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Identification of a nerve ending-enriched 29-kDa protein, labeled with [3-^{32}P]1,3-bisphosphoglycerate, as monophosphoglycerate mutase: inhibition by fructose-2,6-bisphosphate via enhancement of dephosphorylation

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Abstract

Glucose metabolism is of vital importance in normal brain function. Evidence indicates that glycolysis, in addition to production of ATP, plays an important role in maintaining normal synaptic function. In an effort to understand the potential involvement of a glycolytic intermediate(s) in synaptic function, we have prepared [3-^{32}P]1,3-bisphosphoglycerate and [^{32}P]3-phosphoglycerate and sought their interaction with a specific nerve-ending protein. We have found that a 29-kDa protein is the major component labeled with either [3-^{32}P]1,3-bisphosphoglycerate or [^{32}P]3-phosphoglycerate. The protein was identified as monophosphoglycerate mutase (PGAM). This labeling was remarkably high in the brain and synaptosomal cytosol fraction, consistent with the importance of glycolysis in synaptic function. Of interest, fructose-2,6-bisphosphate (Fru-2,6-P_2) inhibited PGAM phosphorylation and enzyme activity. Moreover, Fru-2,6-P_2 potently stimulated release of [^{32}P]phosphate from the ^{32}P-labeled PGAM (EC_{50} = 1 μM), suggesting that apparent reduction of PGAM phosphorylation and enzyme activity by Fru-2,6-P_2 may be due to stimulation of dephosphorylation of PGAM. The significance of these findings is discussed.

Keywords: 1,3-bisphosphoglycerate, fructose-2,6-bisphosphate, glycolysis, nerve ending, phosphoglycerate mutase.

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Glucose metabolism is of paramount importance in brain function. Cerebral energy is derived from glucose under normal conditions (Siesjo 1978). Hence, the necessity for glucose in brain function had been considered solely due to ATP production. However, evidence has accumulated indicating that hypoglycemia leads to neuronal dysfunction, as judged by abnormal electroencephalogram and population spikes, as well as to abnormal behavior, long before cerebral ATP is depleted (Dirks *et al*. 1980; Cox and Bachelard 1982; Ghajar *et al*. 1982). This suggests that sustaining global cellular ATP at the normal level would not substitute for the glucose metabolism requirement. Morino *et al*. (1991) have shown that [1-^{32}P]1,3-bisphosphoglycerate (1,3-BPG), a high energy glycolytic intermediate, can serve as a [^{32}P] phosphate donor to a 72-kDa protein. These lines of evidence suggest that glycolysis plays an important role, distinct from that in ATP production, in maintaining normal synaptic function. In this study, we have devised a method for preparation of [3-^{32}P]1,3-BPG, as well as [^{32}P]3-phosphoglycerate (3-PG), and sought their interaction with a specific protein(s), in particular in the nerve ending, since

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Abbreviations used: 1,3-BPG, 1,3-bisphosphoglycerate; 2,3-BPG, 2,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenol pyruvate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; PGAM, monophosphoglycerate mutase; Pi, phosphoric acid (inorganic phosphate); synsol, synaptosomal cytosol.

activity-dependent increase in glucose metabolism largely occurs in the nerve terminal. We demonstrate that a 29-kDa protein is labeled as the major component in the synaptosomal cytosol (synsol) fraction, and present evidence that the labeled 29-kDa protein is monophosphoglycerate mutase (PGAM). Among non-substrate substances, fructose-2,6-bisphosphate (Fru-2,6-P_2) was found to affect the state of phosphorylation of the 29-kDa protein and PGAM, as well as PGAM enzyme activity. The significance of a unique subcellular and tissue distribution of the labeled 29-kDa protein observed here is also discussed, together with a potential regulatory role of Fru-2,6-P_2 in glycolysis via PGAM.

Materials and methods

Materials

[γ-^{32}P]ATP (6000 Ci/mM) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). [^{32}P]Phosphoric acid (Pi, carrier-free) was purchased from ICN (Irvine, CA). Glycolytic enzymes, their intermediates and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). The affinity-purified polyclonal antibody (rabbit IgG) specific to human recombinant PGAM type B (anti-PGAM pAb) was kindly provided by Oriental Yeast Co., Ltd. (Tokyo, Japan).

Synthesis of [^{32}P]DHAP, [3-^{32}P]1,3-BPG and [^{32}P]3-PG

[^{32}P]Dihydroxyacetone phosphate (DHAP) was prepared by phosphorylation of dihydroxyacetone by glycerol kinase with [γ-^{32}P]ATP in a mixture containing 5 mM Tris-HCl (pH 7.0), 40 μM $MgSO_4$, 10 mM dihydroxyacetone, 20 μM [γ-^{32}P]ATP (6000 Ci/mM) and 0.5 U glycerol kinase (*Bacillus stearothermophilus*). The reaction was performed in a volume of 40 μL at 37°C for 20 min. [3–^{32}P]1,3-BPG was prepared from [^{32}P]DHAP by conversion to [^{32}P]glyceraldehyde-3-phosphate (GAP) with triose phosphate isomerase, followed by a GAP dehydrogenase reaction in the presence of the lactate dehydrogenase-coupled NAD regenerating system. The reaction mixture (400 μL) contained 12.5 mM triethanolamine (pH 8.0), 0.2 mM EDTA, 2 mM NAD, 2 mM Na pyruvate, 2 mM KH_2PO_4, 50 μM DHAP, 3.2 U GAP dehydrogenase (rabbit muscle), 20 U triose phosphate isomerase (rabbit muscle), 10 U lactate dehydrogenase (rabbit muscle) and 40 μL of the [^{32}P]DHAP reaction mixture. The entire mixture was incubated at 25°C for 5 min. For synthesis of [^{32}P]3-PG, a reaction was performed under the same conditions, except that 0.5 U 3-PG kinase (*Saccharomyces cerevisiae*) was added to, and EDTA removed from, the above reaction mixture. Each reaction mixture was filtered to remove the enzymes by an Amicon Centricon-10 concentrator (Amicon, Danvers, MA, USA) and put onto a DEAE-cellulose column (Whatman DE32, 1.2 × 2.2 cm; Watman Inc., Clifton, NJ, USA) previously equilibrated with 10 mM glycylglycine, pH 7.4. Elution was carried out with stepwise increases in the NaCl concentration: 50 mM, 75 mM, 100 mM, 125 mM, 150 mM and 200 mM. DHAP, GAP and inorganic phosphate (Pi) were eluted with 75 mM NaCl in the same buffer. 3-PG and 1,3-BPG were eluted with 125 and 150 mM NaCl, respectively, in the same buffer. All the compounds thus prepared were stored at − 80°C until use.

Analysis of [^{32}P] compounds by HPLC

Radioactive compounds were analyzed by HPLC on a Whatman Partisil 10 SAX WCS column (4.6 × 250 mm; Beckman Coulter Inc., Fullerton, CA, USA), comparing their retention times with those of non-radioactive authentic standards monitored at 214 nm. The column was equilibrated with 0.4 M sodium phosphate buffer (pH 3.2), and glycolytic intermediates and nucleotides were eluted isocratically, as described previously (Morino *et al*. 1991). Retention times for glycolytic intermediates were 4.1 min for DHAP and GAP, 5.3 min for Pi, 5.9 min for 2-phosphoglycerate (2-PG) and 3-PG, 6.0 min for ADP, 7.4 min for phosphoenolpyruvate (PEP), 15.4 min for 1,3-BPG and 30.0 min for ATP.

Preparation of subcellular fractions

Subcellular fractions of bovine brain were prepared as described previously (Ueda *et al*. 1979). The plasma membrane preparation used in this study was the synaptic plasma membrane M-1 (0.9) fraction obtained upon sucrose density gradient centrifugation. Cytosolic fractions of various rat tissues were prepared as described previously (Ueda and Plagens 1987). Protein concentration was determined by the method of Bradford (1976) with a Coomassie Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, USA) with bovine serum albumin as standard protein.

