Phosphocreatine-dependent Glutamate Uptake by Synaptic Vesicles

A COMPARISON WITH ATP-DEPENDENT GLUTAMATE UPTAKE

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ATP-dependent uptake of glutamate into synaptic vesicles has been well documented. Stimulation of glutamate uptake into synaptic vesicles by other high-energy phosphates has not been described. In this paper, we examine the stimulation of phosphocreatine (PCr)-induced glutamate uptake and determine whether this stimulation is secondary to conversion of PCr to ATP. We found the following. 1) PCr stimulates glutamate uptake into synaptic vesicles in the absence of added ATP. 2) At a glutamate concentration of 50 \( \mu \)M, no concentration of added ATP could produce the degree of stimulation seen in the presence of PCr. 3) 0.5 \( \mu \)M iodoacetamide completely inhibits synaptic vesicle creatine kinase activity but does not inhibit PCr-stimulated glutamate uptake. 4) PCr-dependent glutamate uptake, unlike ATP-dependent uptake, is not magnesium- or chloride-dependent. 5) 0.5 \( \mu \)M N-ethylmaleimide, a selective \( \text{H}^-\text{-ATPase} \) inhibitor, completely inhibits ATP-dependent glutamate uptake but only slightly inhibits PCr-dependent glutamate uptake. 6) PCr-dependent glutamate uptake is sensitive to valinomycin, a \( \text{K}^-\text{/H}^+ \) translocator, whereas the ATP-dependent uptake is not. Therefore, it appears that in addition to the well-known ATP-dependent glutamate uptake system, there is a previously unreported PCr-dependent glutamate uptake system in synaptic vesicles. The total glutamate uptake by synaptic vesicles is likely the sum of both ATP- and PCr-dependent glutamate uptake.

Glutamate is the major excitatory neurotransmitter found in the vertebrate central nervous system. Naito and Ueda (1) have demonstrated an ATP-dependent uptake of glutamate into synaptic vesicles. The driving force for this process is an electrochemical proton gradient across the vesicle membrane generated by Mg\(^{2+}\)-ATPase (2, 3). This vesicular uptake system is highly specific for glutamate and is markedly stimulated by physiologically relevant, low millimolar concentrations of chloride (4–6).

PCr, an intracellular energy buffer, is a cosubstrate for creatine kinase in the following reaction.

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\text{PCr} + \text{ADP} \rightarrow \text{Cr} + \text{ATP} + \text{H}^+ \text{ATPase inhibitor}
\]

Materials—L-[3,4-\( ^3\)H]Glutamate (46 Ci/mmol) was obtained from Amersham Corp. All the enzymes and the chemicals were from Sigma. All test solutions were adjusted to pH 7.2–7.4 prior to use in the experiments. Cytosciintillation counting solution was from ICN (Costa Mesa, CA). HAWP filters (13 mm, 0.45 \( \mu \)m) were from Millipore (Bedford, MA).

Purification of Synaptic Vesicles—Synaptic vesicles from bovine brain were purified according to the procedure of Kish and Ueda (34). The purified synaptic vesicles were stored at –80°C until use. Synaptic vesicle protein was determined according to Lowry et al. (15) with bovine serum albumin as standard.

Assay of Vesicular \([^{3}H]\) Glutamate Uptake—The ATP-dependent uptake of glutamate into synaptic vesicles was assayed according to a published procedure (34). The standard incubation mixture contained 50 \( \mu \)g of protein from synaptic vesicles, 5 \( \mu \)M Tris maleate (pH 7.4), 4 \( \mu \)M MgSO\(_4\), 0.25 \( \mu \)M sucrose, 4 \( \mu \)M KCl, either 2 \( \mu \)M Tris-ATP neutralized with Tris base or 30 \( \mu \)M PCr, either 5 or 10 \( \mu \)Ci of [\( ^3\)H]glutamate (specific activity, 46 Ci/mmol) and either 50 \( \mu \)M or 1 \( \mu \)M glutamate as indicated in the figure legends in a final volume of 100 \( \mu \)l. Although the \( K_m \) for glutamate uptake is near 1 \( \mu \)M, most previous studies of ATP-dependent vesicular glutamate uptake were performed using 50 \( \mu \)M glutamate with high specific activity to increase the signal to noise ratio (2, 5, 11, 23). In order to compare our results with these studies, we use both 50 \( \mu \)M and 1 \( \mu \)M glutamate in the assay system. Similarly, 30 \( \mu \)M PCr (3 times higher than the EC\(_{50}\)) was used to optimize the uptake.

