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## Synaptic vesicle-bound pyruvate kinase can support vesicular glutamate uptake

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### Abstract

Glucose metabolism is essential for normal brain function and plays a vital role in synaptic transmission. Recent evidence suggests that ATP synthesized locally by glycolysis, particularly via glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase, is critical for synaptic transmission. We present evidence that ATP generated by synaptic vesicle-associated pyruvate kinase is harnessed to transport glutamate into synaptic vesicles. Isolated synaptic vesicles incorporated [<sup>3</sup>H]glutamate in the presence of phosphoenolpyruvate (PEP) and ADP. Pyruvate kinase activators and inhibitors stimulated and reduced PEP/ADP-dependent glutamate uptake, respectively. Membrane potential was also formed in the presence of pyruvate kinase activators. “ATP-trapping” experiments using hexokinase and glucose suggest that ATP produced by vesicle-associated pyruvate kinase is more readily used than exogenously added ATP. Other neurotransmitters such as GABA, dopamine, and serotonin were also taken up into crude synaptic vesicles in a PEP/ADP-dependent manner. The possibility that ATP locally generated by glycolysis supports vesicular accumulation of neurotransmitters is discussed.

### Keywords

glycolysis; energy metabolism; neurotransmitter; refilling; VGLUT; nerve terminal

### Introduction

Glucose metabolism is required for normal brain function and synaptic transmission (1). Recent evidence demonstrates a direct correlation between glucose utilization and cognitive function as well as dynamic neural activity (2,3). Memory function is impaired by hypoglycemia (4). Under normal conditions, glucose serves as the major substrate for cerebral energy (5). Thus, the glucose requirement has been attributed to ATP production. However, hypoglycemia-induced pathophysiological states and aberrant synaptic transmission precede significant reduction in brain tissue ATP levels (6-12). Moreover, substitution of pyruvate for glucose

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does not support normal evoked neuronal activity, despite the fact that normal tissue ATP levels are maintained (13-15). Fleck et al. (10) have provided evidence that evoked release of glutamate, the major excitatory neurotransmitter, is diminished by lowered extracellular glucose. These observations suggest that either a certain glycolytic intermediate(s) or a minor ATP pool, possibly maintained by local synthesis of ATP, might be critical for normal synaptic transmission. Ikemoto et al. (16) have provided evidence suggesting that ATP produced locally by synaptic vesicle-associated glyceraldehyde-3-phosphate dehydrogenase (GAPDH)<sup>3</sup>/3-phosphoglycerate kinase (3-PGK) may play a critical role in filling synaptic vesicles with glutamate.

The major excitatory neurotransmitter glutamate is involved in learning and memory formation (17,18), as well as being required for basic neuronal communication (19-23). Glutamate accumulation into synaptic vesicles is the first critical step in glutamate synaptic transmission (22,24-28). This process requires ATP, harnessed to generate an electrochemical proton gradient by V-ATPase, the direct driving force for glutamate transport into the vesicle (29-36). The glycolytic requirement for normal synaptic transmission could result from the spatial distance between synaptic vesicles and mitochondria in the nerve ending. When mitochondria are not located within the nerve ending, glycolytically made ATP would be even more critical for maintaining neurotransmission. Morphometric analysis at the electron microscopic level has revealed that half of nerve endings lack mitochondria (37). In order to maintain continuous neurotransmission, the energy demand for rapid vesicle refilling might be met by ATP synthesized locally by glycolysis.

In this study, we have explored the possibility that the glycolytic ATP-generating enzyme pyruvate kinase, in addition to GAPDH/3-PGK, might also be associated with synaptic vesicles and that it can support vesicular glutamate and other neurotransmitter uptake in the presence of its substrates. Evidence is presented to support this notion. Based upon this and additional evidence presented here, as well as previous studies, the possibility is discussed that glycolytic ATP-generating enzymes may be important for the transport of neurotransmitters into synaptic vesicles.

## Experimental procedures

### Materials

L-[G-<sup>3</sup>H]Glutamic acid (43 Ci/mmol), 4-amino-n-[2, 3-<sup>3</sup>H]butyric acid (GABA, 88 Ci/mmol), [7,8-<sup>3</sup>H]dopamine (41 Ci/mmol), and 5-hydroxy-[G-<sup>3</sup>H]tryptamine creatinine sulfate (serotonin, 21 Ci/mmol) were purchased from GE Healthcare Bio-Sciences. Goat anti-pyruvate kinase antibody was purchased from Research Diagnostics Inc. Affinity-purified chicken polyclonal (IgY) anti-human V-ATPase subunit A antibodies (anti-ATP6A1) were from GenWay Biotech, Inc. Monoclonal anti-VGLUT1, anti-VGLUT2, and anti-synaptophysin 1 antibodies were from Synaptic Systems. A monoclonal anti-SV2 antibody-producing hybridoma clone was provided by Dr. Kathleen Buckley (38). An anti-SV2 antibody in hybridoma culture supernatant was utilized for Western blotting. (*Z*)-3-chlorophosphoenolpyruvate (Cl-PEP) and (*E*)-Cl-PEP (39) were kind gifts from Dr. Bernhard Erni and Dr. Luis Garcia-Alles (University of Bern). Hexokinase and all other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

### Preparation of subcellular fractions

Synaptic vesicles were prepared from frozen rat brains (unstripped, Pel-Freeze) by ultracentrifugation through a discontinuous sucrose gradient as previously described (40), with the following modifications. The rat crude synaptic vesicles were prepared as described (40), except that 4 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose and 0.1 mM DTT, and 6 mM

Tris-maleate (pH 8.1) containing 0.1 mM DTT were used as solution B and solution F, respectively. Crude vesicles obtained from 50-100 rat brains were then layered over a discontinuous sucrose gradient consisting of 3.5 ml (per 25 brains) of 0.2 M, 0.3 M, and 0.4 M sucrose in 4 mM Hepes-KOH (pH 7.4) and 0.1 mM DTT. The crude synaptic vesicles were centrifuged at 24,800 rpm ( $109,400 \times g_{\max}$ ) in a Beckman SW40.1 rotor for 2 h. The interface between the sample layer and the 0.2 M sucrose layer, enriched with pyruvate kinase, was diluted with 4 mM Hepes-KOH (pH 7.4) containing 0.1 mM DTT, and then centrifuged at 55,000 rpm ( $278,000 \times g_{\max}$ ) in a Beckman Ti70 rotor for 60 min. The synaptic vesicle-containing pellets were resuspended in a solution containing 0.32 M sucrose, 1 mM  $\text{NaHCO}_3$ , and 1 mM DTT. The purified synaptic vesicles were stored in liquid  $\text{N}_2$ . A synaptosomal cytosolic fraction, a plasma membrane fraction, and a microsomal fraction were prepared from frozen rat brains (Pel-Freeze) as described (41). Protein concentration was determined by the method of Bradford (42), with bovine serum albumin as a protein standard.

### Assay for neurotransmitter accumulation into synaptic vesicles

Uptake of  $^3\text{H}$ -labelled neurotransmitters including [ $^3\text{H}$ ]glutamate was measured by a filtration-based assay using Whatman GF/C filters, as described previously (29,30), with minor modifications, as described previously by Kish and Ueda (40). In the standard assay for [ $^3\text{H}$ ]glutamate uptake, aliquots (15  $\mu\text{g}$  protein) of rat synaptic vesicles were incubated at 30°C for 20 min with 50  $\mu\text{M}$  [ $^3\text{H}$ ]glutamate (0.5 Ci/mmol) in 0.1 ml of 20 mM Hepes-Tris (pH 7.4) containing 50 mM sucrose, 100 mM potassium gluconate, 4 mM KCl, 4 mM  $\text{MgSO}_4$ , 2 mM aspartate, 1 mM PEP, and 0.1 mM ADP. In the GABA uptake assay, 50  $\mu\text{M}$  [ $^3\text{H}$ ]glutamate were replaced with 50  $\mu\text{M}$  [ $^3\text{H}$ ]GABA (0.6 Ci/mmol). To examine dopamine uptake and serotonin uptake, synaptic vesicles were incubated with carrier-free [ $^3\text{H}$ ]dopamine (49 nM, 41 Ci/mmol) or [ $^3\text{H}$ ]serotonin (95 nM, 21 Ci/mmol) at 30°C for 20 min in 12 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl, 4 mM  $\text{MgSO}_4$ , and 1 mM ascorbic acid, in the presence or absence of a mixture of 1 mM PEP and 0.1 mM ADP.