Protein labeling with [3-^{32}P]1,3-BPG and [^{32}P]3-PG

For the assay for ^{32}P-labeling of protein, the synsol fraction (30 μg protein) was pre-incubated at 37°C for 30 s in 27 μL 5 mM Tris-maleate (pH 7.4). The reaction was initiated by the addition of 3 μL [3-^{32}P]1,3-BPG or [^{32}P]3-PG (240 Ci/mM) to the final concentration of 140 nM, unless otherwise stated, and allowed to continue for 10 s. Whenever [^{32}P]3-PG was used, 125 nM 2,3-BPG and 1 mM $MgSO_4$ were included. For the dephosphorylation assay of ^{32}P-labeled protein, protein was labeled with [^{32}P] compounds under the same conditions, followed by removal of unreacted [^{32}P] compounds with an Amicon Centricon-10 concentrator. ^{32}P-labeled protein (30 μg) was incubated at 37°C for 10 s in a mixture (final volume 30 μL) containing 5 mM Tris-maleate (pH 7.4), and indicated concentrations of fructose-1,6-bisphosphate (Fru-1,6-P_2) or fructose-2,6-bisphosphate (Fru-2,6-P_2). The reaction was terminated by addition of 10 μL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and aliquots (25 μL) were subjected to SDS–PAGE (12% acrylamide) according to the method of Laemmli (1970), except for omission of sample boiling. Autoradiography was carried out as described previously (Morino *et al*. 1991) and the amounts of ^{32}P-labeled protein estimated using an imaging analyzer (Bio-Rad Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA, USA). In some experiments, purified rabbit muscle PGAM (Sigma-Aldrich, 0.48 U) was used instead of 30 μg synsol protein, and incubated in a reaction mixture containing 5 mM Tris-maleate (pH 7.4), 0.1 mM $MgSO_4$, 125 nM 2,3-bisphosphoglycerate (2,3-BPG) and 140 nM (240 Ci/mM) [^{32}P]3-PG.

Immunoprecipitation

Anti-PGAM pAb (10 μg) was absorbed on immobilized protein G (0.1 mL as 50% slurry) and chemically cross-linked using the Seize X mammalian immunoprecipitation kit (Pierce). The synsol fraction (100 μg protein) was incubated at 37°C for 10 s in a mixture (final volume 100 μL) containing 5 mM Tris-maleate (pH 7.4) and

140 nM (240 Ci/mM) [3-^{32}P]1,3-BPG or [^{32}P]3-PG. When [^{32}P]3-PG was used, 125 nM 2,3-BPG and 1 mM $MgSO_4$ were included. The reaction mixtures were subjected to immunoprecipitation with immobilized anti-PGAM pAb according to the manufacturer's protocol. Aliquots (20 μL) were subjected to SDS–PAGE (12% acrylamide) according to the method of Laemmli (1970), except for omission of sample boiling, followed by autoradiography as described previously (Morino *et al*. 1991).

Assay for PGAM activity

PGAM activity was determined by coupling the formation of 2-PG from 3-PG with the enolase-, pyruvate kinase- and lactate dehydrogenase-catalyzed reactions, according to the method recommended by the International Committee for Standardization in Haematology (Beutler *et al*. 1977). Briefly, 100 μg protein of synsol or 0.2 U purified PGAM were incubated in a reaction mixture (final volume, 1 mL) containing 0.1 M Tris-HCl/0.5 mM EDTA (pH 8.0), 2 mM $MgCl_2$, 100 mM KCl, 0.2 mM NADH, 1.5 mM neutralized ADP (with NaOH), 10 μM 2,3-BPG, 0.1 mM 3-PG, 1.2 U lactate dehydrogenase, 1.0 U pyruvate kinase and 1.0 U enolase, at 25°C for 10 min. The enzyme activity was calculated from the rate of reduction of NADH by monitoring absorbance at 340 nm.

Radioactive product analysis of the ^{32}P-labeled PGAM de-labeling reaction

PGAM (1.9 mg/mL) was labeled at 37°C for 30 s in a buffer containing 5 mM Tris-maleate (pH 7.4), 125 nM 2,3-BPG, 1 mM $MgSO_4$ and 0.5 μM [^{32}P]3-PG (1600 cpm/pM), followed by removal of unreacted [^{32}P]3-PG with an Amicon Centricon-10 concentrator. [^{32}P]PGAM (175 μg, 40 042 cpm/mg protein) was pre-incubated at 37°C for 30 s in 90 μL 5 mM Tris-maleate (pH 7.4). The reaction was initiated by the addition of 10 μL Fru-2,6-P_2 or 3-PG to indicated final concentrations and allowed to continue for 10 s. The reaction was terminated by addition of 100 μL 30% trichloroacetic acid and centrifuged at 14 000 g_{max} at 4°C. Aliquots (180 μL) of the supernatant fluid were analyzed by HPLC as described above. To analyze the effect of various concentrations of Fru-2,6-P_2, [^{32}P]PGAM (25 μg) was incubated with indicated concentrations of Fru-2,6-P_2 under the same conditions.

Time course of [^{32}P]phosphate release from [^{32}P]PGAM

[^{32}P]PGAM (25 μg) was incubated in the absence or presence of indicated concentrations of Fru-2,6-P_2 at 0°C for various periods. Radioactivity of the supernatant fluid obtained upon centrifugation of the trichloroacetic acid-treated reaction mixture was determined in a Beckman LS 6500 scintillation spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

Kinetics study of PGAM ^{32}P-labeling

PGAM (100 μg) was pre-incubated at 37°C for 30 s in 90 μL of reaction mixture containing 5 mM Tris-maleate (pH 7.4), 125 nM 2,3-BPG, 1 mM $MgSO_4$ and indicated concentrations of Fru-2,6-P_2. The reaction was initiated by the addition of 10 μL [^{32}P]3-PG to indicated final concentrations and allowed to continue for 10 s. The reaction was terminated by addition of 100 μL 30% trichloroacetic acid and centrifuged at 14 000 g_{max} at 4°C. The pelleted protein was washed with 70% ethanol and radioactivity determined in a scintillation spectrophotometer.

Results

Labeling synsol protein with [3-^{32}P]1,3-BPG and time course

When the synsol fraction was incubated with [3-^{32}P]1,3-BPG, it exhibited the 29-kDa protein as the major labeled protein, as shown in Fig. 1. Incorporation of ^{32}P into the protein is dependent on the concentration of [3-^{32}P]1,3-BPG and protein concentration (Fig. 1). As glucose depletion-induced abnormal changes in electroencephalogram and behavior occur within minutes, as does recovery from these deficits observed upon glucose infusion, modification of a protein(s) involved in the mechanism underlying glycolysis-dependent synaptic transmission would have to be relatively rapid, as would its de-modification. Time course of 29-kDa protein labeling and de-labeling is shown in Fig. 2. While the initial rate of labeling is temperature-dependent, the maximal level of labeling is about the same with both incubations at 37°C and 0°C (Fig. 2). At 37°C, ^{32}P-labeling occurs rapidly with $\tau_{1/2}$ of approximately 4 s, whereas the reaction at 0°C is about 10 times slower, with $\tau_{1/2}$ of approximately 45 s. Upon prolonged incubation, the 29-kDa ^{32}P-protein is de-labeled; at 37°C, de-labeling starts at 2 min, with $\tau_{1/2}$ of approximately 20 min, and at 0°C, it takes place after 15 min.