The synaptic vesicle, sucrose, Tris maleate, and MgSO\(_4\) mixture in 80 \( \mu \)l was preincubated at 30°C for 5 min, and then 20 \( \mu \)l of a mixture containing [\( ^3\)H]glutamate, KCl, and ATP or PCr was added, and the samples were incubated for an additional 2.5 min at 30°C. The reaction was terminated by the addition of 2.5 ml of ice-cold 0.15 M KCl, followed by immediate filtration through Millipore HAWP filters (13 mm, 0.45 \( \mu \)m). The incubation tube was rinsed with 3 ml of 0.15 M KCl three times, and the filter was then washed with an additional 15 ml of cold 0.15 M KCl. Radioactivity retained on the filters was counted in a Rack
Beta liquid scintillation counter (Wallac-Pharmacia, Turku, Finland) with 8 ml of Cytosint scintillation mixture.

Controls were incubated in the presence of synaptic vesicles and in the absence of either ATP or PCr, and the radioactivity retained on the filter was subtracted from mixtures containing synaptic vesicles and ATP or PCr.

**31P NMR Analysis—** Samples of the synaptic vesicle preparation containing 2.5 mg of protein were extracted by perchloric acid according to a published procedure (16). The perchloric acid extract was neutralized and lyophilized; 1 ml of D$_2$O (99.8%) was added, and the pH of the solution was adjusted to 9.6. 31P NMR spectra were recorded on a Bruker AM500 spectrometer by a previously described technique (17).

**Determination of Creatine Kinase Activity—** Creatine kinase activity in the synaptic vesicles was determined according to a published procedure (18). The reaction mixture contained 30 mM phosphocreatine, 2 mM ADP, 100 mM imidazole, 2 mM EDTA, 10 mM magnesium acetate, 20 mM N-acetyl-L-cysteine, 5 mM AMP, 0.1 mM $p$,$p$-di(adenosine-5') pentaphosphate, 20 mM D-glucose, 2 mM NADP$^+$, glucose-6-phosphate dehydrogenase (2 units/ml), hexokinase (3 units/ml), adjusted to pH 6.6, and 5 ml of synaptic vesicles in a total volume of 1 ml. The reaction was initiated by adding phosphocreatine. The rate of absorbance change at 339 nm at room temperature was recorded continuously with a Perkin-Elmer Lambda 3B spectrophotometer after a 2-min lag phase.

**Curve Fitting—** Curve fitting for inhibitory effects shown in Figs. 1, 5, 8, and 9 was performed by a least-squares fit to the following equation which is derived from the Hill equation (19):

$$ F_0 = M K^N (X^N + K^N) $$  (Eq. 1)

where $X$ = the test compound concentration, $M$ = maximal percent inhibition, $K$ = the IC$_{50}$ (concentration of the compound at half-maximal inhibition), and $N$ is the Hill coefficient. For stimulatory effects seen in Figs. 1 and 2 the equation

$$ F_0 = M X^N (X^N + K^N) $$  (Eq. 2)

was used. In this equation the variables have the same meaning except that $K$ = the EC$_{50}$ (concentration of the compound at half-maximal stimulation). The RS/1 statistical package (BBN Software products Corp., Cambridge, MA) was used for this curve-fitting analysis.

**RESULTS**

**PCr Enhances ATP-dependent Glutamate Uptake Activity by Synaptic Vesicles—** When assayed at 50 M$\mu$M glutamate, the ATP-dependent [3H]glutamate uptake activity of synaptic vesicles could be increased up to 40% in the presence of 5 mM PCr, a concentration close to its intracellular level (12, 13). The degree of stimulation of [3H]glutamate uptake activity by PCr is dependent upon the glutamate concentration in the assay system. For example, the glutamate uptake activity is stimulated by 300, 40, and 24%, when assayed in the presence of 1 M, 50 M, and 1 M glutamate, respectively. PCr stimulated 50 M$\mu$M glutamate uptake into synaptic vesicles with a maximum increase of 76% and an EC$_{50}$ of 9.5 mM (Fig. 1). Unlike the stimulation of glutamate uptake by addition of PCr, increasing the concentration of added ATP above 2 M$\mu$M did not increase glutamate uptake any further. In contrast, it actually leads to inhibition (Fig. 1). This is consistent with a previous report (1).