### HPLC analysis of tritiated compounds in synaptic vesicles

Purified synaptic vesicles (30  $\mu\text{g}$ ) were incubated with 50  $\mu\text{M}$  [ $^3\text{H}$ ]glutamate at 30°C for 40 min in the presence of 1 mM PEP/ 0.1 mM ADP under the standard conditions for glutamate uptake as described above, followed by filtration with Whatman GF/C as described (40). The GF/C filter was further washed with 12 ml of ice-cold 0.32 M sucrose to remove KCl. The washed filter was then immersed in 2 ml of 80% ethanol, sonicated on ice for 4 min, and placed on a rotating orbital shaker at 4°C for 2 hr to extract the vesicular content. The extract was vacuum-concentrated to about 100  $\mu\text{l}$  and filtrated with an Ultrafree®-MC filter unit (0.1  $\mu\text{m}$ , Millipore). The filtrate was diluted with one volume of HPLC medium and loaded at room temperature onto a Nucleosil 100-5C18 reversed phase column (Macherey-Nagel, 4.8  $\times$  250 mm) attached to an HPLC system (Beckman). The column was eluted isocratically at a flow rate of 1 ml/min, with 13 mM trifluoroacetic acid containing 1 mM 1-octanesulfonate as a mobile phase (43). Elution of glutamate and other compounds was detected spectrophotometrically at 214 nm. Fractions were collected every 6 sec, and radioactivity determined with a Beckman LS 6500 liquid scintillation spectrophotometer. The retention times of aspartate, glutamine, glutamate, and GABA were 8.4, 9.9, 12.5, and 17.4 min, respectively, under these experimental conditions.

### Assay of pyruvate kinase activity

Vesicle-associated pyruvate kinase activity was measured spectrophotometrically by coupling pyruvate formation to lactate dehydrogenase reaction (NADH utilization) (44). Synaptic vesicles (15-30  $\mu\text{g}$  protein) were incubated in a reaction mixture (0.8 ml) containing 20 mM Hepes-Tris (pH 7.4), 50 mM sucrose, 100 mM potassium isethionate, 4 mM LiCl, 4 mM

MgSO<sub>4</sub>, 2 mM aspartate, 0.1 mM or 1 mM PEP, 0.1 mM ADP, 50 μM glutamate, 4.7 unit/ml lactate dehydrogenase, and 0.22 mM NADH. The reaction was started by addition of ADP, and pyruvate kinase activity was calculated from the rate of NADH decrease as monitored by changes in absorbance at 340 nm at 30°C. One unit of pyruvate kinase activity was defined as the amount of pyruvate kinase required to convert 1 μmol of the substrate to product per minute at 30°C.

### Other biochemical analyses

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (45). Two-dimensional electrophoresis was carried out by the Michigan Proteome Consortium as follows. Samples were solubilized in 200 μl of Destreak Rehydration solution (GE Healthcare Bio-Sciences) containing 1% (v/v) IPG buffer pH 3-10 (GE Healthcare Bio-Sciences). Isoelectric focusing in the first dimension was performed at 25°C using Immobiline DryStrip (pH 3-10, 11 cm, GE Healthcare Bio-Sciences) and a Protean IEF cell (Bio-Rad), essentially according to Bio-Rad's instructions. After isoelectric focusing, Immobiline strips were equilibrated for 25 min with gentle rocking in a solution containing 6 M urea, 20% (v/v) glycerol, 2% SDS, 2.5% (v/v) tributylphosphine, and 50 mM Tris-HCl (pH 8.8), and for an additional 25 min in the same solution as above except for the replacement of 2.5% tributylphosphine with 2.5% iodoacetamide. Equilibrated strips were placed on top of a Criterion XT Bis-Tris Gel (4-12%, 11 cm IPG Strip, Bio-Rad) for the second dimension. SDS-polyacrylamide gel electrophoresis was carried out to separate proteins based on molecular weight.

Western blotting analysis was performed as described by Harlow and Lane (46) using peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated rabbit anti-goat IgG as secondary antibodies (Cappel/MP Biomedicals). The Western blots were subjected to an enhanced chemiluminescence detection procedure (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce).

Generation of membrane potential across the synaptic vesicle membrane was monitored by fluorescence quenching of the membrane potential-sensitive dye oxonol V (Molecular Probes, Inc.) using a Fluorolog III fluorospectrophotometer (Horiba Jobin Yvon Co. Ltd.) as described previously (34,47).

## Results

### PEP/ADP-dependent glutamate uptake into synaptic vesicles

When incubated with [<sup>3</sup>H]glutamate, synaptic vesicles exhibited PEP/ADP-dependent glutamate uptake (Fig. 1). At ADP concentrations above 0.1 mM, ADP alone showed some uptake, but the uptake was markedly enhanced by PEP. HPLC analysis of radioactive vesicular content obtained in the presence of PEP and ADP demonstrated that greater than 95% of radioactivity taken up into synaptic vesicles was glutamate (data not shown).

Figure 2 shows Western blotting analysis of the isolated synaptic vesicles used in this study, with antibodies raised against synaptic vesicle-specific proteins including VGLUT1/2 (29, 31,48-50), SV2 (38), and synaptophysin 1 (51). These vesicle-specific proteins were highly enriched in synaptic vesicle fractions (SV), but minimally detected in plasma membrane (PM) or microsomal fractions (M) prepared from rat brain. Moreover, the catalytic subunit of V-ATPase, functionally linked to glutamate uptake into synaptic vesicles through VGLUT (25, 29,31,36), was also enriched in synaptic vesicle fractions, but not in plasma membrane or microsomal fractions.

## Involvement of vesicle-associated pyruvate kinase in PEP/ADP-dependent glutamate uptake

Because PEP and ADP induced vesicular uptake of [ $^3\text{H}$ ]glutamate, we conducted experiments to determine if pyruvate kinase associated with synaptic vesicles is involved in PEP/ADP-dependent glutamate uptake. In the glycolytic pathway, pyruvate kinase utilizes PEP to produce ATP from ADP. Mammalian pyruvate kinase is known to require certain monovalent cations for activity (52). Thus,  $\text{K}^+$  is far more effective than  $\text{Na}^+$  or  $\text{Li}^+$  as an activator at 100 mM, and the effect of  $\text{K}^+$  is uniquely mimicked by a low concentration (3 mM) of  $\text{TI}^+$ . We compared the effects of  $\text{K}^+$  and  $\text{Na}^+$  on PEP/ADP-dependent glutamate uptake. We also compared the effect of  $\text{TI}^+$  with the effect of  $\text{K}^+$ . As shown in Fig. 3A, PEP/ADP-dependent glutamate uptake was significantly attenuated, when 100 mM  $\text{K}^+$  were replaced with 100 mM  $\text{Na}^+$ . In addition, 3 mM  $\text{TI}^+$  was able to substitute for 100 mM  $\text{K}^+$ . As shown in Fig. 3B, 3 mM  $\text{TI}^+$  also markedly activated PEP/ADP-dependent glutamate uptake. The effect of these monovalent cations on vesicle-associated pyruvate kinase activity was examined. Significant pyruvate kinase activity ( $0.071 \pm 0.008$  unit/mg protein) was found associated with isolated synaptic vesicles when assayed in the presence of 100 mM  $\text{K}^+$  (Fig. 3C). When 100 mM  $\text{K}^+$  was replaced with 100 mM  $\text{Na}^+$ , pyruvate kinase activity was markedly reduced, as observed with PEP/ADP-dependent glutamate uptake (Figs. 3A and 3C).  $\text{TI}^+$  (3 mM) had a similar stimulatory effect to that of  $\text{K}^+$  on pyruvate kinase activity (Fig. 3D), consistent with the data for glutamate uptake (Fig. 3B) and the previously reported data on muscle pyruvate kinase (52).

In an effort to obtain further evidence in support of the notion mentioned above, we examined the effect of the highly selective pyruvate kinase inhibitor (*Z*)-Cl-PEP on PEP/ADP-dependent glutamate uptake. Garcia-Alles and Erni (39) reported that (*Z*)-Cl-PEP, but not (*E*)-Cl-PEP, is a potent inhibitor of pyruvate kinase activity. Therefore, we tested (*Z*)-Cl-PEP and (*E*)-Cl-PEP for the ability to inhibit PEP/ADP-dependent glutamate uptake. (*Z*)-Cl-PEP (Fig. 4A, filled circles) inhibited PEP/ADP-dependent glutamate uptake in a concentration-dependent manner, whereas (*E*)-Cl-PEP (Fig. 4A, open circles) had no effect on glutamate uptake at the concentrations tested. (*Z*)-Cl-PEP (Fig. 4B, filled circles) inhibited vesicle-associated pyruvate kinase activity in a manner similar to that observed with glutamate uptake, whereas (*E*)-Cl-PEP (Fig. 4B, open circles) was ineffective. These observations further support a role for vesicle-associated pyruvate kinase in PEP/ADP-dependent glutamate uptake.

We analyzed the synaptic vesicle fraction and the synaptosomal cytosol fraction for pyruvate kinase by 2-dimensional Western blotting using an anti-pyruvate kinase antibody. A Western blot of the synaptic vesicle fraction revealed 4 distinct spots, designated as spots 1-4; their approximate isoelectric points were 7.1, 6.9, 6.7, and 6.5, respectively (Fig. 5A). In contrast, the cytosol fraction showed several spots, including spots 2, 3, and 4, but spot 1 was barely detected (Fig. 5B). This is not due to poor resolution of the 2D gel for the latter; the amount of the cytosol fraction applied was much less than that of the vesicle fraction. The presence of spot 1 in the vesicle fraction could raise the possibility that vesicle-bound pyruvate kinase might be different in some respect from the enzyme in the cytosol fraction. The difference in spot pattern suggests that cytosolic pyruvate kinase is not bound to synaptic vesicles in a non-specific manner.