Subcellular and tissue distribution

In order to determine the subcellular distribution of the 29-kDa ^{32}P-protein, the synsol, pericaryal cytosol,

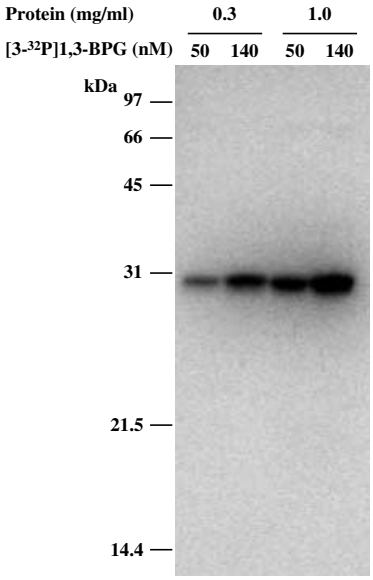

Fig. 1 Labeling of 29-kDa protein with [3-^{32}P]1,3-BPG in bovine synsol. The synsol fraction was incubated with [3-^{32}P]1,3-BPG at 37°C for 10 s and aliquots (18.8 μL) were subjected to SDS–PAGE (12% acrylamide), followed by autoradiography, as described in Materials and methods. The autoradiogram shown is representative of results obtained from three separate experiments.

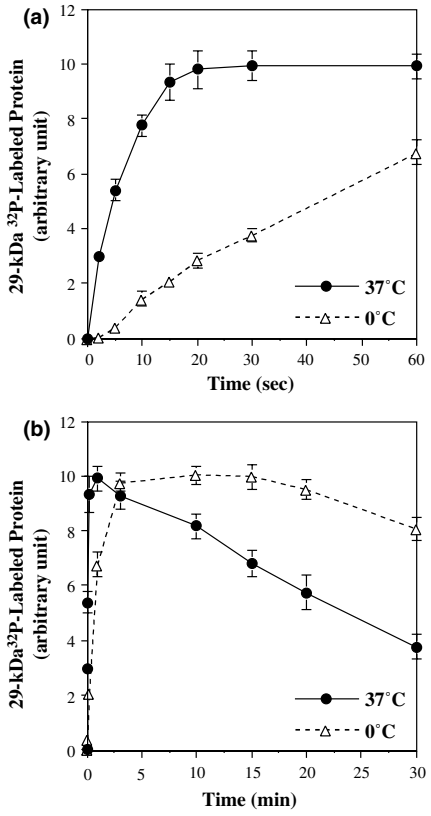

Fig. 2 Time course and temperature dependency of labeling of 29-kDa protein with [3-^{32}P]1,3-BPG. The synsol fraction (1 mg/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG at 37°C or 0°C for the indicated periods. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of 29-kDa ^{32}P-labeled protein during short [(a) approximately 60 s] and long [(b) approximately 30 min] incubation periods was estimated using an image analyzer as described in Materials and methods. Values are the mean ± SD of three experiments.

microsomal, synaptic vesicle and plasma membrane fractions were analyzed for labeling with [3-^{32}P]1,3-BPG. As shown in Fig. 3(a), the highest level of labeling was observed in the synsol fraction, followed by the pericaryal (cell body) cytosol fraction and the microsomal fraction. The synaptic vesicle and plasma membrane fractions labeled the 29-kDa protein to a minimal extent. In order to determine tissue distribution, various tissues including brain, heart, lung, spleen, pancreas, kidney, liver, muscle and adrenal were homogenized under hypotonic conditions, and the soluble fraction obtained upon ultracentrifugation was analyzed for 29-kDa protein labeling with [3-^{32}P]1,3-BPG. Brain and the fast-twitch muscle extensor digitorum longus (EDL) exhibited the most intense labeling of 29-kDa protein among all the tissues examined, followed by pancreas (Fig. 3b). The protein was labeled to a much smaller extent in the spleen, the slow-twitch muscle soleus (SOL) and adrenal. The 29-kDa protein in the heart, lung, kidney and liver was minimally labeled. Similar labeling patterns were observed when tissues were incubated with [^{32}P]3-PG in the presence of 125 nM 2,3-BPG (data not shown).

Effect of glycolytic intermediates and related compounds on labeling of 29-kDa protein

In an effort to explore the possibility that the interaction of the 29-kDa protein with 1,3-BPG, the most minor glycolytic intermediate, is subject to regulation by a step in glucose metabolism, we tested various glycolytic intermediates for the ability to affect 29-kDa protein labeling. As shown in Fig. 4, the 29-kDa protein labeling was greatly reduced by 3-PG, 2-PG and PEP. Interestingly, Fru-2,6-P_2 also exhibited a marked inhibition; however, its analog Fru-1,6-P_2 was less effective. The 3-PG close analogs GAP, glycerol-3-phosphate and glycerate had no significant effect, however. The effect of PEP could be due to its conversion to 2-PG by enolase present in the synsol preparation.

The strong diminishing effect of 3-PG and 2-PG on 29-kDa protein labeling suggested that this might be PGAM (for review, see Rose 1980; Fothergill-Gilmore and Watson 1989). If that were the case, [3-^{32}P]1,3-BPG should have been converted to [^{32}P]3-PG by 3-PG kinase present in the synsol, which contains some endogenous ADP. In order to determine whether this is the case, we analyzed potential reaction products derived from [3-^{32}P]1,3-BPG upon incubation for various times, as shown in Fig. 5. Indeed, [3-^{32}P]1,3-BPG was rapidly metabolized to [^{32}P]3-PG/[^{32}P]2-PG and [^{32}P]PEP, indicating the presence of 3-PG kinase, PGAM and enolase in the synsol. This suggests that non-radioactive 2-PG and PEP, when added to the synsol, would be converted to 3-PG, causing isotopic dilution of [^{32}P]3-PG. Thus, the 'apparent inhibitory' effect of 3-PG, 2-PG and PEP on labeling of the 29-kDa protein would be due to isotopic dilution of [^{32}P]3-PG. These observations and considerations support the notion that the 29-kDa protein is PGAM interacting with [^{32}P]3-PG. In order to provide further supporting evidence, the ability of [^{32}P]3-PG to label the 29-kDa protein was compared with that of [3-^{32}P]1,3-BPG. As shown in Fig. 6, [^{32}P]3-PG was more effective than [3-^{32}P]1,3-BPG in labeling the 29-kDa protein. This differential effect is larger at 0°C than at 37°C, probably due to a decreased rate of conversion of [3-^{32}P]1,3-BPG to [^{32}P]3-PG at 0°C. These results argue for the notion that [^{32}P]1,3-BPG is converted to [^{32}P]3-PG and then interacts with PGAM, forming the phosphorylated enzyme intermediate.

Identification of the 29-kDa labeled protein as PGAM

The relative molecular weight (M_r) of the labeled 29-kDa protein in the synsol fraction was first compared with that of purified PGAM phosphorylated with [^{32}P]3-PG. As shown in Fig. 7(a), the M_rs of both proteins were indistinguishable, as judged by SDS–PAGE. The labeling of both proteins was