**PCr Stimulates Glutamate Uptake into Synaptic Vesicles in the Presence of ATP—** Glutamate uptake into synaptic vesicles has been reported to be an ATP-dependent process (1). It is possible that PCr functions to maintain a specific level of ATP within the synaptic vesicles, but the above data suggest that PCr has other actions. In the absence of added ATP, PCr stimulated glutamate uptake activity in a dose-dependent manner with an EC$_{50}$ about 10 mM (Fig. 2).

Characterization of the PCr-activated Glutamate Uptake System—The time courses of L-[3H]glutamate uptake by purified bovine brain synaptic vesicles either in the presence of 2 mM ATP or 30 mM PCr are shown in Fig. 3. An incubation time of 2.5 min was chosen for the assay as a compromise between adequate sensitivity (adequate counts taken up) and being near the linear portion of the uptake curve. The effect of varying the concentration of glutamate on either the ATP- or the PCr-dependent [3H]glutamate uptake activity is shown in Fig. 4A. From the Lineweaver-Burk plot (Fig. 4B), a single K$\text{m}$ value for glutamate was determined to be 0.39 M for the PCr-dependent glutamate uptake and 1.34 M for the ATP-dependent glutamate uptake. The latter value is comparable with previous reports (4, 5, 20–22). The V$\text{m}$ is determined to be 1.23 and 0.24 nmol/min/mg for the ATP- and the PCr-dependent glutamate uptake, respectively. The glutamate uptake activity in the presence of 2 mM ATP (29.6 ± 3.3 pmol/min/mg; n = 12) is about 69% of that in the presence of 30 mM PCr (43.0 ± 5.9 pmol/min/mg; n = 12), when assayed in the presence of 50 M$\mu$M glutamate. It was reported that the concentrations of ATP and PCr in the brain are about 3 and 4.5 mM, respectively (12, 13). The glutamate uptake activities in the presence of 3 mM ATP or 4.5 mM PCr or both were measured in the presence of 1 mM glutamate. The results shown in Table I indicate that PCr-dependent uptake activity and ATP-dependent uptake activity are additive. The glutamate uptake activity in the presence of both 3 mM ATP and 4.5 mM PCr is very close to the sum of the ATP-dependent and the PCr-dependent glutamate uptake ac-

**FIG. 1. Effect of PCr and ATP on ATP-dependent [3H]glutamate uptake into synaptic vesicles.** Uptake of 50 M$\mu$M L-[3H]glutamate (specific activity, 1 Ci/mmol) was determined in the absence of added ATP as described under "Experimental Procedures" in the presence of 2 mM ATP and varying concentrations of added PCr (○) and ATP (■). L-[3H]Glutamate uptake activity was expressed as the percent of the activity in the presence of 2 mM ATP and in the absence of PCr, which was 32.46 ± 2.56 pmol/min/mg.

**FIG. 2. Vesicular uptake of glutamate as a function of PCr concentration.** Uptake of 50 M$\mu$M L-[3H]glutamate (specific activity, 1 Ci/mmol) was determined in the absence of added ATP as described under "Experimental Procedures" in the presence of varying concentrations of PCr either in the presence of 4 mM Mg$^{2+}$ and 4 mM Cl$^{-1}$ (○) or in the absence of added Mg$^{2+}$ and Cl$^{-1}$ (□).
The glutamate uptake activities measured in the presence of 3 mM ATP and 30 mM PCr also are additive. Under physiologic conditions, glutamate uptake into synaptic vesicles is far too low to generate the millimolar levels of ATP that are required for maximal \(^{3}H\)glutamate uptake activity. Therefore, it is important to determine if the stimulatory effect of PCr on glutamate uptake activity is simply due to ATP formation, the additional ATP being the actual species responsible for the increased uptake activity. The concentration of ADP, ATP, and PCr in perchloric acid extracts of purified synaptic vesicles was determined by \(^{31}P\) NMR and found to be below the detection limit of this method (3 \(\mu M\)). Creatine kinase activity in the synaptic vesicle preparation was 2.87 ± 0.17 \(\mu mol/min/mg\). The combined concentration of ADP and ATP in the synaptic vesicles is far too low to generate the millimolar levels of ATP that are required for maximal \(^{3}H\)glutamate uptake activity even in the presence of high concentrations of exogenously added PCr and adequate activity of endogenous creatine kinase.