## Characterization of PEP/ADP-dependent glutamate uptake

Next, we examined the effect of various compounds on PEP/ADP-dependent glutamate uptake (Fig. 6). PEP/ADP-dependent uptake was inhibited by the proton ionophore carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP) (12.5  $\mu\text{M}$ ), as well as by the V-ATPase inhibitor bafilomycin (1 mM) (33,53). These observations suggest that PEP/ADP-dependent glutamate uptake is driven by an electrochemical proton gradient formed by V-ATPase. ATP-dependent vesicular glutamate uptake is known to be stimulated by low millimolar  $\text{Cl}^-$  (30, 33,36,54) and inhibited by Rose Bengal (47), as well as by *trans*-1-aminocyclopentane-1,3-

dicarboxylic acid (ACPD) (55,56); however, this uptake is not affected by aspartate (30,33, 36) or *cis*-ACPD (55,56). As shown in Fig. 6, Rose Bengal (10  $\mu$ M) and *trans*-ACPD (2 mM) both significantly inhibited glutamate uptake, whereas *cis*-ACPD (2 mM) had little if any effect. Omission of aspartate from the uptake medium also had no effect on glutamate uptake, but omission of 4 mM  $\text{Cl}^-$  from the uptake medium significantly reduced PEP/ADP-dependent glutamate uptake. Taken together, these data strongly suggest that PEP/ADP-dependent glutamate uptake into synaptic vesicles is mediated by VGLUT, which functions in the presence of an electrochemical proton gradient formed by V-ATPase (25).

If PEP/ADP-dependent glutamate uptake is mediated by the VGLUT system, an electrochemical proton gradient should be formed in a PEP/ADP-dependent manner. Therefore, we examined whether a membrane potential across the synaptic vesicle membrane is generated upon addition of PEP and ADP, using the membrane potential-sensitive fluorescent probe oxonol V, as described in Experimental procedures. When synaptic vesicles were incubated with PEP plus ADP, a gradual decrease in fluorescence was observed (Figs. 7A and 7B, bold solid line). This fluorescent quenching was reversed by addition of FCCP (12.5  $\mu$ M), which indicates that PEP/ADP leads to membrane potential formation. Formation of the membrane potential was not observed when synaptic vesicles were incubated with ADP alone (Fig. 7A, dotted line), PEP alone (Fig. 7B, dotted line), or PEP/ADP plus FCCP (Figs. 7A and 7B, thin solid line). We also examined the effect of monovalent cations on membrane potential formation in the presence of PEP/ADP. In the presence of 100 mM  $\text{K}^+$  (bold solid line), but not  $\text{Na}^+$  (dotted line), membrane potential formation was observed, as shown in Fig. 7C.  $\text{Ti}^+$  was found to mimic the stimulatory effect of  $\text{K}^+$ , as expected (Fig. 7D, bold solid line). These data were expected based on the monovalent cation-dependence of pyruvate kinase activity and glutamate uptake shown in Fig. 3.

### Effects of hexokinase on ATP-dependent and PEP/ADP-dependent vesicular glutamate uptake

In an attempt to determine whether ATP produced by vesicle-bound pyruvate kinase is readily used by V-ATPase coupled to VGLUT, we carried out "ATP-trapping experiments" using hexokinase (57), as shown in Fig. 8. In this experiment, ATP-dependent glutamate uptake and PEP/ADP-dependent glutamate uptake into isolated synaptic vesicles were compared in the presence of varying amounts of hexokinase, which would trap and consume ATP to phosphorylate glucose in competition with the glutamate uptake system. PEP/ADP-dependent glutamate uptake was somewhat reduced by hexokinase in a dose-dependent manner (Fig. 8, closed circles), suggesting that ATP produced by vesicle-bound pyruvate kinase diffuses to the surrounding medium to some degree. However, ATP-dependent glutamate uptake driven by 60  $\mu$ M ATP, which gave about the same glutamate uptake activity as that observed in the presence of 1 mM PEP and 0.1 mM ADP, was much more markedly reduced in a dose-dependent manner by hexokinase (Fig. 8, open circles). There was a clear difference in sensitivity to hexokinase between PEP/ADP-induced uptake and ATP-dependent uptake. These data suggest that the exogenously added hexokinase is less accessible to ATP endogenously generated by vesicle-bound pyruvate kinase.

### PEP/ADP-dependent uptake of other neurotransmitters into synaptic vesicles

We conducted experiments to determine if neurotransmitters other than glutamate can be taken up into synaptic vesicles in a PEP/ADP-dependent manner. As shown in Fig. 9 (filled bars), vesicular uptake of GABA (Fig. 9B), dopamine (Fig. 9C), and serotonin (Fig. 9D) in the presence of PEP and ADP was all higher than in their absence (hatched bars). These results suggest that vesicle-associated glycolytic ATP-generating enzyme pyruvate kinase could support vesicular uptake of not only glutamate but also other classical neurotransmitters.

## Discussion

We have presented evidence that the glycolytic ATP-generating enzyme pyruvate kinase is bound to synaptic vesicles (Fig. 5A) and that its substrates can support vesicular glutamate uptake (Fig. 1 and Fig. 9A). This study represents the first functional evidence, to our knowledge, suggesting that vesicle-bound pyruvate kinase may play a role in synaptic transmission. The synaptic vesicle fraction used here is enriched with synaptic vesicle marker proteins such as SV2, synaptophysin 1, VGLUT1, and VGLUT2, which occur minimally in plasma membrane and microsomal fractions (Fig. 2). Our finding is in agreement with the recent demonstrations by Morciano et al. (58) and Takamori et al. (59) that pyruvate kinase is associated with synaptic vesicles purified by immunoisolation and by sucrose density gradient centrifugation in combination with size exclusion chromatography, respectively. Pyruvate kinase has also been associated with brain clathrin-coated vesicles (60). The monovalent cations  $K^+$  and  $Tl^+$ , agents known to activate pyruvate kinase, stimulate glutamate uptake into isolated synaptic vesicles, in the presence of PEP and ADP (Fig. 3). The highly selective pyruvate kinase inhibitor (*Z*)-Cl-PEP, but not its geometric isomer (*E*)-Cl-PEP, diminishes vesicular glutamate uptake in the presence of PEP and ADP (Fig. 4). Pharmacological characterization suggests that PEP/ADP-dependent uptake is mediated by VGLUT, which functions in the presence of an electrochemical proton gradient generated by V-ATPase (Fig. 6). Moreover, addition of PEP and ADP to the isolated synaptic vesicle fraction results in formation of a membrane potential (Fig. 7). These observations indicate that synaptic vesicle-associated pyruvate kinase, when activated, can provide ATP necessary to generate an electrochemical proton gradient, the driving force for glutamate transport into vesicles.

In ATP trap experiments (Fig. 8), hexokinase at high concentrations somewhat consumed ATP generated by vesicle-bound pyruvate kinase to reduce PEP/ADP-dependent glutamate uptake, suggesting that ATP produced by vesicle-bound pyruvate kinase is not directly transferred to V-ATPase, and diffuses into the surrounding milieu. However, PEP/ADP-dependent glutamate uptake was much more refractory to hexokinase than glutamate uptake driven by exogenously added ATP. This suggests that ATP produced by vesicle-bound pyruvate kinase is restricted to the microenvironment around the vesicle where V-ATPase is more accessible to ATP than exogenously added hexokinase. In the close quarters of the nerve ending *in vivo*, ATP concentration may increase much more rapidly than we observed for the *in vitro* assay conditions in this study, since the diffusion of cytosolic components is limited due to the presence of rather concentrated protein constituents (61). Thus, ATP locally produced by vesicle-bound pyruvate kinase might be more readily utilized *in vivo* by V-ATPase to achieve vesicular uptake faster than that observed under our experimental conditions.

Previously, we showed that inhibitors of GAPDH reduce vesicular glutamate uptake in synaptosomes, while inhibitors of mitochondrial ATP synthase have little effect, despite the fact that both treatments substantially reduce total synaptosomal ATP (16). This provided evidence suggesting that under normal conditions in which glucose serves the major substrate for energy metabolism, mitochondrially produced ATP has a minimal role in vesicular accumulation of glutamate in synaptosomes. Recent proteomic studies (58,59,62) indicate that glycolytic enzymes including GAPDH and pyruvate kinase are present in isolated synaptic vesicle preparations. Moreover, in the nerve ending, mitochondria are not always localized close to synaptic vesicle clusters or neurotransmitter release sites (63). Temporarily, glycolytic ATP production precedes mitochondrial ATP synthesis. Thus, it is conceivable that a pool of ATP produced by glycolytic enzymes associated with synaptic vesicles is predominantly utilized at the site of production to carry out certain cellular processes, such as neurotransmitter refilling of synaptic vesicles, which occurs rapidly *in vivo* (64). Shephard and Harris (37) reported that half of nerve endings in certain brain regions lack mitochondria. Dependence on glycolytic ATP would be even greater in these nerve endings devoid of mitochondria. This

notion is further supported by the observation reported here that activation of synaptic vesicle-associated pyruvate kinase is capable of vesicular glutamate accumulation. Glycolytic ATP locally produced by vesicle-bound enzymes could diffuse to the microenvironment around the vesicle to be utilized by V-ATPase in proximity to them, efficiently generating an electrochemical proton gradient to transport glutamate into synaptic vesicles. This concept could be applied to vesicular transport of other classical neurotransmitters such as GABA, dopamine, and serotonin, as these transmitters were also shown to be taken up upon activation of vesicle-bound pyruvate kinase (Fig. 9). In some other nerve endings, however, ATP utilized for neurotransmitter uptake could also come from “soluble” glycolytic enzymes as well as from neighboring mitochondria. The specific activity of pyruvate kinase activity in the soluble fraction was found to be much higher than that in the vesicle fraction studied here. Moreover, Pellerin and Magistretti (65) and Rikhy et al (66) provided evidence supporting the importance of mitochondrial ATP in synaptic transmission; however, the role of mitochondrial ATP in neurotransmitter vesicular accumulation is not addressed. The extent to which vesicle-bound pyruvate kinase contributes to the ATP requirement for neurotransmitter uptake into synaptic vesicles *in vivo* remains to be clarified.