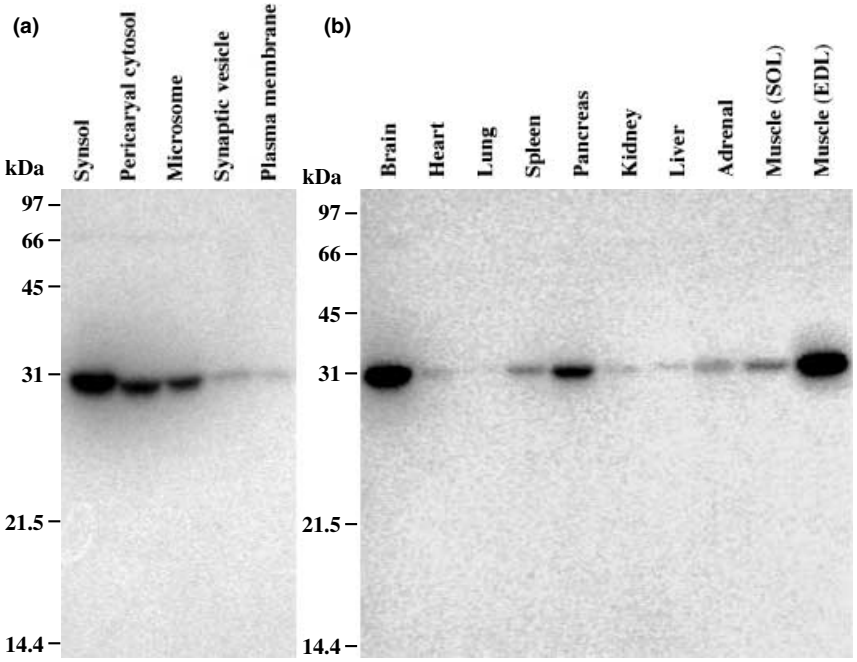

Fig. 3 Subcellular and tissue distributions of 29-kDa protein labeling with [3-^{32}P]1,3-BPG. Various subcellular fractions of bovine brain (a) and total cytosolic fractions of rat tissue (b) as indicated, each containing 1 mg/mL), were incubated with 140 nM [3-^{32}P]1,3-BPG at 37°C for 10 s and aliquots (18.8 μL) subjected to SDS–PAGE (12%), followed by autoradiography as described in Materials and methods. The autoradiograph shown is representative of results obtained from three separate experiments. SOL, soleus; EDL, extensor digitorum longus.

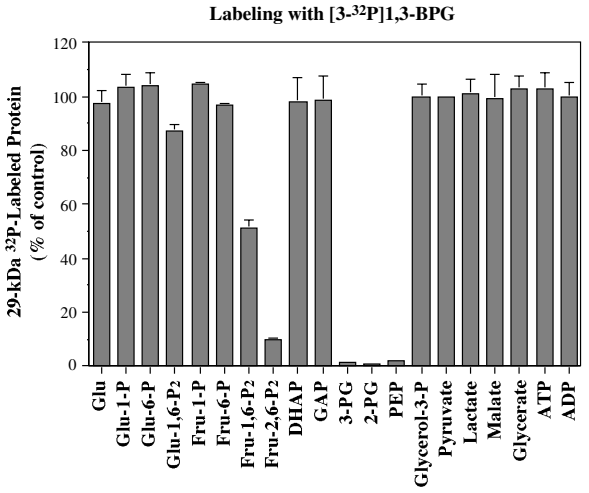

Fig. 4 Effect of various glycolytic intermediates and related compounds on 29-kDa protein labeling in bovine synsol with [3-^{32}P]1,3-BPG. The synsol fraction (1 mg/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG at 37°C or 0°C for 10 s in the absence (control) or presence of various glycolytic intermediates and related compounds (50 μM). Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of the 29-kDa ^{32}P-labeled protein was estimated using an image analyzer as described in Materials and methods, and expressed as the percentage of control value. Values are the mean ± SD of three experiments. Glu, glucose; Glu-1-P, glucose-1-phosphate; Glu-6-P, glucose-6-phosphate; Glu-1,6-P_2, glucose-1,6-bisphosphate; Fru-1-P, fructose-1-phosphate; Fru-6-P, fructose-6-phosphate; glycerol-3-P, glycerol-3-phosphate.

substantially reduced by 3-PG. Fru-2,6-P_2 also affected phosphorylation of both proteins. Moreover, the 29-kDa protein in the synsol fraction labeled with either [3-^{32}P]1,3-BPG or [^{32}P]3-PG was immunoprecipitated by antibodies to recombinant PGAM (Fig. 7b).

The effect of glycolytic intermediates and related compounds on ^{32}P-labeling of purified PGAM was examined, in comparison with the effect on ^{32}P-labeling of the synsol, 29-kDa protein. As shown in Fig. 8, the inhibition pattern with various glycolytic intermediates and related compounds is very similar to that observed with labeling the 29-kDa protein in the synsol (Fig. 4), with the exception of the effect of PEP. Although phosphorylation of purified PGAM was inhibited by 3-PG and 2-PG, it was not affected by PEP (Fig. 8). This is most likely because purified PGAM is largely free of enolase; thus, PEP would not have been converted to 2-PG and hence, not to 3-PG. As a result, isotopic dilution of [^{32}P]3-PG would not have occurred.

Tissue distribution in ^{32}P-labeling of the cytosolic 29-kDa protein (Fig. 3) was quite striking. In order to determine whether this represents the tissue distribution of PGAM, the cytosolic fractions of various tissues were assayed for PGAM activity. As shown in Fig. 9, the specific enzyme activity pattern among various tissues tested was similar to the specific ^{32}P-labeling activity pattern of the cytosolic 29-kDa protein. EDL muscle revealed the highest specific enzyme activity, followed by brain and pancreas; heart, lung, kidney and liver showed the lowest enzyme activity. These observations are consistent with the notion that the cytosolic ^{32}P-labeled 29-kDa protein is PGAM.

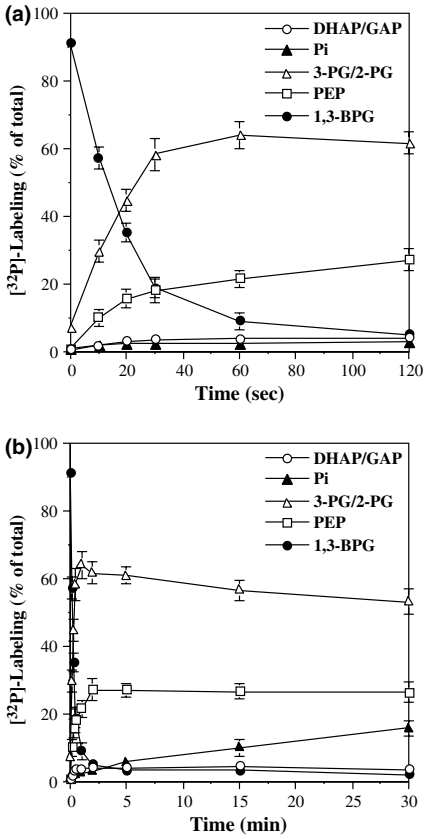

Fig. 5 Metabolism of [3-^{32}P]1,3-BPG upon bovine synsol. The synsol fraction (1 mg/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG at 37°C for the indicated periods [(a) approximately 120 s; (b) approximately 30 min]. After the protein was removed by use of an Amicon Centricon-10 concentrator, radioactive compounds were analyzed by HPLC as described in Materials and methods. The level of each radioactive compound was calculated as the percentage of total radioactive compounds. Values are the mean ± SD of three experiments. Pi, inorganic phosphate; the other abbreviations are shown in the Fig. 4 legend.

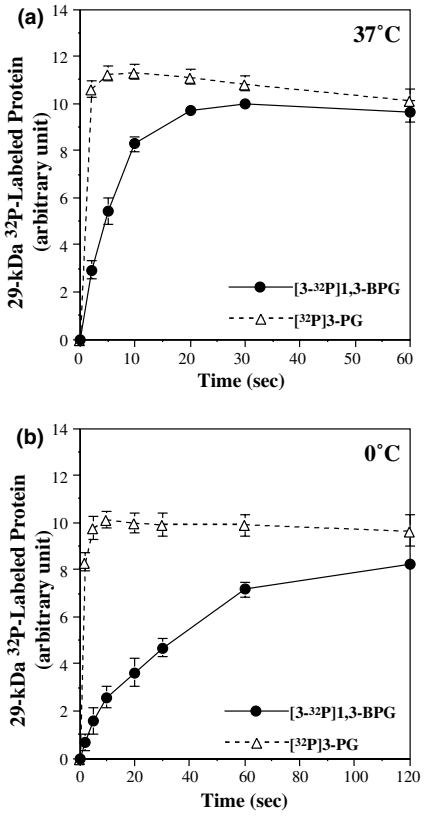

Fig. 6 Labeling of 29-kDa protein with [^{32}P]3-PG in comparison with [3-^{32}P]1,3-BPG. Synsol (1 mg/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG or 140 nM [^{32}P]3-PG at 37°C (a) or 0°C (b) for the indicated periods. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of 29-kDa ^{32}P-labeled protein was estimated using an image analyzer as described in Materials and methods. Values are the mean ± SD of three experiments.

