 1979), show no Na^+-dependent uptake of ^{3}H-kainic acid (Fig. 7).

Brew and Atwell (1987) concluded that the inward current they measured in axolotl retina Muller cells (236 pA at 3×10^{-5} M L-glutamate) was due to electrogenic uptake of L-glutamate with Na^+ since no reversal potential could be obtained over a range of −120 to +100 mV. This inward current in axolotl retina Muller cells differed from the depolarizations measured for rat brain primary astrocyte cultures since kainic acid had an insignificant effect in the Muller cells. Whole-cell currents of 40–300 pA at 10^{-4} M L-glutamate and the normal astrocyte resting membrane potential have recently been measured for astrocytes in primary culture (Sontheimer et al., 1988), and these authors also found a clear reversal potential that appeared to be due to increased conductances to both Na^+ and K^+. On the basis of our flux measurements, we can calculate an approximate

current of 6.0 pA/cell.[4] This is about 40-fold less than the current measured in axolotl Muller cells by Brew and Atwell (1987) and 7-fold less than the smallest current measured in primary astrocyte cultures by Sontheimer et al. (1988). It is possible that the whole-cell currents in astrocytes may represent the conductance of several cells as it is known that astrocytes in culture form syncytia (Fischer and Kettenmann, 1985). We can also estimate the depolarization expected from an inward current of 6 pA/cell to be around 6 mV,[5] although a major uncertainty in this calculation is the resistance of an individual cell. This is actually about 5-fold smaller than the observed depolarizations. In view, however, of the assumptions underlying these calculations, we cannot assert that electrogenic uptake cannot account for the depolarizations. In contrast to glutamate, the increased Na^+ influx due to kainic acid is not likely to be involved in any uptake mechanism, and since this flux is 2- to 3-fold greater than the glutamate-stimulated flux (compare Figs. 4 and 6 and see Table 5), this is more likely to be able to account for the depolarization obtained with this agonist.

Effects of temperature

Decreasing the temperature clearly dissociated glutamate-stimulated $^{22}Na^+$ uptake from Na^+-dependent ^{3}H-glutamate uptake (Figs. 8 and 9). This strongly supports the concept that the $^{22}Na^+$ uptake does not represent only cotransport with L-glutamate. Indeed, the calculated $^{22}Na^+$:^{3}H-glutamate ratios were very dependent on the temperature of the experiment (Fig. 9*B*). This suggests that a receptor-mediated mechanism would predominate at lower temperatures.

In conclusion, this study represents the only published description known to the authors, since the work of Stallcup et al. (1979), to attempt to directly measure the stoichiometry of Na^+: glutamate uptake in cultured cells known to have an active glutamate uptake system. In the last 10 years, however, a number of specific receptors for glutamate and other excitatory amino acids have been recognized (see Watkins and Olverman, 1987). The coexistence of depolarizing effects for excitatory amino acids such as L-glutamate that are also taken up, as well as for analogs such as kainic acid that are not taken up, implies complex effects on ion transport that do not allow an uptake stoichiometry to be readily determined. This study has examined this question for glutamate and kainic acid effects on Na^+ transport in rat brain primary astrocyte cultures, and we conclude that it is not possible to ascribe unequivocally all the glutamate-stimulated $^{22}Na^+$ uptake to cotransport with glutamate; also, it appears that both uptake and receptor-mediated components are involved. The elucidation of these interrelationships is likely to be important in considerations of astroglial function, as well as the more fundamental question of the relationship of Na^+-dependent cotransport systems to receptor stimulation of Na^+ transport when both these systems coexist in the same cell.

[4] An uptake at 10^{-4} M L-glutamate of 7.4 nmol/mg protein/min, in the absence of ouabain (Fig. 1), and assuming 1 positive charge per glutamate, would give a flux of 7.13×10^{-4} coulombs/mg protein min or 11.9 μA. There are around 2×10^6 cells/mg protein (Kimelberg et al., 1979), giving a current of 6.0×10^{-12} A, or 6.0 pA/cell.

[5] Astrocytes in primary culture have input impedances of 2–10 MΩ (Kimelberg and Walz, 1988), but these cells form syncytia (Fischer and Kettenmann, 1985). The usual area-specific membrane resistances of around 1000 Ωcm^2 and an average area per cell of approximately 10^{-6} cm^2, gives a resistance of 1000 MΩ/cell (Kimelberg and Walz, 1988). With a current of 6 pA, this would give a depolarization of $6.0 \times 10^{-12} \times 10^9$ or 6 mV at 10^{-4} M L-glutamate.

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