Since the brain usually metabolizes ketone bodies for energy during prolonged periods of fasting, and functional mitochondria are required to generate ATP from ketone bodies, it has been assumed that mitochondrial ATP has more significant roles than glycolytic ATP in the fasting brain. Under calorically restricted conditions, early glycolytic intermediates such as glucose 6-phosphate and fructose 6-phosphate are reduced as expected. However, most of the downstream metabolites, as well as 3-phosphoglycerate kinase, are not decreased in the liver; they include fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate (67). Rather, the first three of these metabolites are increased under caloric restriction conditions. Thus, it is likely that the amount of ATP produced by 3-phosphoglycerate kinase under caloric restriction is no less than that generated under normal conditions. In the brain, it has been argued that caloric restriction could lead to an increase in 3-phosphoglycerate kinase activity in aged rats, because of less inactivation due to formation of smaller amounts of nitrotyrosine residues (68). These lines of evidence support the notion that vesicular glutamate uptake driven by ATP generated by 3-phosphoglycerate kinase would be hardly compromised under caloric restriction. On the other hand, pyruvate kinase activity in the liver is reduced resulting in an increase in phosphoenol pyruvate and a decrease in pyruvate under caloric restriction (67). In contrast, in the brain, caloric restriction has been thought to increase pyruvate kinase activity, since it produces smaller amounts of carbonylation, resulting in less inactivation of the enzyme (68). This suggests that vesicular glutamate uptake fueled by pyruvate kinase-generated ATP would not be suppressed under restricted diet. Thus, overall glutamate accumulation in synaptic vesicles achieved by glycolytic ATP may not be significantly affected under caloric restriction conditions.

There is growing evidence that membrane-bound glycolytic ATP-generating enzymes are linked to the transport of other cations (69). These lines of evidence altogether suggest a common theme: local glycolytic ATP production is coupled to the transport of cations including Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> as well as H<sup>+</sup>, playing vital roles in various cellular processes. Further investigation is warranted into the role of glycolytic ATP, in particular that locally produced by membrane-associated enzymes, in maintaining normal synaptic transmission and other cellular processes.

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## Abbreviations used

ACPD, 1-aminocyclopentane-1,3-dicarboxylic acid; Cl-PEP, 3-chlorophosphoenolpyruvate; DTT, dithiothreitol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEP, phosphoenolpyruvate; 3-PGK, 3-phosphoglycerate kinase; SDS, sodium dodecyl sulfate; V-ATPase, V-type proton-pump ATPase; VGLUT, vesicular glutamate transporter.

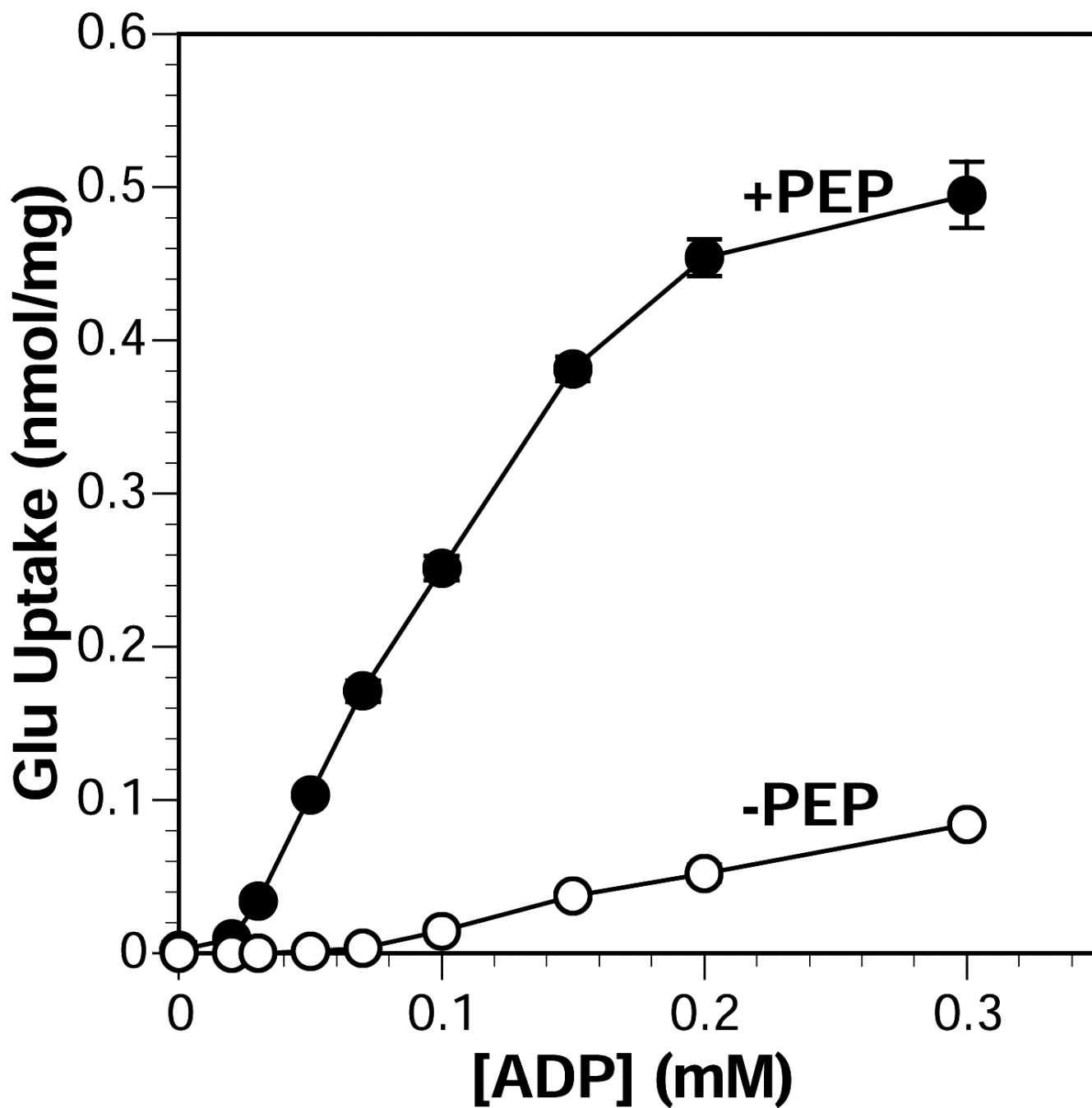
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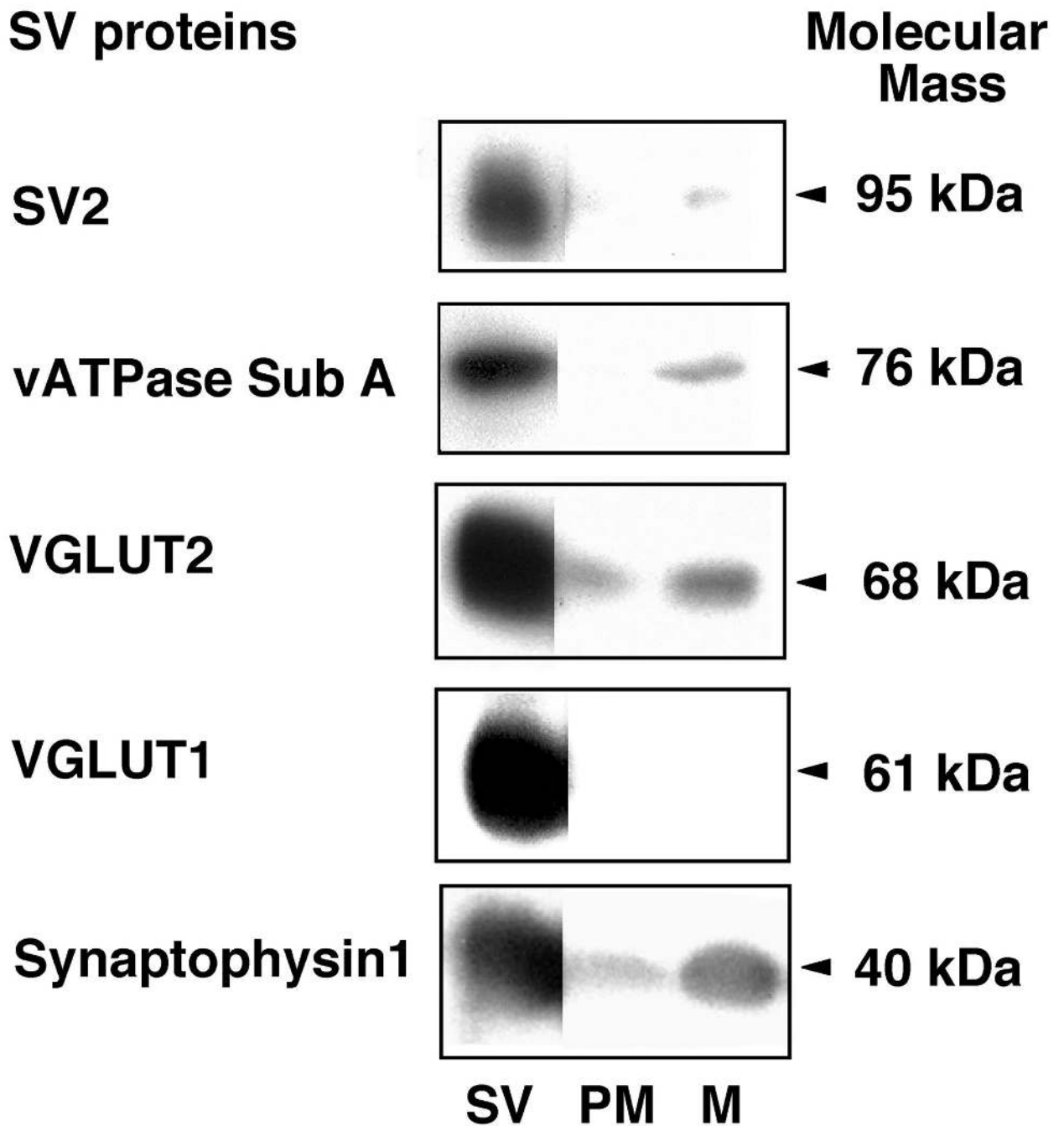
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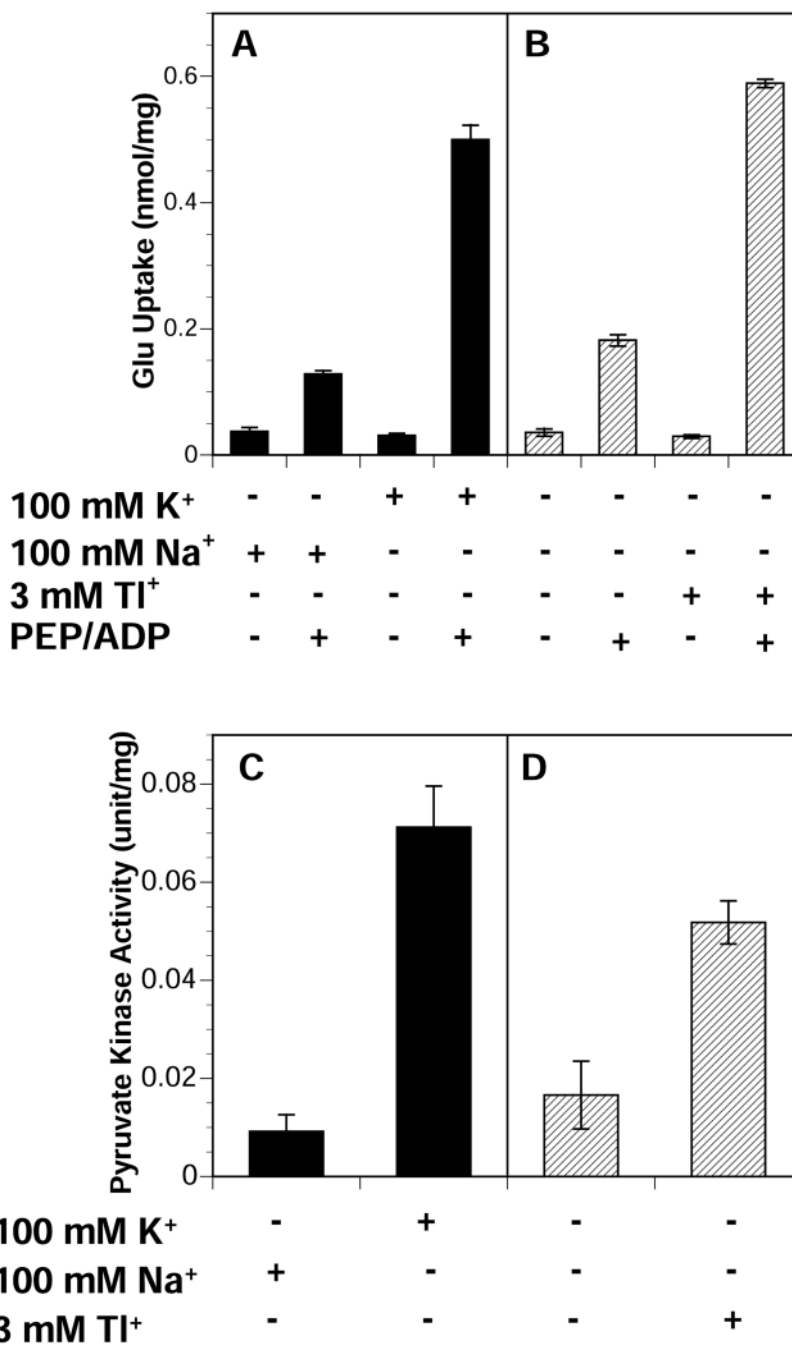
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**Fig. 1.** Effect of various concentrations of ADP on PEP/ADP-dependent glutamate uptake in synaptic vesicles. Purified synaptic vesicles (15  $\mu$ g) were incubated at 30°C for 20 min with 50  $\mu$ M [ $^3$ H]glutamate in 20 mM HEPES-Tris (pH 7.4) containing 50 mM sucrose, 100 mM potassium gluconate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, 2 mM aspartate, and the indicated concentrations of ADP in the presence (filled circles) or absence (open circles) of 1 mM PEP. Glutamate uptake was measured as described in Experimental procedures. Values are the mean  $\pm$  S.D. of three separate experiments.