Fru-2,6-P_2 reduces ^{32}P-labeling of PGAM by enhancing dephosphorylation

As the inhibitory effect of Fru-2,6-P_2 on phosphorylation of PGAM has not been reported, we further studied this property of the enzyme, in comparison with its effect on the 29-kDa protein in the synsol. Shown in Fig. 10 is the effect of various concentrations of Fru-2,6-P_2 on PGAM phosphorylation. The data indicate that Fru-2,6-P_2 quite potently inhibits labeling of the synsol 29-kDa protein and purified PGAM with similar potencies (IC_{50} = 8–9 μM). In contrast, Fru-1,6-P_2 was less effective but exhibited similar potencies (IC_{50} = 70–80 μM). Fru-2,6-P_2 also inhibited overall enzyme activity in the purified PGAM preparation and in the synsol fraction in a similar manner (Fig. 11); however, the IC_{50} value of 50 μM was higher than that observed for inhibition of ^{32}P-labeling. Fru-1,6-P_2 also showed similar inhibitory effects, but with a much lower potency (IC_{50} = 1 mM). These observations further argue for the notion that the synsol 29-kDa protein labeled with [3-^{32}P]1,3-BPG or [^{32}P]3-PG is PGAM.

In an effort to understand the mechanism of inhibition by F-2,6-P_2, kinetic experiments were carried out on ^{32}P-labeling and enzyme activity of PGAM, each in the absence or presence of two concentrations of Fru-2,6-P_2, as shown in Fig. 12. The data indicate that Fru-2,6-P_2 inhibits both ^{32}P-labeling and enzyme activity of PGAM in a non-competitive manner with respect to the substrate 3-PG. The K_i was calculated to be 42 μM in the ^{32}P-labeling reaction and 36 μM in the overall enzyme reaction. These data suggest that Fru-2,6-P_2 produces the inhibitory effect by binding to an allosteric site.

To further understand the action of Fru-2,6-P_2, we explored the possibility that Fru-2,6-P_2 enhances ^{32}P-de-labeling of PGAM. As shown in Fig. 13, the ^{32}P-labeled 29-kDa protein was found to be rapidly de-labeled in the

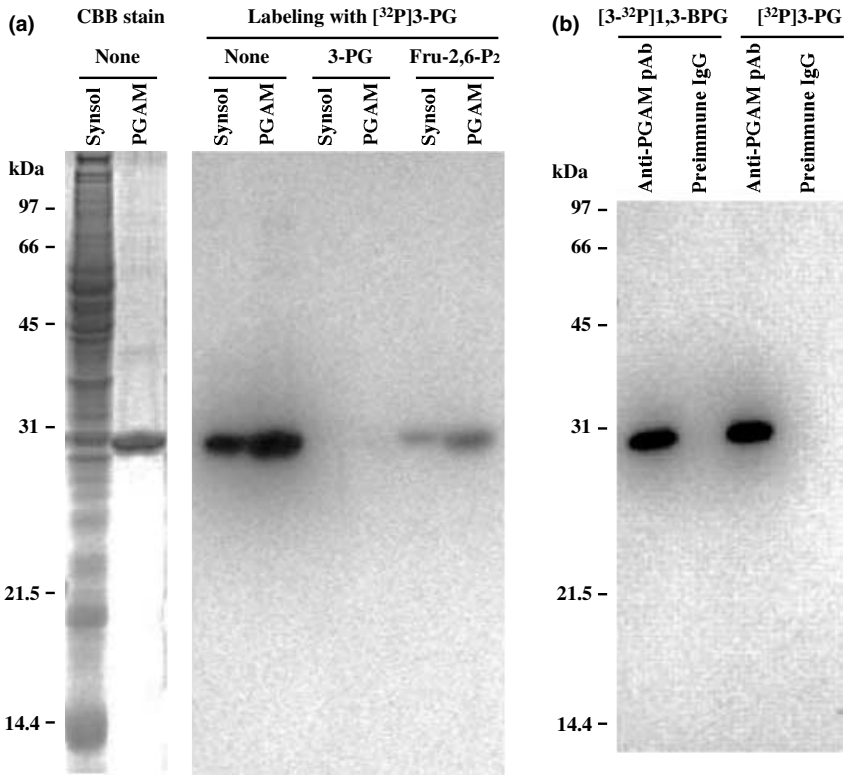

Fig. 7 Identification of the 29-kDa protein as PGAM. (a) Comparison of the 29-kDa protein in the synsol labeled with [^{32}P]3-PG and purified PGAM labeled with [^{32}P]3-PG. The synsol (1 mg/mL) or purified PGAM (PGAM; 16 unit/mL) was incubated with 140 nM [^{32}P]3-PG at 37°C for 10 s in the absence or presence of 50 μM 3-PG or Fru-2,6-P_2. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography as described in Materials and methods. CBB, Coomassie Brilliant Blue. (b) Immunoprecipitation of the 29-kDa protein with anti-PGAM polyclonal antibodies. The synsol (1 mg/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG or [^{32}P]3-PG at 37°C for 10 s and immunoprecipitated with anti-PGAM polyclonal antibodies (anti-PGAM pAb) or non-immunized rabbit IgG, followed by autoradiography as described in Materials and methods. The result shown is representative of three separate experiments.

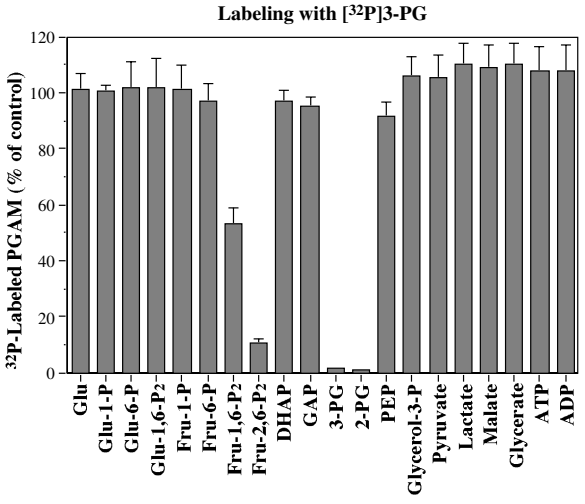

Fig. 8 Effect of various glycolytic intermediates and related compounds on ^{32}P-labeling of purified PGAM with [^{32}P]3-PG. Purified PGAM (16 unit/mL) was incubated with 140 nM [3-^{32}P]1,3-BPG at 37°C for 10 s in the absence (control) or presence of various glycolytic intermediates and related compounds (50 μM). Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of ^{32}P-labeled PGAM was estimated as the percentage of control value using an image analyzer as described in Materials and methods. Values are the mean ± SD of three experiments. Abbreviations are shown in the Fig. 4 legend.