Furthermore, iodoacetamide (IAA), an inhibitor of creatine kinase (24), inhibited creatine kinase activity in the synaptic vesicle preparation with an \(IC_{50}\) of 0.034 \(mM\) and completely inhibited activity at a concentration of 1 \(mM\) (Fig. 5). In contrast, 1 \(mM\) IAA inhibited PCr-stimulated \(^{3}H\)glutamate uptake activity in the synaptic vesicles by only 2%. IAA inhibited PCr-stimulated \(^{3}H\)glutamate uptake with an \(IC_{50}\) of 75 \(mM\) (Fig. 5). In addition, two other creatine kinase inhibitors, 0.5 \(mM\) \(S\)-methyl methanethiosulfonate and 0.5 \(mM\) 2,4-dinitrofluorobenzene, completely inhibited creatine kinase activity (25–27) but only inhibited \(^{3}H\)glutamate uptake by 21 and 25% respectively.

\(Mg^{2+}\) and \(Cl^{-1}\) Stimulate ATP-dependent \(^{3}H\)glutamate Uptake, but Not PCr-dependent Uptake—It was previously reported that both \(Mg^{2+}\) and \(Cl^{-1}\) could stimulate ATP-dependent \(^{3}H\)glutamate uptake into synaptic vesicles. The maximal stimulation of the ATP-dependent uptake by chloride was approximately 3.3-fold at 4 \(mM\) (Fig. 6A), and the concentration of magnesium giving maximal stimulation was around 4 \(mM\) (Fig. 6B). These results are consistent with previous reports (1, 14). The PCr-dependent \(^{3}H\)glutamate uptake activity is stimulated only 20% at 4 \(mM\) KCl compared with 230% for the ATP-dependent uptake system. Furthermore, magnesium does not have any significant effect on PCr-dependent glutamate uptake activity, in contrast to the stimulatory effect on the ATP-dependent uptake system (Fig. 6B). PCr also stimulated \(^{3}H\)glutamate uptake in the absence of magnesium and chloride in a dose-dependent manner, with an \(EC_{50}\) of 4.6 \(mM\) (Fig. 2).

Inhibitors of ATP-dependent Glutamate Uptake Have Little Effect on PCr-dependent Uptake Activity—It is known that the

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**Table I**

| Compounds | Uptake activity (pmol/min/mg) |
|-----------|-------------------------------|
| 4.5 \(mM\) PCr | 84.15 ± 10.56 |
| 3 \(mM\) ATP | 281.2 ± 33.57 |
| 4.5 \(mM\) PCr + 3 \(mM\) ATP | 362.1 ± 5.74 |
| 30 \(mM\) PCr | 105.8 ± 14.0 |
| 30 \(mM\) PCr + 3 \(mM\) ATP | 482.7 ± 25.74 |

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**Fig. 3.** Time course of vesicular glutamate uptake by synaptic vesicles. Uptake of 50 \(\mu M\) \(L\)\(^{3}H\)glutamate (specific activity, 1 \(Ci/mmol\)) was determined as described under “Experimental Procedures” in the presence of 30 \(mM\) PCr (○) or 2 \(mM\) ATP (□) or in the absence of both exogenous PCr and ATP (●).

**Fig. 4.** A, rate of ATP- and PCr-dependent vesicular uptake of glutamate as a function of glutamate concentration. Uptake into synaptic vesicles was determined in the presence of varying concentrations of \(L\)glutamate (0.05–4 \(mM\)) and either 30 \(mM\) PCr (○) or 2 \(mM\) ATP (□) as described under “Experimental Procedures.” Radioactive \(L\)\(^{3}H\)glutamate was kept constant at 10 \(\mu Ci\). B, a Lineweaver-Burk plot of the kinetic data of A.