**Fig. 2.**

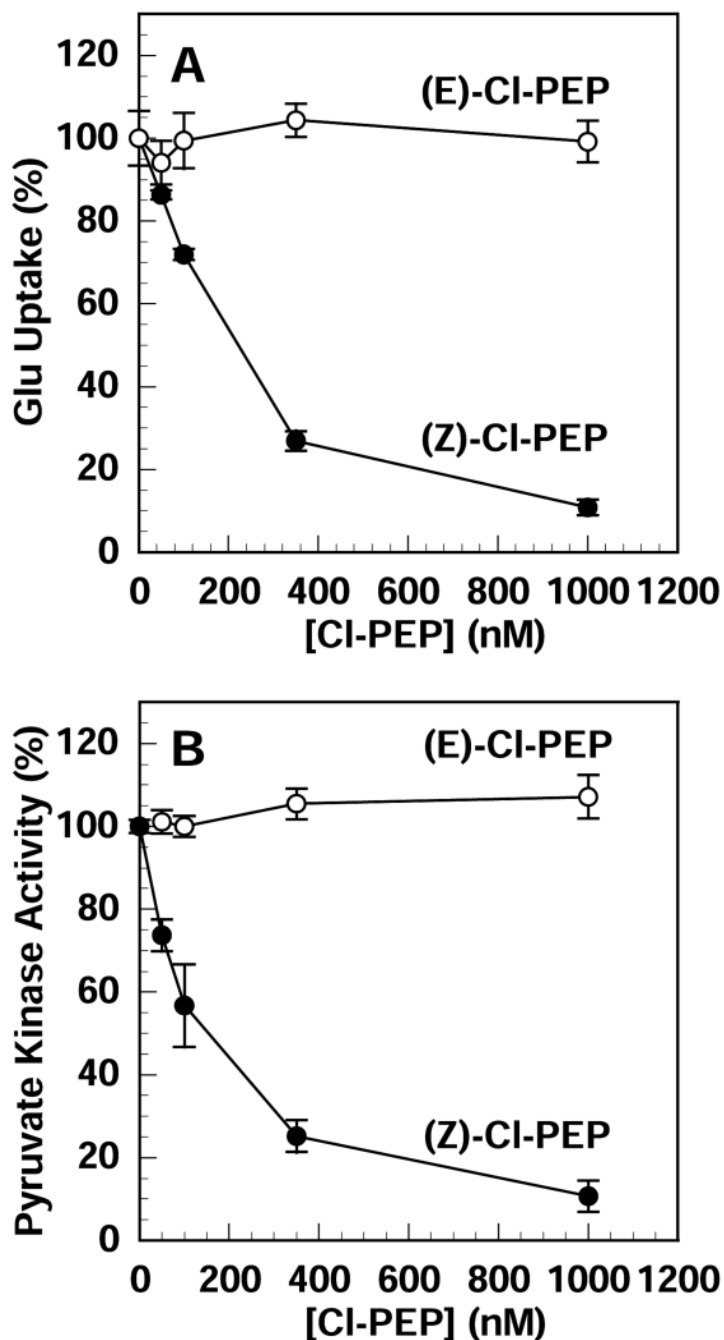
Western blotting analysis of purified synaptic vesicles. Crude synaptic vesicles were purified by sucrose density gradient centrifugation as described in Experimental procedures. The plasma membrane and microsomal fractions were prepared as described in Experimental procedures. The purified synaptic vesicle (SV, 15  $\mu$ g), plasma membrane (PM, 15  $\mu$ g), and microsomal (M, 15  $\mu$ g) fractions were analyzed by Western blotting, using antibodies raised against the indicated proteins. *Sub A*, subunit A.