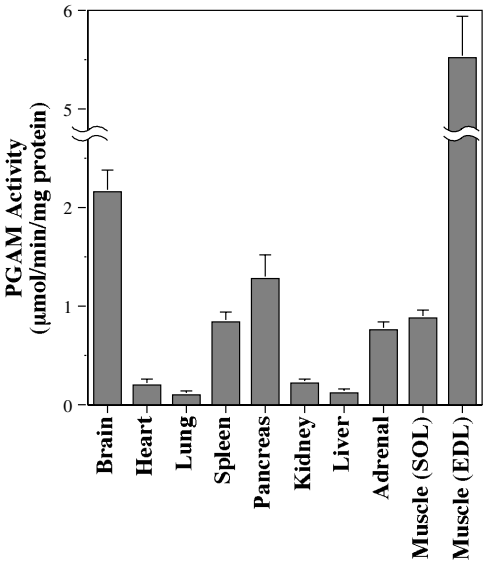

Fig. 9 PGAM activity in various rat tissues. Aliquots (100 μg) of various rat tissue whole cytosolic fractions were assayed for PGAM activity, as described in Materials and methods. Values are the mean ± SD of three experiments. SOL, soleus; EDL, extensor digitorum longus.

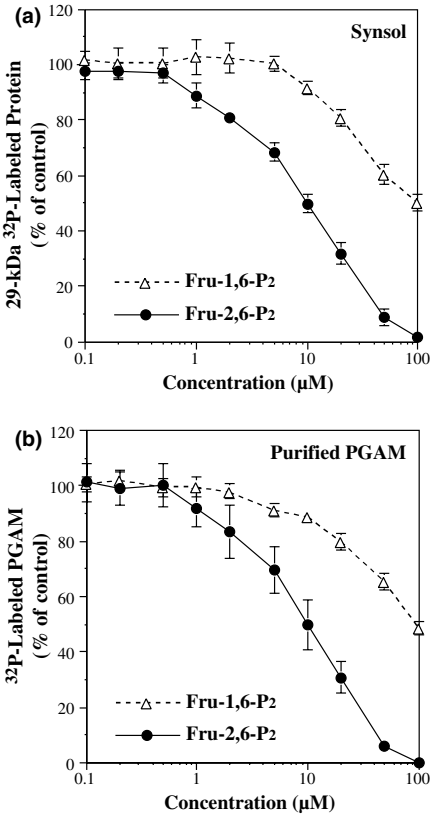

Fig. 10 Effect of various concentrations of fructose bisphosphates on ^{32}P-labeling of the 29-kDa protein in synsol and purified PGAM with [^{32}P]3-PG. The synsol fraction [1 mg/mL (a)] or PGAM [16 unit/mL (b)] was incubated with 140 nM [^{32}P]3-PG at 37°C for 10 s in the absence (control) or presence of Fru-2,6-P_2 or Fru-1,6-P_2 at the indicated concentrations. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of ^{32}P-labeled protein was estimated as the percentage of control value using an image analyzer as described in Materials and methods. Values are the mean ± SD of three experiments.

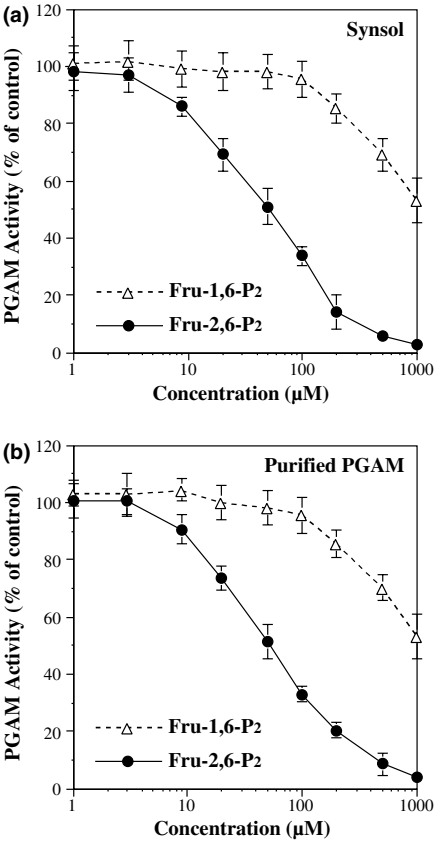

Fig. 11 Effect of various concentrations of fructose bisphosphates on PGAM activity in synsol and purified PGAM. PGAM activity in the synsol fraction [0.1 mg/mL, approximately 3 unit/mg protein (a) or purified PGAM 0.2 unit/mL (b)] was measured in the absence (control) or presence of Fru-1,6-P_2 or Fru-2,6-P_2 at the indicated concentrations, as described in Materials and methods. Values are the mean ± SD of three experiments.

presence of 50 μM Fru-2,6-P_2, as well as in the presence of 3-PG, not only at 37°C but also at 0°C. The effect of various concentrations of Fru-2,6-P_2 and Fru-1,6-P_2 on de-labeling of the [^{32}P]-labeled 29-kDa protein was examined and compared with the effect on de-labeling of purified ^{32}P-labeled PGAM (Fig. 14). Fru-2,6-P_2 was equally potent in the de-labeling of both the 29-kDa protein and PGAM; the concentration required to cause 50% de-labeling was determined to be 1 μM in both cases. Thus, Fru-2,6-P_2 is far more effective in causing de-labeling than in inhibiting [^{32}P]-labeling of the 29-kDa protein.

The radioactive product of the de-labeling reaction was analyzed by HPLC. As shown in Fig. 15(a), incubation of ^{32}P-labeled purified PGAM in the presence of Fru-2,6-P_2 resulted in release of [^{32}P]P_i, whereas the presence of unlabeled 3-PG produced [^{32}P]3-PG or [^{32}P]2-PG, or both. The amount of [^{32}P]P_i released in the presence of 50 μM Fru-2,6-P_2 was essentially the same as the amount of [^{32}P]3-PG/2-PG formed in the presence of 50 μM 3-PG. [^{32}P]P_i was released in a time-dependent manner (Fig. 15b). In order to correlate [^{32}P]P_i release with PGAM de-labeling, the effect of various concentrations of Fru-2,6-P_2 on [^{32}P]P_i release was examined (Fig. 15b) in comparison with the effect on PGAM de-labeling. The figure shows that the sensitivity of [^{32}P]P_i release from ^{32}P-labeled PGAM to Fru-2,6-P_2 is indistinguishable from that of PGAM de-labeling to Fru-2,6-P_2 (Fig. 14b). The concentration required for 50% maximal release was 1 μM. These results indicate that ^{32}P-labeled PGAM represents [^{32}P]phosphorylated PGAM, and that Fru-2,6-P_2 stimulates transfer of [^{32}P]phosphate to water, thus causing a phosphatase reaction, while 3-PG-stimulated de-labeling represents the mutase reaction.

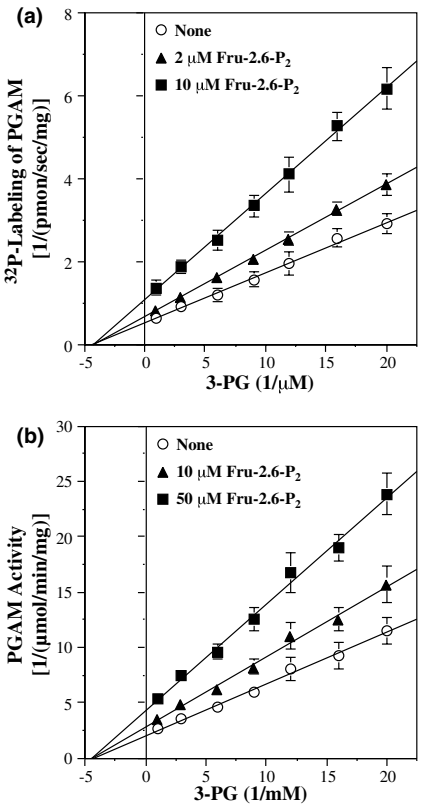

Fig. 12 Kinetics of the ^{32}P-labeling and activity of purified PGAM in the absence or presence of Fru-2,6-P_2. (a) PGAM (1 mg/mL) was incubated with various concentrations of [^{32}P]3-PG at 37°C for 10 s in the absence or presence of indicated concentrations of Fru-2,6-P_2, and the level of ^{32}P-labeled protein determined. (b) PGAM activity was determined with various concentrations of 3-PG in the absence or presence of indicated concentrations of Fru-2,6-P_2, as described in Materials and methods. Values are the mean ± SD of three experiments.