**Fig. 5.** Effect of IAA on PCr-dependent glutamate uptake and creatine kinase activity. Uptake of 50 \(\mu M\) \(L\)\(^{3}H\)glutamate (specific activity, 1 \(Ci/mmol\)) (□) and creatine kinase activity (○) was measured as described under “Experimental Procedures” in the presence of varying concentrations of IAA. In the absence of IAA, the control glutamate uptake activity was 27.65 ± 4.5 pmol/min/mg and the control creatine kinase activity was 2.87 ± 0.17 \(\mu mol/min/mg\).
ATP-dependent [$^3$H]glutamate uptake by synaptic vesicles is driven by an electrochemical proton gradient generated by Mg$^{2+}$-ATPase. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 25 μM), a proton translocator and 0.5 mM N-ethylmaleimide (NEM), a V-type ATPase inhibitor, completely inhibits the ATP-dependent [$^3$H]glutamate uptake activity but only inhibits the PCr-dependent glutamate uptake by 18% (FCCP) and 27% (NEM) (Fig. 7). NEM inhibits the PCr-dependent glutamate uptake with an IC$_{50}$ of 6.32 mM, which is 70 times higher than for the ATP-dependent glutamate uptake (0.09 mM) (Fig. 8). 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 5 μM), a potent inhibitor of anion translocators (3), inhibits ATP-dependent [$^3$H]glutamate uptake by 83% but has no detectable effect on PCr-dependent glutamate uptake activity. Oligomycin (10 μM), a mitochondrial H$^+$-ATPase inhibitor (F-type ATPase), and 1 mM ouabain, a Na$^+$/K$^+$-ATPase inhibitor, have no significant effect on either the ATP-dependent uptake or the PCr-dependent glutamate uptake (Fig. 7). In contrast, 10 μM valinomycin, a potassium ion ionophore, strongly inhibits (55%) PCr-dependent glutamate uptake but has no significant effect on ATP-dependent glutamate uptake.

The valinomycin-induced inhibition of PCr-dependent glutamate uptake suggests that this uptake system could be potassium-dependent. To test this possibility directly, the effect of potassium isethionate, an impermeant anion which has no effect on ATP-dependent uptake (14), was studied in the PCr-dependent system. Fig. 9 shows that potassium actually inhibited PCr-stimulated glutamate uptake into synaptic vesicles at low concentrations. The inhibitory effect reached a maximum at about 2 mM potassium isethionate with little additional inhibition being seen up to 150 mM. Sodium isethionate had no effect on PCr-dependent uptake (data not shown).

FIG. 6. Effect of varying concentrations of chloride and magnesium on the ATP- and PCr-dependent vesicular uptake of glutamate. Uptake of 50 μM [$^3$H]glutamate (specific activity, 1 Ci/mmol) was determined as described under “Experimental Procedures” in the presence of varying concentrations of chloride (A) and magnesium (B) and in the presence of either 30 mM PCr (□) or 2 mM ATP (○). The data are expressed as percent of control (without chloride in A and without magnesium in B). The controls in A for PCr- and ATP-dependent glutamate uptake activity are 62.26 ± 11.87 and 16.23 ± 4.19 pmol/min/mg, respectively. The controls in B for PCr- and ATP-dependent glutamate uptake activity are 37.11 ± 1.35 and 11.05 ± 1.36 pmol/min/mg, respectively.

FIG. 7. Effect of PCr on ATP- and PCr-dependent vesicular uptake of glutamate. Uptake of 50 μM [$^3$H]glutamate (specific activity, 1 Ci/mmol) was determined as described under “Experimental Procedures” in the presence of either 0 or 2 mM ATP (open bars) and in the presence of 0 (filled bars) or 2 mM PCr (empty bars). The data are expressed as percent of control (without inhibitor). The controls in the absence of inhibitors for PCr- and ATP-dependent glutamate uptake activity were 67.15 ± 12.5 and 28.11 ± 3.67 pmol/min/mg, respectively.
A synaptic vesicle preparation cannot generate the millimolar levels of ATP that would be required for significant ATP-dependent glutamate uptake through creatine kinase-catalyzed conversion of PCr and ADP to ATP. Furthermore, the nonselective creatine kinase inhibitor, IAA, had no effect on PCr-dependent glutamate uptake activity at an IAA concentration known to completely inhibit creatine kinase activity (Fig. 5). This suggests that in addition to the well-known ATP-dependent uptake system, there is a PCr-dependent glutamate uptake system located in synaptic vesicles.