**Fig. 3.**

Effect of monovalent cations on PEP/ADP-dependent glutamate uptake and vesicle-associated pyruvate kinase activity. (A) Purified synaptic vesicles (15  $\mu$ g) were incubated at 30°C for 20 min with 50  $\mu$ M [<sup>3</sup>H]glutamate in 20 mM HEPES-Tris (pH 7.4) containing 50 mM sucrose, 100 mM potassium isethionate, 4 mM LiCl, 4 mM MgSO<sub>4</sub>, and 2 mM aspartate, in the presence or absence of 1 mM PEP plus 0.1 mM ADP. Glutamate uptake was measured by a filtration-based assay using Whatman GF/C filters, as described in Experimental procedures. Sodium isethionate was used in place of potassium isethionate when the effect of 100 mM Na<sup>+</sup> was examined. (B) When the effect of 3 mM thallium acetate was examined, the medium containing 50 mM sucrose and 100 mM isethionate was replaced by 250 mM sucrose. (C, D) Vesicle-

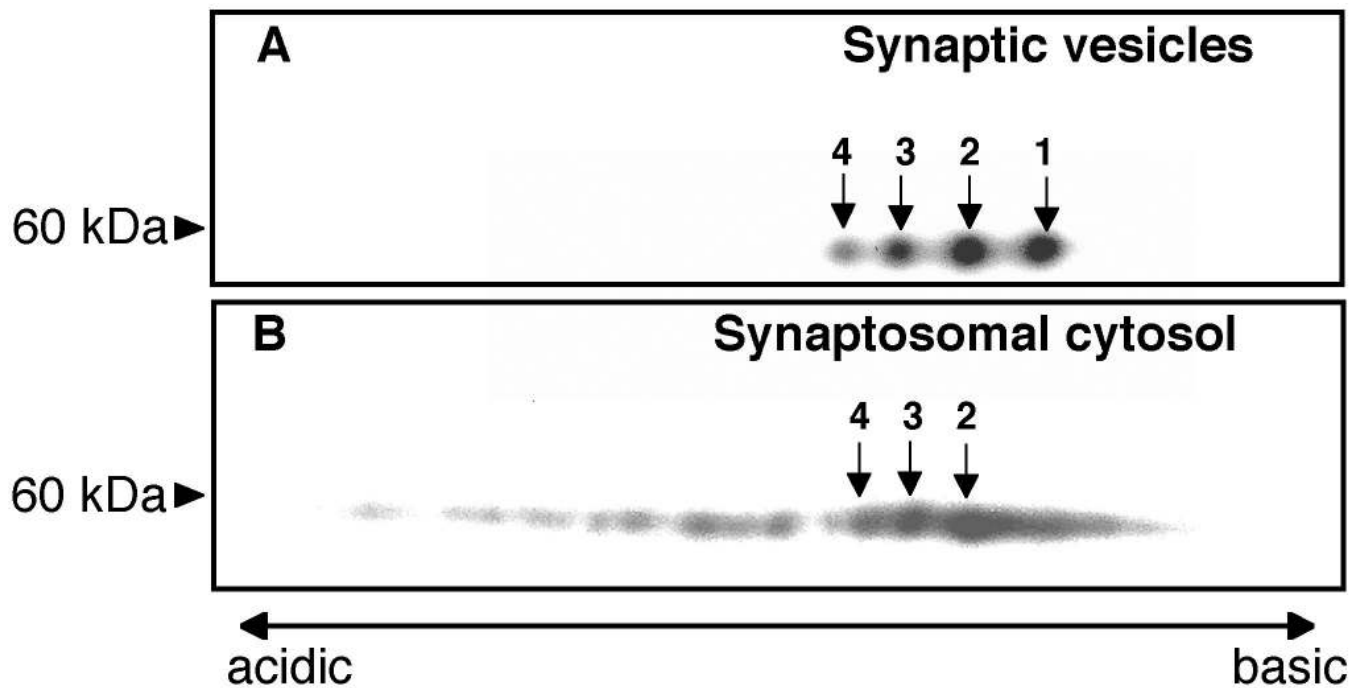
associated pyruvate kinase activities were measured in the presence of 1 mM PEP plus 0.1 mM ADP, with addition or omission of the indicated monovalent cations as described in Experimental procedures. The effect of 100 mM Na<sup>+</sup> was examined as in (A). (D) When the effect of 3 mM thallium acetate was examined, the medium containing 50 mM sucrose and 100 mM isethionate was replaced by 250 mM sucrose. Values are the mean ± S.D. of three separate experiments.



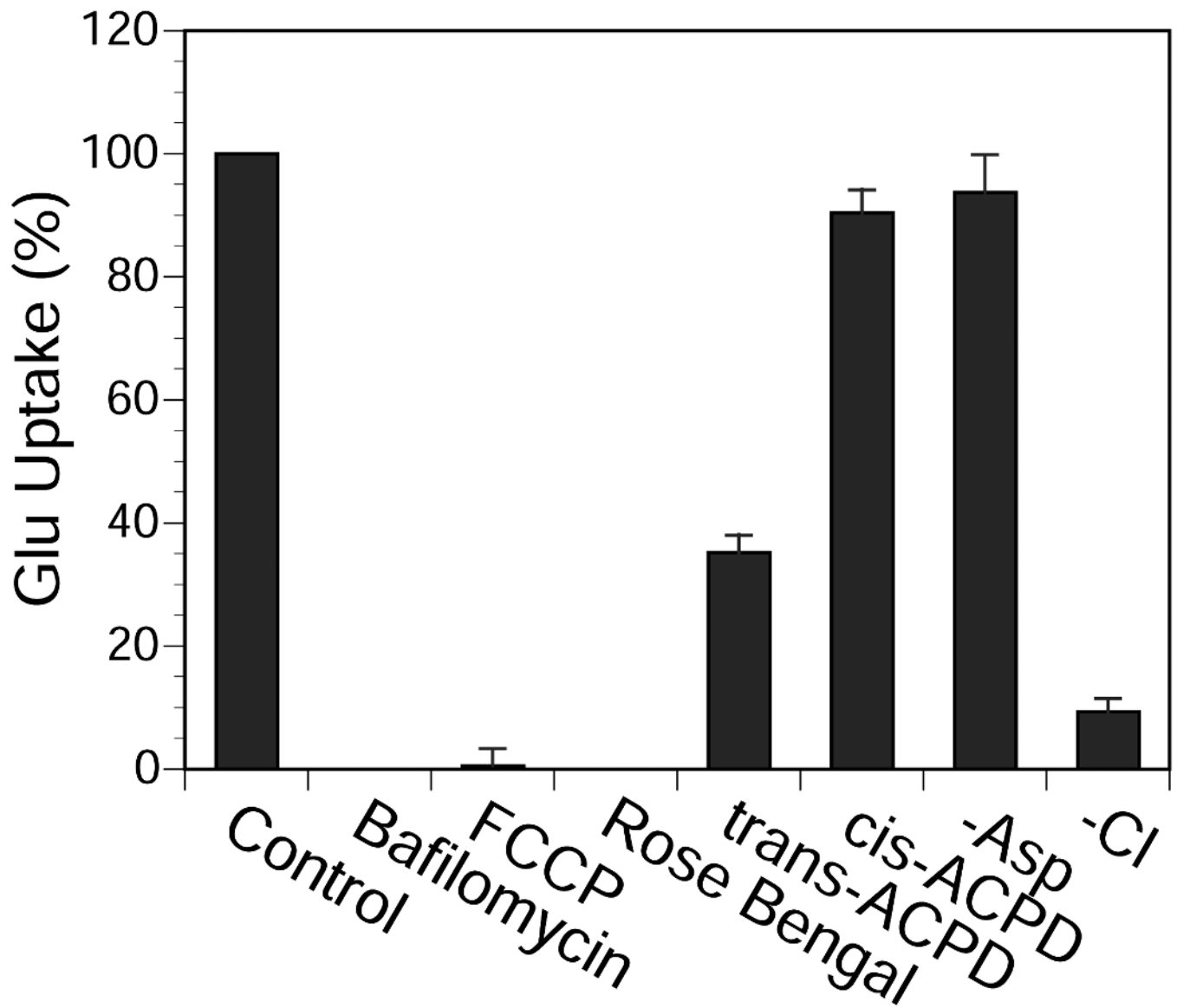


**Fig. 4.** Effect of (Z)-CI-PEP and (E)-CI-PEP on PEP/ADP-dependent glutamate uptake and vesicle-associated pyruvate kinase activity. (A) Purified synaptic vesicles (15  $\mu$ g) were incubated at 30°C for 20 min with 50  $\mu$ M [ $^3$ H]glutamate in 20 mM HEPES-Tris (pH 7.4), containing 50 mM sucrose, 100 mM potassium isethionate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, 2 mM aspartate, 0.1 mM PEP, 0.1 mM ADP, and the indicated concentrations of (Z)-CI-PEP (filled circles) or (E)-CI-PEP (open circles). Glutamate uptake was measured as described in Experimental procedures. (B) Vesicle-associated pyruvate kinase activity was determined in the presence of the indicated concentrations of (Z)-CI-PEP (filled circles) or (E)-CI-PEP (open circles), as described in Experimental procedures, except that 4 mM KCl were used in place of 4 mM LiCl. The results