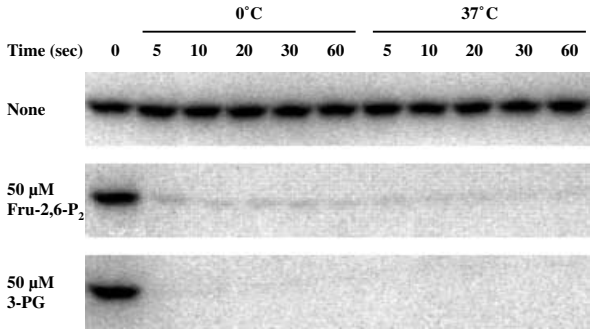

Fig. 13 Fru-2,6-P_2 as well as 3-PG stimulates de-labeling of ^{32}P-labeled 29-kDa protein in synsol. The synsol fraction was incubated with 140 nM [^{32}P]3-PG at 37°C for 10 s. After unreacted [^{32}P]3-PG was removed by use of an Amicon Centricon-10 concentrator, ^{32}P-labeled protein (1 mg/mL) was incubated at 0° or 37°C for the indicated time in the absence or presence of 50 μM Fru-2,6-P_2 or 3-PG. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography, as described in Materials and methods. The result shown is representative of three separate experiments.

Discussion

In an effort to understand a potential biochemical link between glycolysis and synaptic function, in a previous study we sought a glycolytic intermediate-dependent protein phosphorylation system using [γ-^{32}P]ATP. Evidence was obtained for the existence of phosphoglycerate-dependent phosphorylation of 72-kDa and 155-kDa proteins in mammalian brain (Ueda and Plagens 1987; Morino *et al*. 1991). The 72-kDa protein was subsequently shown to be directly phosphorylated with [1-^{32}P]1,3-BPG and identified as glucose 1,6-bisphosphate synthase (Morino *et al*. 1991). In contrast, the 155-kDa protein was not subject to direct phosphorylation with [1-^{32}P]1,3-BPG; hence it is thought to be phosphorylated at the expense of [γ-^{32}P]ATP by '3-PG-dependent kinase.' Although both proteins are enriched in the synsol fraction, their functional role in synaptic function remains to be elucidated.

In the present study, we synthesized [3-^{32}P]1,3-BPG and sought a nerve terminal protein(s) which may be modified by a mechanism involving the transfer of [3-^{32}P]phosphate or the phosphoglyceroyl moiety. We provided evidence that the major protein labeled with [3-^{32}P]1,3-BPG, under the assay conditions used, is PGAM; the 72-kDa and 155-kDa proteins were not labeled with [3-^{32}P]1,3-BPG, in contrast to labeling with [1-^{32}P]1,3-BPG or [γ-^{32}P]ATP, indicating that neither 3-phosphate nor the 3-phosphoglyceroyl group of 1,3-BPG is transferred onto these proteins. This labeled protein has a subunit molecular weight of approximately 29 000 and is rapidly labeled by both [3-^{32}P]1,3-BPG and [^{32}P]3-PG. However, this protein was not labeled with [1-^{32}P]1,3-BPG (Morino *et al*. 1991), indicating that 1-phosphate of 1,3-BPG is not transferred onto the 29-kDa protein. The labeling with [3-^{32}P]1,3-BPG is most potently reduced by 3-PG, 2-PG and Fru-2,6-P_2 of all the agents tested. [^{32}P]3-PG-labeled PGAM undergoes fast de-labeling in the presence of 3-PG or 2-PG. This is in agreement with Rose and Dube (1976), who have shown that both PGAM phosphorylation and dephosphorylation occur rapidly. Moreover, the labeled protein is subject to immunoprecipitation with affinity-purified anti-PGAM IgG. These observations indicate that the [3-^{32}P]1,3-BPG or [^{32}P]3-PG-labeled 29-kDa protein is identical to PGAM. Thus, labeling and de-labeling of the synsol 29-kDa protein would represent phosphorylation and dephosphorylation of the active site histidine residue of PGAM (Rose 1970, 1980).

An interesting finding in this study is that Fru-2,6-P_2 inhibits both phosphorylation (IC_{50} = 10 μM) and enzymatic activity of PGAM (IC_{50} = 50 μM). Fru-2,6-P_2 also accelerates dephosphorylation of PGAM (EC_{50} = 1 μM). Fru-2,6-P_2 is more effective in dephosphorylating PGAM than in inhibiting its enzymatic activity. Thus, the apparent diminishing

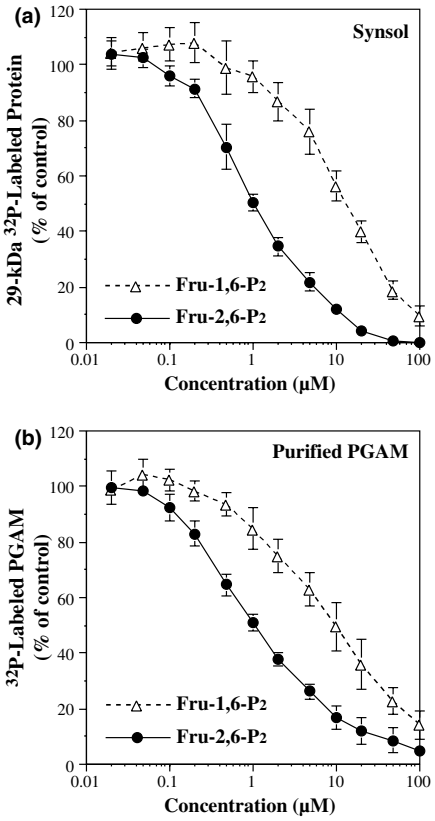

Fig. 14 Effect of various concentrations of fructose bisphosphates on the dephosphorylation of ^{32}P-labeling of 29-kDa protein in synsol and purified PGAM. The synsol fraction [1 mg/mL (a)] or purified PGAM [16 unit/mL (b)] was incubated with 140 nM [^{32}P]3-PG at 37°C for 10 s. After unreacted [^{32}P]3-PG was removed by use of an Amicon Centricon-10 concentrator, ^{32}P-labeled protein of synsol (1 mg/mL) or PGAM (0.016 unit/mL) was incubated at 37°C for 10 s in the absence (control) or presence of Fru-2,6-P_2 or Fru-1,6-P_2 at the indicated concentrations. Aliquots (18.8 μL) were subjected to SDS–PAGE (12%), followed by autoradiography. The level of ^{32}P-labeled protein was estimated as the percentage of control value, using an image analyzer as described in Materials and methods. Values are the mean ± SD of three experiments.

effect of Fru-2,6-P_2 on PGAM phosphorylation could be largely due to its ability to enhance dephosphorylation. In contrast to the case with 3-PG, transfer of radioactive phosphate to Fru-2,6-P_2 was not observed. In fact, Fru-2,6-P_2 stimulated release of inorganic [^{32}P]phosphate from the [^{32}P]phosphoenzyme (Fig. 15). Kinetic experiments (Fig. 12) suggest that Fru-2,6-P_2 binds to an allosteric site. These experiments suggest that, upon binding of Fru-2,6-P_2, PGAM could alter its conformation, exposing the phosphohistidine moiety to the aqueous environment so that the phosphoenzyme is hydrolyzed to inorganic phosphate and dephosphoenzyme.