The mechanism for the PCr stimulation of glutamate uptake into the synaptic vesicles is unclear. It is known that the driving force for glutamate uptake via the ATP-dependent system is an electrochemical proton gradient generated by Mg$^2+$-ATPase. Several pieces of data suggested that this is not the case for the PCr-dependent system. First, ATP-dependent uptake is magnesium-dependent, but magnesium has no significant effect on the PCr-dependent glutamate uptake activity (Fig. 2B). Second, ATP-dependent glutamate uptake activity is strongly stimulated by low concentrations of chloride, whereas the PCr-dependent glutamate uptake is stimulated by only 20% in the presence of 4 mM chloride (Fig. 6A). Third, the inhibitory effects of FCCP, a proton translocator, NEM, a V-type ATPase inhibitor, and DIDS, an inhibitor of anion transporters, are all greater on ATP-dependent glutamate uptake activity than on PCr-dependent glutamate uptake activity (Figs. 7 and 8). Compared with its marked inhibition of ATP-dependent glutamate uptake, NEM is at least 70-fold less potent in inhibiting PCr-dependent uptake.

It is possible that PCr donates its high energy phosphate group to a novel kinase or acts as an allosteric effector for a novel enzyme that regulates the activation of the PCr-dependent glutamate transporter. Unlike ATP, PCr has not been shown to be a direct donor of its high energy phosphate bond. However, it is known to compete with phosphoenolpyruvate, another molecule containing a high energy phosphate bond, as a substrate for pyruvate kinase (7). It is also possible that PCr can donate its high energy phosphate bond to an intermediate other than ATP, and this intermediate could function as a substrate for a kinase or as an allosteric effector to modulate an enzyme necessary for the stimulation of the PCr-dependent synaptic vesicle glutamate transporter. Precedent for PCr-dependent phosphorylation has been reported in two proteins of molecular masses 18 and 29 kDa in dialyzed cell-free extracts of rat skeletal muscle (28).

It is interesting that valinomycin, a potassium translocator, strongly inhibits PCr-dependent glutamate uptake (Fig. 7). This suggests that PCr-dependent glutamate uptake could be potassium-dependent. However, this hypothesis is not supported by the potassium-induced inhibition of PCr-dependent uptake (Fig. 9). Therefore, the effect of valinomycin appears not to be mediated by changes in potassium gradients across the vesicular membrane.

Glutamate neurotoxicity has been proposed as a cause of neuronal death in a variety of diseases including Alzheimer's disease (29). Certain phosphomonoesters, such as inositol 1-monophosphate and phosphodiester such as L-α-glycerophosphoryl ethanolamine and L-α-glycerophosphoryl cholnine are elevated in AD brain (30, 31). An in vivo $^{31}$P magnetic resonance spectroscopy study of AD patients showed that PCr levels are initially low and then become increased as the dementia worsens (20). Xu et al. (22) reported that inositol 1-monophosphate and α-glycerophosphate significantly stimulate glutamate uptake into synaptic vesicles. In this study we found that PCr also strongly stimulates glutamate uptake. Enhanced uptake of glutamate into synaptic vesicles could produce excessive glutamate release. This could result in glutamate-induced neurotoxicity and eventually induce the neuronal degeneration that is observed in Alzheimer's disease. Therefore, elevated levels of phosphomonoesters and PCr in Alzheimer's disease brain could play a role in the pathogenesis of this dementia.

In summary, we have demonstrated that in addition to an ATP-dependent glutamate uptake system, there exists a previously unreported PCr-dependent glutamate uptake system. The PCr-dependent glutamate uptake system differs from the ATP-dependent glutamate uptake system in several respects, including substrate affinity, requirement for magnesium and chloride, and mechanism of glutamate transport. Total glutamate transport into synaptic vesicles is likely the sum of both ATP-dependent and PCr-dependent glutamate uptake, with the PCr-dependent system accounting for approximately 25-50% of the total under physiologic conditions.

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Vesicular Phosphocreatine-dependent Glutamate Uptake

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