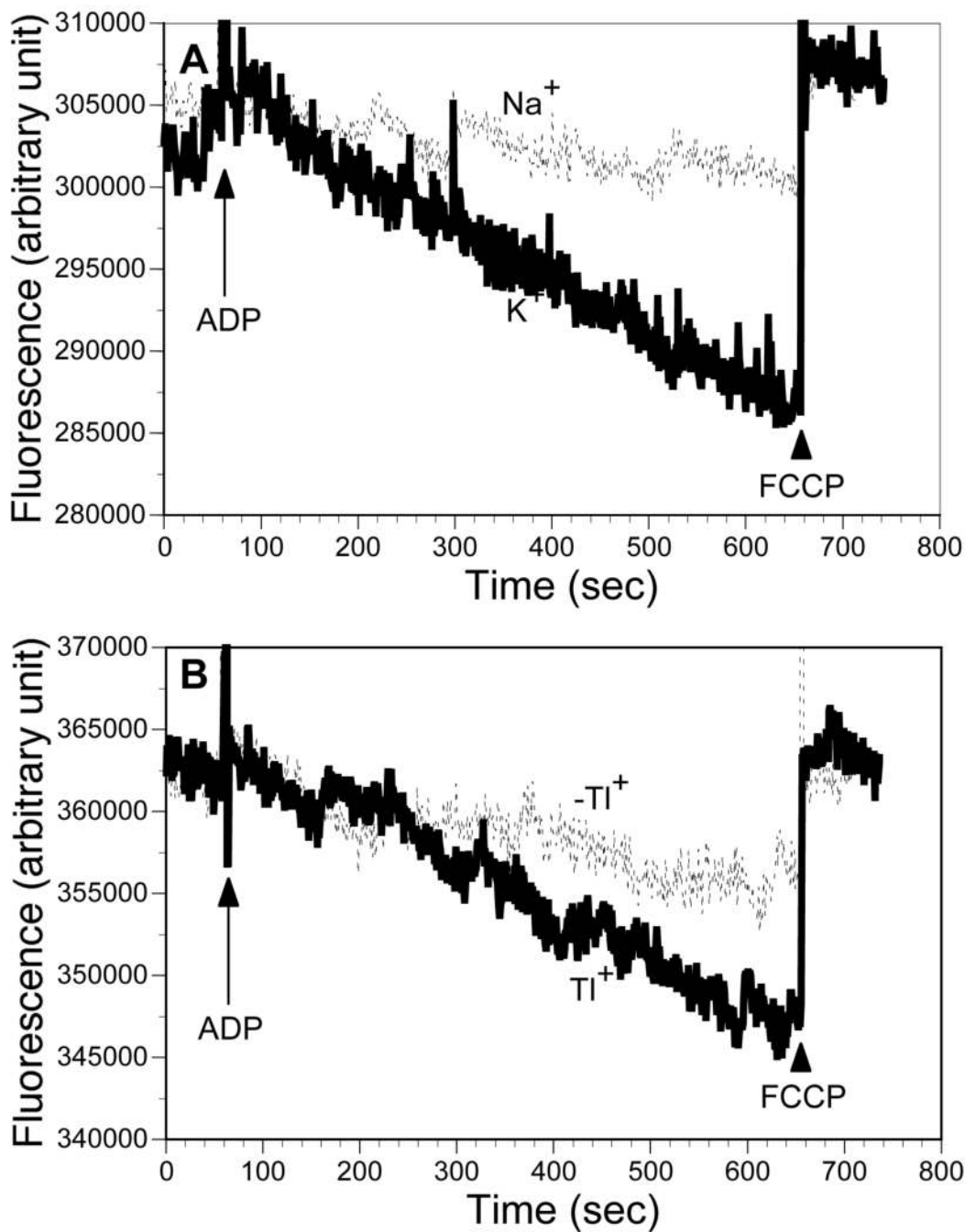
are expressed as a percentage of glutamate uptake or pyruvate kinase activity observed in the absence of Cl-PEP, and each value represents the mean  $\pm$  S.D. of three separate experiments.



**Fig. 5.** Two-dimensional Western blotting analysis of vesicle-associated pyruvate kinase and synaptosomal cytosolic pyruvate kinase. Purified synaptic vesicles (20  $\mu$ g, *A*) and a synaptosomal cytosolic fraction (5.3  $\mu$ g, *B*) were subjected to 2-dimensional electrophoresis as described in Experimental procedures. Western blotting analysis was carried out as described, using goat anti-pyruvate kinase antibodies (1:1,000 dilution) and peroxidase-conjugated anti-goat IgG (1:5,000 dilution) as primary and secondary antibodies, respectively.

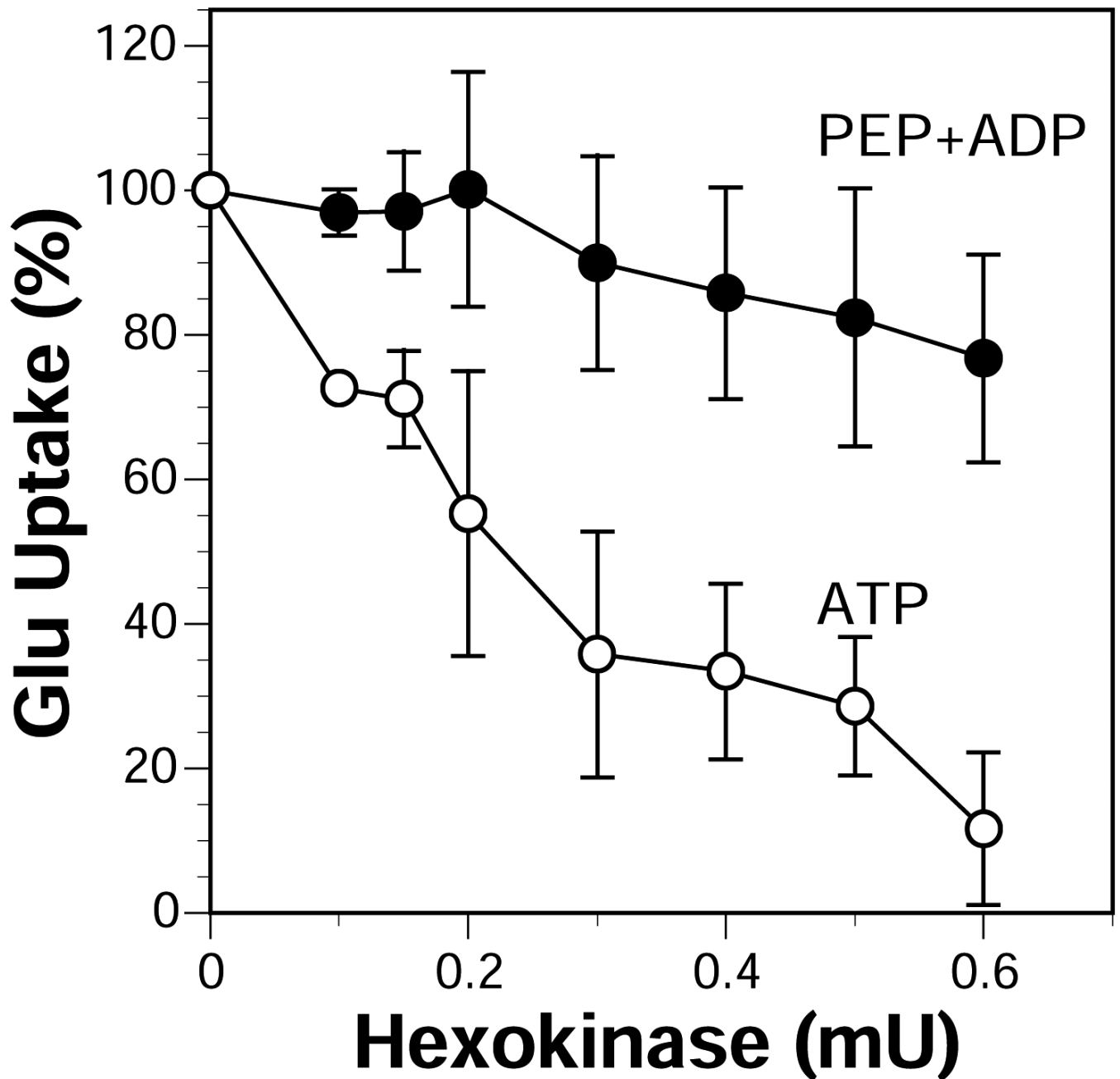


**Fig. 6.** Pharmacological characterization of PEP/ADP-dependent glutamate uptake. Purified synaptic vesicles (15  $\mu$ g) were incubated at 30°C for 20 min with 50  $\mu$ M [ $^3$ H]glutamate in a solution containing 20 mM HEPES-Tris (pH 7.4), 50 mM sucrose, 100 mM potassium gluconate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, 2 mM aspartate, 1 mM PEP, and 0.1 mM ADP, with addition or omission of the indicated compounds. The concentrations used were: 1  $\mu$ M (bafilomycin); 12.5  $\mu$ M (FCCP); 10  $\mu$ M (Rose Bengal); 2 mM (*trans*-ACPD); 2 mM (*cis*-ACPD). “-Asp” and “-Cl” indicate omission of aspartate and KCl from the incubation medium, respectively. Glutamate uptake was measured as described in Experimental procedures. The results are expressed as a percentage of control (no addition), and each value represents the mean  $\pm$  S.D. of three separate experiments.