Fru-2,6-P_2 is known as a regulator of glycolysis in the liver (Van Schaftingen 1987; Okar and Lange 1999). It acts as an allosteric activator of phosphofructokinase 1 (PFK-1), increasing the enzyme's affinity for fructose 6-phosphate (Van Schaftingen *et al*. 1981). In the liver, the biosynthesis of Fru-2,6-P_2 is achieved by phosphofructokinase-2 (PFK-2), whose activity is regulated via phosphorylation of a serine residue by cAMP-dependent protein kinase (Okar *et al*. 2001). In the brain, however, PFK-2 activity is not altered as a result of phosphorylation by cAMP-dependent protein kinase (Ventura *et al*. 1991; Ventura *et al*. 1992). Nonetheless, Fru-2,6-P_2 acts as a positive allosteric effector of brain PFK-1 and is thought to play an essential role in the basal activation of the brain enzyme under a variety of physiological conditions (Foe and Kemp 1985; Ventura *et al*. 1991). Brain PFK-1, maximally activated with 4–6 μM Fru-2,6-P_2, is saturated with Fru-2,6-P_2 under normal conditions (Foe and Kemp 1984; Yamamoto *et al*. 1990; Ambrosio *et al*. 1991, 1992; Ventura *et al*. 1991; Anglard *et al*. 1992; Kasten *et al*. 1993). Thus, PFK-1 may not be subject to regulation by Fru-2,6-P_2 in the brain under normal conditions (Ventura *et al*. 1992).

Fru-2,6-P_2 inhibits PGAM enzyme activity with a K_i value of 36 μM. The steady-state tissue level of Fru-2,6-P_2 in the brain is 5–16 nM/g tissue (Yamamoto *et al*. 1990; Ambrosio *et al*. 1991, 1992; Ventura *et al*. 1991; Kasten *et al*. 1993). Neuronal Fru-2,6-P_2 concentration can be increased three to fourfold by treatment with either ACTH or 12-*O*-tetradecanoylphorbol 13-acetate (Anglard *et al*. 1992). Under these conditions, Fru-2,6-P_2 might exert an inhibitory effect on PGAM. Thus, PGAM could be subject to regulation by Fru-2,6-P_2 under certain conditions. Under normal conditions, neuronal PFK-1 would most likely be in a fully activated state in the presence of saturating concentrations of Fru-2,6-P_2. When PGAM is inhibited by increased concentrations of Fru-2,6-P_2, the glycolytic intermediate 3-PG would be accumulated. One of the consequences of 3-PG elevation would be to activate 3-PG-dependent protein kinase, leading to phosphorylation of the synsol 155-kDa protein (Ueda and Plagens 1987; Morino *et al*. 1991). Thus, it is plausible that glycolysis up to the step of 3-PG production, Fru-2,6-P_2-induced accumulation of 3-PG and 3-PG-dependent phosphorylation of the 155-kDa protein are all involved in the mechanism underlying glycolysis-dependent synaptic transmission. Further studies are required to determine the significance of PGAM inhibition by Fru-2,6-P_2.

Another interesting observation is that the level of PGAM labeling with [3-^{32}P]1,3-BPG, as well as the specific enzyme activity of PGAM, is remarkably high in the brain and the fast-twitch muscle extensor digitorum longus; of brain subcellular fractions, the synsol exhibited the most prominent labeling. The amount of labeled PGAM in the cell body cytosol fraction is substantially smaller. These observations suggest that the rate of glycolysis might be high in subcellular regions where either neurotransmitters or hormones are actively released. It is feasible that in the nerve

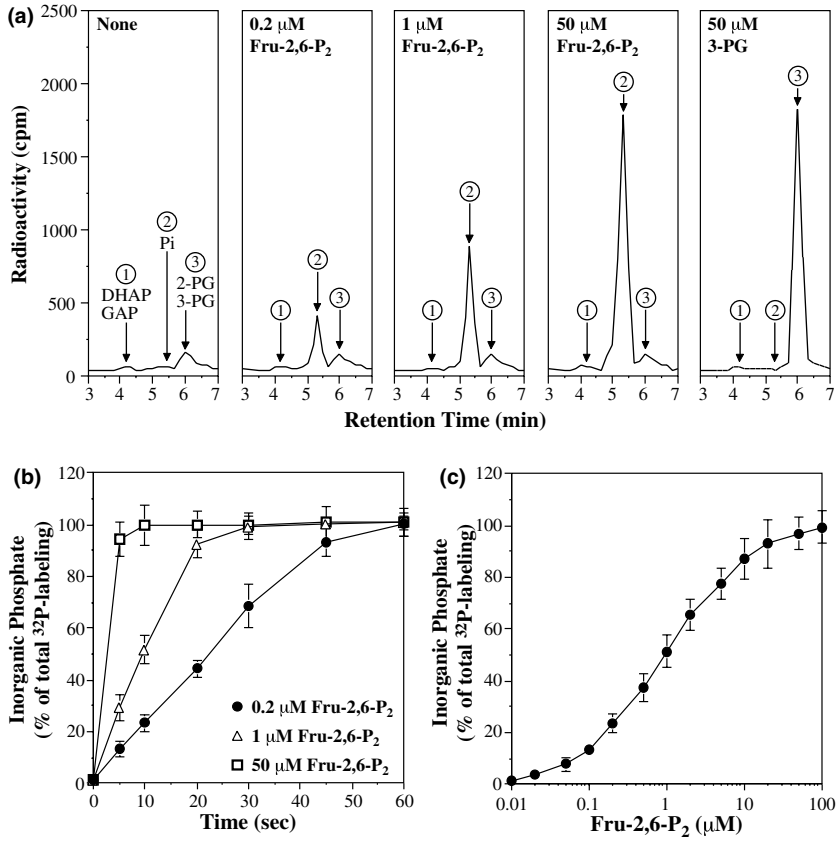

Fig. 15 Analysis of [^{32}P]compounds released from [^{32}P]PGAM and the effect of various concentrations of Fru-2,6-P_2 and incubation time on [^{32}P]P_i release. Purified PGAM was labeled with [^{32}P]3-PG and unreacted [^{32}P]3-PG removed by use of an Amicon Centricon-10 concentrator. ^{32}P-Labeled PGAM was incubated at 37°C for 10 s in the absence or presence of Fru-2,6-P_2 or 3-PG at indicated concentrations, and [^{32}P]compounds released from ^{32}P-PGAM were analyzed by HPLC (a). [^{32}P]Inorganic phosphate (P_i) released from ^{32}P to labeled PGAM after incubation at 0°C for indicated periods (b) or with various concentrations of Fru-2,6-P_2 at 37°C for 10 s (c) was measured as described in Materials and methods. Values represent percentage of total labeling of PGAM and the mean ± SD of three experiments.

ending, there is a high demand for glycolytically-produced ATP, rather than ATP produced in mitochondria, in order to meet quick demands for energy; duly responding transmitter release is thus sustained. In further support of this notion is evidence that the nerve ending is rich in glycolytic enzymes (Knull 1978); some are associated with the synaptic plasma membrane via actin (Knull 1980), which would allow glycolysis to occur efficiently at high energy-requiring presynaptic membrane sites. Compatible with this concept, we have recently obtained evidence indicating that glycolytically-generated ATP is utilized for neurotransmitter uptake into the releasable vesicular pool (Ikemoto *et al*. 2003). Thus, acute depletion of glycolytically-produced ATP, without much affecting the total reserve of ATP which is largely made in mitochondria, would diminish neurotransmitter release. This might provide an explanation for the critical importance of glycolysis in synaptic transmission. The fast-twitch fiber is far richer in glycolytic enzymes, including PGAM, than the slow-twitch fiber of the soleus (Baldwin *et al*. 1973; Andres *et al*. 1989), and is thought to mainly harness glycolytically-produced ATP for rapid muscle movement (Holloszy and Booth 1976). These lines of evidence are consistent with the notion that rapid, energy-consuming, cellular processes rely on glycolytically-produced ATP.

Acknowledgements

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