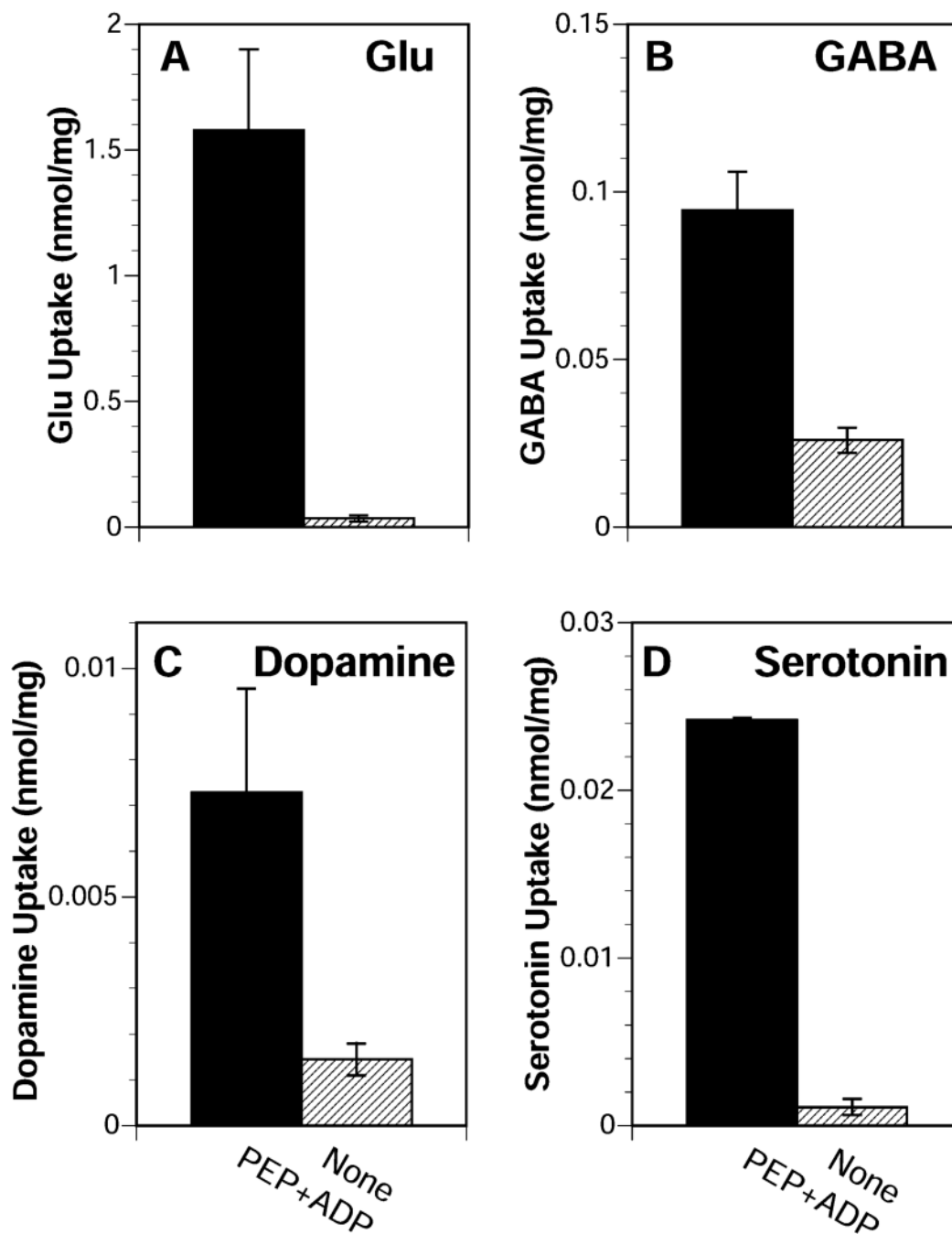
**Fig. 7.** PEP/ADP-induced membrane potential in synaptic vesicles. (A) Purified synaptic vesicles (30  $\mu$ g) were incubated at 30°C in 20 mM HEPES-Tris (pH 7.4) containing 50 mM sucrose, 100 mM potassium gluconate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, 2 mM aspartate, and 1.3  $\mu$ M oxonol V in the presence (bold solid line) or absence (dotted line) of 1 mM PEP. After oxonol V was allowed to equilibrate with synaptic vesicles, ADP (0.1 mM final concentration) was added in order to observe fluorescence quenching, which showed membrane potential formation across the synaptic vesicle membrane (arrow). At the time point indicated by the arrowhead, FCCP (12.5  $\mu$ M) was added to dissipate the membrane potential. FCCP was also added at the start of the reaction, together with 1 mM PEP; ADP was then added (thin solid line). (B) The purified

synaptic vesicles (30  $\mu\text{g}$ ) were incubated at 30°C in 20 mM HEPES-Tris (pH 7.4), containing 50 mM sucrose, 100 mM potassium gluconate, 4 mM KCl, 4 mM  $\text{MgSO}_4$ , 2 mM aspartate, and 1.3  $\mu\text{M}$  oxonol V in the presence of 1 mM PEP. After oxonol V was allowed to equilibrate with synaptic vesicles, ADP (0.1 mM, bold solid line) or  $\text{H}_2\text{O}$  (dotted line) was added at the arrow-indicated time point. At the arrowhead-indicated time point, FCCP (12.5  $\mu\text{M}$ ) was added. FCCP was also added at the start of the reaction; ADP was then added (thin solid line). (C) Purified synaptic vesicles (30  $\mu\text{g}$ ) were incubated as in (B) except that 4 mM LiCl were used in place of 4 mM KCl (bold solid line). Synaptic vesicles were also incubated in the medium containing 100 mM sodium gluconate in place of potassium gluconate (dotted line). ADP (0.1 mM) and FCCP (12.5  $\mu\text{M}$ ) were added at the arrow-indicated and arrowhead-indicated time points, respectively. (D) The purified synaptic vesicles (30  $\mu\text{g}$ ) were incubated at 30°C in 20 mM HEPES-Tris (pH 7.4), containing 250 mM sucrose, 4 mM LiCl, 4 mM  $\text{MgSO}_4$ , 2 mM aspartate, 1 mM PEP, and 1.3  $\mu\text{M}$  oxonol V in the presence (bold solid line) or absence (dotted line) of 3 mM thallium acetate. ADP (0.1 mM) and FCCP (12.5  $\mu\text{M}$ ) were added at the arrow-indicated and arrowhead-indicated time points, respectively.



**Fig. 8.**

Effect of “ATP-trapping” by use of hexokinase. Purified synaptic vesicles (15  $\mu$ g) were incubated with 1 mM PEP plus 0.1 mM ADP (filled circles) or 60  $\mu$ M ATP (open circles) for 20 min at 30°C in 20 mM HEPES-Tris (pH 7.4), containing 150 mM sucrose, 50 mM potassium gluconate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, 2 mM aspartate, 5 mM [<sup>3</sup>H]glutamate, 10 mM glucose, and the indicated amounts of hexokinase. Glutamate uptake was determined as described in Experimental procedures. Under these experimental conditions, PEP/ADP-dependent Glu uptake and ATP-dependent Glu uptake in the absence of hexokinase showed similar level of uptake (7.2 nmol/20 min/mg and 6.1 nmol/20 min/mg, respectively). The results are expressed as a percentage of control (no hexokinase), and each value represents the mean  $\pm$  S.D. of three separate experiments.



**Fig. 9.** PEP/ADP-dependent uptake of glutamate, GABA, dopamine, and serotonin into synaptic vesicles. Crude synaptic vesicles were freshly prepared just prior to the experiment, as described in Experimental procedures. Crude synaptic vesicles (31-35  $\mu$ g) were incubated at 30°C for 20 min with 50  $\mu$ M [ $^3$ H]glutamate (A) or with 50  $\mu$ M [ $^3$ H]GABA (B) in 20 mM HEPES-Tris (pH 7.4), containing 150 mM sucrose, 50 mM potassium isethionate, 4 mM KCl, 4 mM MgSO<sub>4</sub>, and 2 mM aspartate, in the presence (filled bars) or absence (hatched bars) of 1 mM PEP and 0.1 mM ADP. Glutamate uptake and GABA uptake were determined under these conditions, as described in Experimental procedures. For dopamine (C) and serotonin (D) uptake, freshly prepared crude synaptic vesicles (31-35  $\mu$ g) were incubated with 49 nM [ $^3$ H]



dopamine or 95 nM [<sup>3</sup>H]serotonin, respectively, at 30°C for 20 min in 12 mM potassium phosphate buffer (pH 7.4), containing 100 mM KCl, 4 mM MgSO<sub>4</sub>, and 1 mM ascorbic acid, in the presence (filled bars) or absence (hatched bars) of 1 mM PEP and 0.1 mM ADP. Dopamine uptake and serotonin uptake were determined as described in Experimental procedures. Values are the mean ± S.D. of three separate experiments.